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Prevalence and phylogenetic analysis of the current porcine circovirus 2 genotypes after implementation of widespread vaccination programmes in the USA

Hui-Gang Shen, Patrick G. Halbur and Tanja Opriessnig

Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA

Correspondence
Tanja Opriessnig
tanjaopr@iastate.edu

To determine the prevalence of porcine circovirus 2 (PCV2) genotypes in the USA during 2010–2011, 5 years after widespread PCV2 vaccination, serum samples from clinically normal pigs that were PCV2 vaccinated ($n=1177$), non-vaccinated ($n=378$) or of unknown vaccination status ($n=120$), and 100 lung samples from pigs diagnosed with PCV-associated disease (PCVAD) were tested. The presence of PCV2, PCV1, PCV1-2a and porcine parvovirus (PPV) DNA was determined by PCR. Determination of the PCV2 genotype was done by differential PCR and sequencing. The prevalence of PCV2a and PCV2b in serum samples was 7.7% (129/1675) and 8.4% (141/1675), respectively. PCV2a DNA was only detected in non-vaccinated pigs. For the 100 PCVAD pigs, the prevalence of PCV2a and PCV2b in lung tissues was 13.0 and 65.0%, respectively. Partial PCV2 ORF2 sequences (9–563 nt) were obtained from 85 PCV2 DNA-positive samples (24 normal pigs and 61 PCVAD cases). Phylogenetic analysis revealed that 12.9% (11/85) of the sequences belonged to the 2E clade and the PCV2a genotype and 87.1% (74/85) belonged to the 1B clade and the PCV2b genotype. The alignment of putative PCV2 capsid amino acid sequences revealed possible recombination or mutation between PCV2a and PCV2b genotypes. Chimeric PCV1-2a was not detected in any of the samples and the prevalence rates of PCV1 and PPV were low. Our results suggest PCV2b is more prevalent than PCV2a in PCVAD cases and in vaccinated herds PCV2b circulation is common. The data generated in this study provide novel information on the distribution of PCV2 genotypes in vaccinated pig populations.

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INTRODUCTION

Porcine circovirus (PCV) is a circular ssDNA virus in the family *Circoviridae* (Tischer *et al.*, 1982). Two types of PCV have been described up to now, PCV type 1 (PCV1) and PCV type 2 (PCV2). PCV1 was first isolated from a porcine kidney cell line (PK-15) (Tischer *et al.*, 1974) and was not pathogenic to pigs (Tischer *et al.*, 1986), whereas PCV2 is associated with a number of disease manifestations (porcine circovirus-associated disease, PCVAD) including post-weaning multisystemic wasting syndrome (PMWS), porcine respiratory disease complex (PRDC), porcine dermatitis and nephropathy syndrome (PDNS), pneumonia, diarrhoea in grow-finish pigs and reproductive failure (Opriessnig *et al.*, 2007).

The overall DNA sequence homology within PCV1 or PCV2 isolates is greater than 90%, while the homology between PCV1 and PCV2 isolates is 68 to 76% (Hamel *et al.*, 1998; Larochelle *et al.*, 2002; Meehan *et al.*, 1998; Zhou *et al.*, 2006).

Three ORFs were recognized in PCV2 with ORF1 located on the viral plus-strand, and ORF2 and ORF3 on the counterclockwise strand. PCV2 ORF1 encodes a 35.7 kDa replication protein involved in virus replication (Mankertz *et al.*, 1998). PCV2 ORF2 encodes a 27.8 kDa capsid protein (Cap) involved in viral immunogenicity (Nawagitgul *et al.*, 2000). PCV2 ORF3 protein is not essential for PCV2 replication, but is involved in PCV2-induced apoptosis (Liu *et al.*, 2005).

A unified nomenclature for PCV2 genotypes (PCV2a, PCV2b and PCV2c) has been proposed (Segalés *et al.*, 2008). PCV2 has two major genotypes: PCV2a (or group 2), which was further subdivided into five clusters (2A–2E), and PCV2b (or group 1) subdivided into three clusters (1A–1C) (Olvera *et al.*, 2007). A third genotype (PCV2c) was identified in Denmark (Dupont *et al.*, 2008). PCV2d and PCV2e have been proposed to be PCV2 novel genotypes emerging in China (Wang *et al.*, 2009); however, they were later demonstrated to belong to the PCV2a and PCV2b clusters (Cortey *et al.*, 2011). Severe PCVAD

The GenBank/EMBL/DDBJ accession numbers are shown in Table S1 (available in JGV Online).

Supplementary tables are available with the online version of this paper.

outbreaks emerged in North America during 2005–2006 (Cheung *et al.*, 2007); the main PCV2 subtype isolated during these outbreaks was found to be PCV2b, which was different from investigations conducted prior to 2005 that only identified PCV2a (Cheung *et al.*, 2007; Fenaux *et al.*, 2000; Laroche *et al.*, 2002).

PCV2 vaccination was introduced in 2006 in the USA (Opriessnig *et al.*, 2007). The currently available commercial PCV2 vaccines in North America include an inactivated vaccine based on a chimeric PCV2, two subunit vaccines based on the PCV2 Cap expressed in the baculovirus system, and an inactivated vaccine based on a PCV2 virus (Opriessnig *et al.*, 2007). Because of the detection of a novel chimeric PCV2 virus (PCV1-2a) containing PCV1 ORF1 and PCV2a ORF2 in Canada in 2010 (Gagnon *et al.*, 2010), the chimeric PCV2 vaccine (Suvaxyn PCV; Fort Dodge Animal Health Inc.) was voluntarily removed from the market in May 2010 due to concerns about the inactivation process. A reformulated version of the chimeric PCV2 vaccine re-entered the market in August 2011 under a new brand name (Fostera PCV; Pfizer Animal Health Inc.). All the currently available vaccines, regardless of the type, are based on PCV2a isolates (Opriessnig *et al.*, 2007). It is estimated that today 99% of all growing pigs in the USA are vaccinated against PCV2 at or around the time of weaning.

To the best of our knowledge, an investigation of PCV2 subtype distribution and prevalence of PCV2 in USA herds after implementation of widespread PCV2 vaccination is lacking. The objectives of this study were (i) to investigate the prevalence of PCV2a and PCV2b in serum samples of grow-finish herds with no known history of PCVAD, and (ii) to investigate the predominant PCV2 subtype in cases diagnosed as PCVAD by histopathology. Additionally, the prevalence of PCV1, PCV1-2a and porcine parvovirus (PPV) was also investigated.

RESULTS

Prevalence of major PCV2 subtypes

Serum samples. Within the 1675 serum samples, 1177 were from vaccinated pigs, 378 were from non-vaccinated pigs and 120 were from pigs with unknown PCV2 vaccination history. Among the 1675 serum samples, 129 (7.7%) samples were positive for PCV2a and 141 (8.4%) were positive for PCV2b DNA (Table 1). PCV2 DNA was not detected in samples from 45.9% (28) of the 61 sites from which 33.1% (554 serum samples) of the 1675 serum samples were received. PCV2a was identified in four sites and six barns with 2–49 samples positive in each barn [mean, 21.5; confidence interval (CI), –0.5, 43.5]. PCV2b was identified on 28 sites in 29 barns with 1–26 samples being positive in each barn (mean, 4.9; CI, 2.9, 6.9). For PCV2a, samples derived from non-vaccinated pigs had a higher prevalence (34.1 versus 0%) than those derived

from vaccinated pigs (Table 2). Interestingly, for PCV2b, samples from vaccinated pigs had significantly higher viral DNA loads (0.64 ± 0.06 versus 0.21 ± 0.05 \log_{10} copies ml^{-1}) than those found in non-vaccinated pigs (Table 2). Site and barn nested within site had no effect on the results based on vaccination status (vaccinated; non-vaccinated) for both \log_{10} PCV2a and PCV2b DNA loads.

For the 1177 samples from herds known to utilize PCV2 vaccination, 922 samples were from herds using one of the three commercial vaccines (Table 3) and 255 samples were from herds that were vaccinated, but it was not absolutely clear which of the products had been used on the group of pigs. When the results were compared based on vaccine products used, pigs vaccinated with Suvaxyn PCV had lower PCV2b DNA loads compared with pigs vaccinated with Ingelvac CiroFLEX and Circumvent PCV (Table 3). Site and barn nested within site had no effect on the results and were controlled for in the model. An association of age and prevalence of PCV2 DNA was not identified (data not shown).

PCVAD cases. For the PCVAD cases, the prevalence of PCV2a and PCV2b was 13.0% (13/100) and 65.0% (65/100), with PCV2 DNA loads of 1.21 ± 0.32 and 6.47 ± 0.52 \log_{10} copies ml^{-1} , respectively (Table 1). Specifically, in lung tissues single PCV2 infection was identified in 10 of 100 cases, single PCV2b infection was identified in 62 of 100 cases, concurrent PCV2a and PCV2b infection was identified in 3 of 100 cases and PCV2 DNA was not detected in 25 of 100 cases. Comparison of the prevalence and level of PCV2 DNA load in serum samples revealed the prevalence of PCV2a and PCV2b was significantly ($P < 0.05$) higher in tissue samples, and tissue samples had significantly higher ($P < 0.05$) PCV2a and PCV2b DNA loads (Table 1). When the amount of PCV2 DNA in PCVAD cases was compared, no age-related association was identified (data not shown). The mean number of positive samples on a site was 1–5 pigs. The PCV2a-positive samples were from 11 sites and the PCV2b-positive samples were from 54 sites. Site and barn nested within site had no effect on the results.

PCV2 ORF2 gene sequence analysis

Samples with cycle threshold (C_t) values lower than 32 in real-time PCR were selected for sequencing. Partial PCV2 ORF2 sequences (9–563 nt) were obtained from 85 PCV2 PCR-positive samples, including 24 serum and 61 PCVAD cases; the sequence IDs and GenBank accession numbers are shown in Table S1. Closely related sequences (with distances of less than 0.005) that differed by up to 3 nt and one putative amino acid was represented by a single representative sequence with the numbers of similar sequences in phylogenetic analysis (Table S2); as a result, 17 representative sequences were identified with distances of more than or equal to 0.005. A phylogenetic tree was constructed from the 17 representative sequences, 11 PCV2 reference sequences, as well as one sequence for the PCV1-2a vaccine (GenBank accession no. AF264042) (Fig. 1). The

Table 1. Overall prevalence of PCV2a and PCV2b in the samples from healthy pigs (serum) regardless of vaccination status or from PCVAD pigs (tissue)

Data are presented as number of positive samples, percentage (mean log₁₀ PCV2 DNA copies ml⁻¹ ± SEM).

Sample type	No.	PCV2a	PCV2b
Serum	1 675	129, 7.7 % (0.38 ± 0.03)*	141, 8.4 % (0.52 ± 0.43)*
Tissue	100	13, 13.0 % (1.21 ± 0.32)†	65, 65.0 % (6.47 ± 0.52)†
Total	1 775	142, 8.0 % (0.43 ± 0.04)	206, 11.6 % (0.86 ± 0.06)

*,†Different superscripts within columns represent significant ($P < 0.05$) differences in the mean amount of PCV2a or PCV2b DNA between sample types.

analysis revealed that 11/85 sequences belonged to the PCV2a genotype and 74/85 belonged to PCV2b (Fig. 1).

In pairwise comparisons, the partial PCV2 ORF2 nucleotide sequences had distances of 0.005–0.022 among the PCV2a sequences, 0.005–0.028 among the PCV2b sequences, and 0.050–0.086 between PCV2a and PCV2b sequences. For pairwise comparisons of the amino acid sequences, the distances were 0.005–0.044 within PCV2a, 0.005–0.085 within PCV2b, and 0.073–0.103 between PCV2a and PCV2b. All the 11 PCV2a nucleotide sequences belonged to the 2E clade (Fig. 1) with distances of 0.011–0.016 with the 2E reference strain (GenBank accession no. AF264039). All the PCV2a samples had distances of 0.016–0.033 with the PCV1–2a ORF2 nucleotide sequence (data not shown). All the 74 PCV2b sequences belonged to the 1B clade (Fig. 1). To the prototype of this clade, GenBank accession no. AY678532, the sequences from the samples had distances between 0.005 and 0.016.

Putative PCV2 Cap amino acid sequence analysis

In order to investigate the amino acid variation in Cap of the two major PCV2 genotypes detected in the samples, 185 putative amino acid sequences (positions 4–188) of the PCV2 Cap obtained in this study were aligned with the 11 known PCV2 clades (1A–1C and 2A–2D) and three known genotypes (2a, 2b and 2c) and the PCV1–2a vaccine reference sequence (Fig. 2). Fifty-three of the 74 PCV2b Cap amino acid sequences were closely related to PCV2 clades 1A and 1B reference strains within the PCV2b

genotype with only one amino acid difference. The following amino acid substitutions between PCV2a and PCV2b genotypes were identified: for PCV2b Cap amino acid sequences, 6/74 varied at position 30 (V to L, same as 2E clade reference strains in PCV2a), 3/74 varied at position 76 (I to L, same as 2D and 2E clades reference strains in PCV2a), 3/74 varied at position 131 (T to P, same as 2A, 2C and 2E clade reference strains in PCV2a (white boxes in Fig. 2); for PCV2a Cap amino acid sequences, 90.9 % (10/11) PCV2a amino acid sequences had a T to N substitution at position 134, which was the same as that in 1C clade reference strain in PCV2b genotype (shaded boxes in Fig. 2). Interestingly, one of the 74 PCV2b sequences investigated had a T to P substitution at position 64, which is also present in the PCV2c reference strain (white box in Fig. 2). In addition, there were other substitutions at positions 28–34, 64, 118, 133–134 and 188 that were not identified in any of the references strains (shaded but unboxed in Fig. 2). Compared with the Cap sequence of the chimeric PCV1–2a vaccine, the 11 PCV2a samples had several variations such as at position 131 (T to P in 11/11 samples), 133 (A to V in 10/11 samples), 134 (T to D in 1/11 sample, and T to N in 10/11 samples) and 136 (L to Q in 10/11 samples) (Fig. 2).

Prevalence of PCV1, PCV1-2a and PPV

PCV1 DNA was detected in 2.7 % (45/1675) of the serum samples and 2.0 % (2/100) of the PCVAD cases. PCV1–2a DNA was not identified in any of the tested samples. PPV

Table 2. Prevalence of PCV2a and PCV2b in serum samples from vaccinated or non-vaccinated herds

Data are presented as number of positive samples, percentage (mean log₁₀ PCV2 DNA copies ml⁻¹ ± SEM).

Vaccination status	No.	PCV2a	PCV2b
Vaccinated	1 177	0, 0 % (0.00 ± 0.00)*	117, 9.9 % (0.64 ± 0.06)*
Non-vaccinated	378	129, 34.1 % (1.70 ± 0.13)†	16, 4.2 % (0.21 ± 0.05)†

*,†Different superscripts within columns represent significant ($P < 0.05$) differences in the mean amount of PCV2a or PCV2b DNA between samples obtained from vaccinated and non-vaccinated herds.

Table 3. Comparison of prevalence of PCV2b in serum samples from farms with a history of PCV2 vaccination and vaccine product information

Data are presented as number of positive samples, percentage (mean \log_{10} PCV2 DNA copies $\text{ml}^{-1} \pm \text{SEM}$).

Vaccine	Administration	No.	PCV2b*
Ingelvac CircoFLEX	1 dose	184	32, 17.4 % (1.02 \pm 0.17)
Suvaxyn PCV	1 or 2 dose*	310	15, 4.8 % (0.35 \pm 0.09)
Circumvent PCV	2 doses	428	68, 15.9 % (1.01 \pm 0.12)

*Information on the actual dose used on a site or in a barn was not available.

DNA was detected in 1.0 % (17/1675) of the serum samples and in none of the PCVAD cases.

DISCUSSION

PCV2 vaccination has been demonstrated to be highly efficient in inducing protective immunity against PCV2 infection and PCVAD under both experimental (Fort *et al.*, 2008; Opriessnig *et al.*, 2008a; Shen *et al.*, 2010b) and field (Cline *et al.*, 2008; Fachinger *et al.*, 2008; Segalés *et al.*, 2009) conditions. To the best of our knowledge, this is the first epidemiological report investigating the prevalence of PCV2 genotypes and clades after implementation of widespread PCV2 vaccination programmes in the USA; our data provide novel information on the distribution of PCV2 genotypes and subtypes in the vaccinated pig population, which is impossible to achieve under controlled experimental studies. The aim of this study was not to compare the efficacy of different commercial vaccines, which were demonstrated to be equally effective in previous experimental studies (Shen *et al.*, 2010b). Since many factors such as farm condition, age distribution, vaccination protocol (time of vaccination, doses used) and time of sample collection can influence results obtained under field conditions, our data are not sufficient to draw conclusions regarding possible differences in efficacy of commercial PCV2 vaccines in the field. Moreover, the serum samples and the tissue samples investigated in this study all originated from different sites and geographical variation could explain any differences in the results obtained from the different sample types.

To our knowledge both the Ingelvac CircoFLEX and the Circumvent PCV vaccines are based on PCV2a strains. Since these two vaccines are subunit vaccines, based on ORF2 protein expressed in a baculovirus vector, both vaccines should be positive for PCV2a by the differential PCV2a/PCV2b PCR which was confirmed in our lab (data not shown). For Suvaxyn PCV, the PCV1–2a real-time

PCR assay used in this study contains special primers and a probe for the chimeric region and this vaccine is also positive by the differential PCR utilized in this study (data not shown). Nevertheless, all the serum samples that originated from confirmed vaccinated pigs were negative for PCV2a DNA and potential detection of vaccine virus can be ruled out. This result indicates that the current commercial PCV2 vaccines are efficient in preventing PCV2a viraemia. However, it is unexpected that the diagnostic serum samples from known vaccinated pigs had significantly higher PCV2b DNA loads than samples derived from known non-vaccinated pigs. One explanation could be that the currently available vaccines may be relatively poor in cross-protecting against PCV2b infection due to the PCV2a origin of the vaccine strains. This hypothesis is in disagreement with a previous report, in which a PCV2 subunit vaccine containing PCV2a Cap was found to be equally effective in preventing viraemia, decreasing nasal and faecal virus shedding caused by PCV2a or PCV2b infection (Fort *et al.*, 2008). Alternatively, as the three vaccines available at the time this study was done were licensed for usage as one dose (Ingelvac CircoFLEX), two doses (Circumvent PCV) or one or two dose application (Suvaxyn PCV) and since it is unknown if the manufacturer's instructions were followed, this may also have impacted the results. The discrepancy between the results obtained under experimental conditions and the results obtained in the current USA field investigation could be due to differences in circulating PCV2 isolates, which are controlled strains under experimental conditions compared with uncontrolled strains with possible mutations under field conditions.

For the serum samples from non-vaccinated herds, 34.1 % (129/378) were positive for PCV2a; this prevalence was lower than what was found in 2006 prior to PCV2 vaccination where over 82 % of sera from 185 farms were positive for PCV2 by PCR (Puvanendiran *et al.*, 2011). Furthermore, 3.1 % (4/129) of the positive serum samples from non-vaccinated herds had high PCV2 DNA loads of more than 7 \log_{10} PCV2 DNA copies ml^{-1} (data not shown). Interestingly, Puvanendiran *et al.* (2011) found that 7.2 % (246/3424) of the PCV2 DNA-positive serum samples had similarly high levels prior to PCV2 vaccination. Although differences in DNA extraction and PCR assays could account for some of these differences, these results suggest that the implementation of PCV2 vaccination on the USA pig population substantially reduced the circulation of PCV2a in the population as a whole, including herds that currently are not vaccinated.

In the PCVAD cases, PCV2 DNA was identified in 75 % of lung tissues. The lack of detection of PCV2 DNA in 25 % of the PCVAD cases in the current study could be explained by testing only lung tissue and not pooling tissues or including lymphoid tissues. In cases of enteric or systemic PCVAD without a respiratory component, PCV2 DNA would not necessarily be expected to be present in lung tissues. Overall, we found a higher prevalence and higher

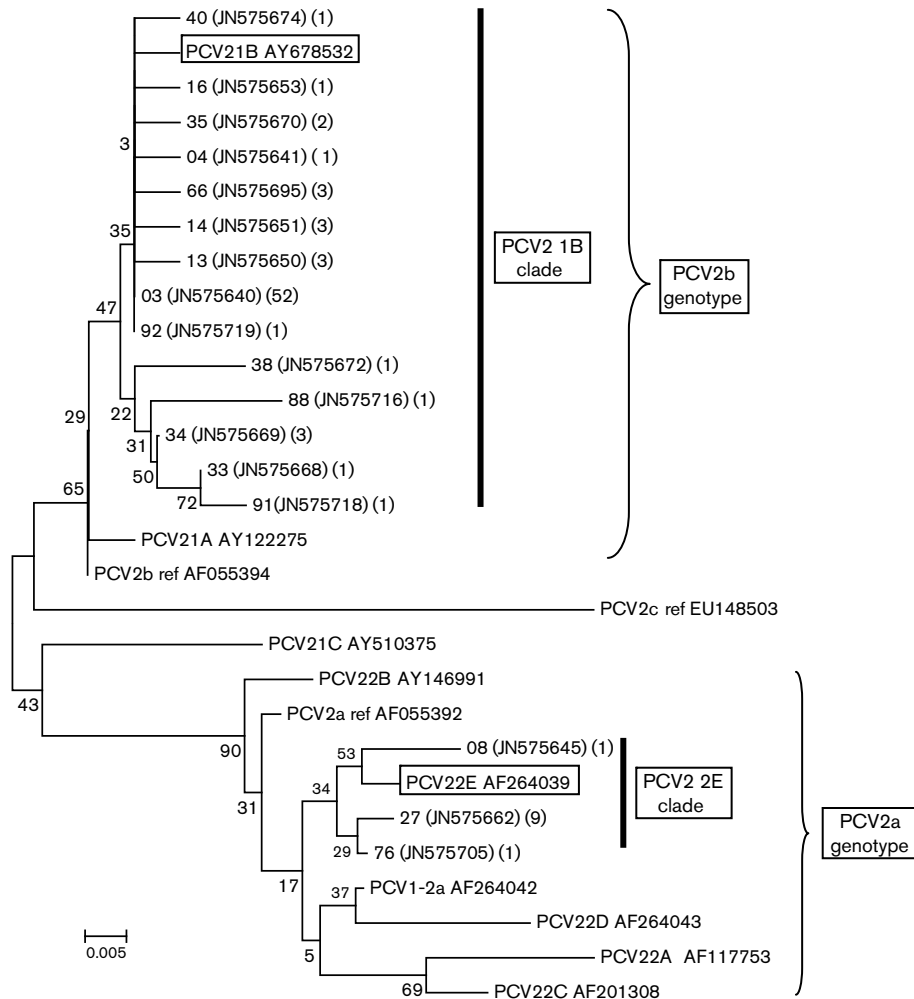


Fig. 1. Phylogenetic analysis of the representative partial PCV2 ORF2 sequences (9–563 nt) obtained in this study and 11 PCV2 clade (1A–1C and 2A–2D) and genotype (2a, 2b and 2c) and PCV1–2a vaccine reference sequences. The phylogenetic tree was constructed by the NJ method. Significant bootstrap values are indicated as a percentage for 1000 replicates. The reference sequences for PCV2 clades related to the sequences in this study are indicated in the boxes. Sequences obtained in the present study are presented as follows: sequence ID (GenBank accession number) (numbers of sequences with distances of <0.005 in pairwise comparisons).

DNA loads of PCV2b than PCV2a. This is consistent with previous findings (Cheung *et al.*, 2007). However, it is still disputable as to whether the PCV2b genotype is more virulent than the PCV2a genotype, since some investigations found links between the PCV2b genotype and occurrence of PCVAD (Chae & Choi, 2010; Cheung *et al.*, 2007; Grau-Roma *et al.*, 2008; Timmusk *et al.*, 2008) and some did not (de Boisséson *et al.*, 2004; Martins Gomes de Castro *et al.*, 2007; Olvera *et al.*, 2007). Under experimental conditions, the virulence of PCV2a and PCV2b isolates is not different in the conventional specific-pathogen-free pig model (Opriessnig *et al.*, 2008b); however, the virulence of isolates within the same cluster differs (Opriessnig *et al.*, 2006, 2008b). Some studies indicate that PCV2a and PCV2b co-infection or recombination may play a role in PCV2 replication and pathogenesis. In a recent study, dual

heterologous PCV2a and PCV2b inoculation 7 days apart induced severe clinical illness, but singular PCV2a or PCV2b appeared to be of equivalent virulence (Harding *et al.*, 2010). Another study performed on tissues from PCVAD cases in Switzerland revealed that both PCV2a and PCV2b were equally present in PCV2 pre-epizootic- and post-epizootic-infected piglets, and superinfections and co-replication of the two major PCV2 genotypes (PCV2a and PCV2b) were observed *in vivo* (Khaiseb *et al.*, 2011). In addition, two recombinant mutants of PCV2 with PCV2b ORF1 and PCV2a ORF2 were shown to have enhanced replication efficiencies compared with the parental strains (Guo *et al.*, 2011). All these studies suggest PCV2a and PCV2b may interact with each other during infection. It is notable that in the present study even though PCV2a was not the prevalent genotype in PCVAD,

	4	10	20	30	40	50	60	70	80	90	96
PCV2b	P	R	R	R	R	R	R	R	R	R	R
03 (52)	R	R	R	R	R	R	R	R	R	R	R
35 (2)	R	R	R	R	R	R	R	R	R	R	R
04 (1)	R	R	R	R	R	R	R	R	R	R	R
13 (3)	R	R	R	R	R	R	R	R	R	R	R
14 (3)	R	R	R	R	R	R	R	R	R	R	R
16 (1)	R	R	R	R	R	R	R	R	R	R	R
40 (1)	R	R	R	R	R	R	R	R	R	R	R
66 (3)	R	R	R	R	R	R	R	R	R	R	R
88 (1)	R	R	R	R	R	R	R	R	R	R	R
33 (1)	R	R	R	R	R	R	R	R	R	R	R
34 (3)	R	R	R	R	R	R	R	R	R	R	R
91 (1)	R	R	R	R	R	R	R	R	R	R	R
92 (1)	R	R	R	R	R	R	R	R	R	R	R
38 (1)	R	R	R	R	R	R	R	R	R	R	R
PCV21A											
PCV21B											
PCV21C											
PCV2b ref											
PCV2a											
08 (1)											
27 (9)											
76 (1)											
PCV1-2a											
PCV22A											
PCV22B											
PCV22C											
PCV22D											
PCV22E											
PCV2a ref											
PCV2c											
PCV2c ref											
PCV2b	R	I	R	K	V	K	V	E	F	W	P
03 (52)	R	I	R	K	V	K	V	E	F	W	P
35 (2)	R	I	R	K	V	K	V	E	F	W	P
04 (1)	R	I	R	K	V	K	V	E	F	W	P
13 (3)	R	I	R	K	V	K	V	E	F	W	P
14 (3)	R	I	R	K	V	K	V	E	F	W	P
16 (1)	R	I	R	K	V	K	V	E	F	W	P
40 (1)	R	I	R	K	V	K	V	E	F	W	P
66 (3)	R	I	R	K	V	K	V	E	F	W	P
88 (1)	R	I	R	K	V	K	V	E	F	W	P
33 (1)	R	I	R	K	V	K	V	E	F	W	P
34 (3)	R	I	R	K	V	K	V	E	F	W	P
91 (1)	R	I	R	K	V	K	V	E	F	W	P
92 (1)	R	I	R	K	V	K	V	E	F	W	P
38 (1)	R	I	R	K	V	K	V	E	F	W	P
PCV21A											
PCV21B											
PCV21C											
PCV2b ref											
PCV2a											
08 (1)											
27 (9)											
76 (1)											
PCV1-2a											
PCV22A											
PCV22B											
PCV22C											
PCV22D											
PCV22E											
PCV2a ref											
PCV2c											
PCV2c ref											
PCV2b	R	I	R	K	V	K	V	E	F	W	P
03 (52)	R	I	R	K	V	K	V	E	F	W	P
35 (2)	R	I	R	K	V	K	V	E	F	W	P
04 (1)	R	I	R	K	V	K	V	E	F	W	P
13 (3)	R	I	R	K	V	K	V	E	F	W	P
14 (3)	R	I	R	K	V	K	V	E	F	W	P
16 (1)	R	I	R	K	V	K	V	E	F	W	P
40 (1)	R	I	R	K	V	K	V	E	F	W	P
66 (3)	R	I	R	K	V	K	V	E	F	W	P
88 (1)	R	I	R	K	V	K	V	E	F	W	P
33 (1)	R	I	R	K	V	K	V	E	F	W	P
34 (3)	R	I	R	K	V	K	V	E	F	W	P
91 (1)	R	I	R	K	V	K	V	E	F	W	P
92 (1)	R	I	R	K	V	K	V	E	F	W	P
38 (1)	R	I	R	K	V	K	V	E	F	W	P
PCV21A											
PCV21B											
PCV21C											
PCV2b ref											
PCV2a											
08 (1)											
27 (9)											
76 (1)											
PCV1-2a											
PCV22A											
PCV22B											
PCV22C											
PCV22D											
PCV22E											
PCV2a ref											
PCV2c											
PCV2c ref											

Fig. 2. Amino acid sequence alignment of the representative partial putative Cap (4–188 aa) obtained in this study and that from and 11 PCV2 clade (1A–1C and 2A–2D) and genotype (2a, 2b and 2c) and PCV1–2a vaccine reference sequences. White boxes indicate PCV2a- or PCV2c-related amino acid substitutions in PCV2b; shaded boxes indicate PCV2b-related amino acid substitutions in PCV2a; shaded but unboxed areas indicate unrecognized amino acid substitutions; the stop codon is presented as asterisk (*).

it was still associated with 13.0% of the cases and the co-existence of PCV2a with PCV2b in the same pig may facilitate the interaction and recombination of PCV2a and PCV2b genotypes in the USA field.

To further characterize the prevalence of the PCV2 strains in the field, we determined the main PCV2 clades (or subgroups) by phylogenetic analysis of partial ORF2 sequences with different clade reference sequences reported previously (Olvera *et al.*, 2007). We found that all the PCV2a nucleotide sequences belonged to the 2E clade and all the PCV2b sequences belonged to the 1B clade. In a previous report on genotypic analysis of PCV2 strains in the swine herds of the USA, the PCV2a strains were included in 2D and 2E clades, and PCV2b in 1A clade (Cheung *et al.*, 2007). It should be pointed out that the 1B clade classification in our study is related to the reference strain we selected. Since there were no amino acid differences in Cap between 1A and 1B clades (Olvera *et al.*, 2007), the 1B clade can be considered the same as 1A or 1A/B. In contrast to the USA, 1A and 1C clades for PCV2b, 2D and 2E for PCV2a have been reported in Republic of Korea (Chae & Choi, 2010); 1 A/B and 1C for PCV2b was reported in Thailand (Jantafong *et al.*, 2011). Up to now, the possible relationship between pathogenicity and PCV2 clades is unclear.

Cap serves as a protective antigen (Blanchard *et al.*, 2003; Fenaux *et al.*, 2004; Shen *et al.*, 2008) and serological marker (Mahé *et al.*, 2000; Truong *et al.*, 2001) for PCV2. Distinct amino acid sequences have been suggested as signature motifs for PCV2a and PCV2b genotypes (Cheung *et al.*, 2007). In the present study, the putative amino acid sequences revealed that 100% (74/74) of the PCV2b contained the signature motif of SNPRSV, and 90.9% (10/11) of the PCV2a contained the signature motif of TNKISI at positions 86–90. However, 9.1% (1/11) of PCV2a had PCV2b substitution (T to S) at position 86 in the signature motif. In addition, we found PCV2a amino acid substitutions at positions 30, 76 and 131 in PCV2b Cap, and PCV2b amino acid substitutions at position 134 in PCV2a. These substitutions may be caused by possible recombination between PCV2a and PCV2b or mutations. Position 30 was located in one of the two dominant regions (22–41 and 71–86) (Wen *et al.*, 2005), and positions 76, 131 and 134 were located in the variant regions (Larochelle *et al.*, 2002; Wang *et al.*, 2009) as well as antigenic domains (Lekcharoensuk *et al.*, 2004; Mahé *et al.*, 2000; Truong *et al.*, 2001). Since PCV2 is subject to selective pressure exerted by the pre-existing acquired immunity in the population, it can be deduced that these selected amino acid substitutions between PCV2a and PCV2b are favourable

for viral evasion from the immunity elicited by the currently used vaccines. In a previous study, a transition of amino acid from T to P at position 151 of Cap was found to be due to positive selection (Firth *et al.*, 2009); however, we did not identify mutations at this position in our study. Previous studies also suggest that the immunoreactive regions of Cap of PCV2 are potential candidate regions involved in the emergence of novel PCV2 variants (Larochelle *et al.*, 2002; Wellenberg *et al.*, 2000; Wen *et al.*, 2005).

In the present study, PCV1 DNA was detected in 2.7% of the serum samples and 2.0% of the PCVAD cases. This result is consistent with an investigation in USA finishing swine herds in 2006 prior to PCV2 vaccination where only 2.4% of sera from 185 farms were positive for PCV1 by PCR (Puvanendiran *et al.*, 2011). For detection of possible PCV1 and PCV2 recombination and vaccine derivatives, we utilized a real-time PCR method that is capable of detecting chimeric PCV1–2a or PCV1–2b DNA containing PCV1 ORF1 and PCV2 ORF2 that originated from either PCV2a or PCV2b. PCV1–2a DNA was not identified in any of the samples, suggesting that this kind of recombination between PCV1 ORF1 and PCV2 ORF2 was rare or undetectable in the USA field. In 2010, PCV1–2a containing the ORF1 of PCV1 and the ORF2 of PCV2a was identified in Canada with a prevalence of 2.5% (Gagnon *et al.*, 2010). The origin of PCV1–2a was thought to be the result of natural genetic recombination between PCV1 and PCV2a or a virus originating from a chimeric inactivated vaccine strain (Gagnon *et al.*, 2010). In the present study, we had 310 serum samples confirmed to be from pigs (with ages of 10–23 weeks) vaccinated with an inactivated chimeric PCV2 vaccine (Suvaxyn PCV). The negative results for PCV1–2a chimeric DNA on these serum samples and on all other serum samples collected from non-vaccinated farms or farms that used a different PCV2 vaccine in addition to the negative results for PCV1–2a chimeric DNA on all tissue samples collected from PCVAD pigs suggested that this vaccine is not likely to cause emergence of a PCV1–2a recombinant virus in the USA. However, it is difficult to compare the absence of PCV1–2a in the current survey to the presence of PCV1–2a reported in Canada (Gagnon *et al.*, 2010), due to the differences in pig ages, sample types and laboratory methods between the two studies and different batches of the Suvaxyn PCV vaccine used in these two countries.

PCV2 Cap is capable of inducing PCV2 neutralizing antibody (McNeilly *et al.*, 2001; Shen *et al.*, 2008; Zhou *et al.*, 2005). The antigenetic diversity of PCV2 Cap was demonstrated by differentiation of different PCV2 strains

by certain mAbs (Lefebvre *et al.*, 2008; Shang *et al.*, 2009). In challenge models, 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 (Opriessnig *et al.*, 2008b, 2010). The live-attenuated chimeric PCV2 vaccine based on subtype PCV2a was found to be effective in reducing PCV2b viraemia (Opriessnig *et al.*, 2011a; Shen *et al.*, 2010b). And vice versa, the live-attenuated chimeric PCV2b vaccine is effective in producing protective immunity against PCV2a infection (Beach *et al.*, 2010; Opriessnig *et al.*, 2011b). All these results suggest that cross-protection between PCV2a and PCV2b exists. However, the present study indicated that the vaccinated herds still had higher level of PCV2b viraemia than the non-vaccinated herds. This observation may suggest a need for a new generation of PCV2 vaccines based on the PCV2b genotype or both PCV2a and 2b. In addition, we detected exclusive 1B and 2E clades in this study. Based on this, we speculate that introducing vaccines based on the strains and the same clade will increase the vaccine efficacy against PCV2a or PCV2b infection. The effect of amino acid substitutions in Cap on the pathogenicity and antigenicity of the virus is still unclear. Therefore, further studies need to be performed to better understand the importance of these observations.

METHODS

Samples. A total of 1675 serum samples from 61 USA sites (with one to five barns on each site and a total of 105 barns) located in seven States (IA, IL, IN, MO, NC, ND and OH) were obtained in groups of 5–97 samples for each barn (mean: 16.2 samples, CI: 13.5, 19.1). These samples were either selected as a convenience sample based on availability through the Iowa State University (ISU) Veterinary Diagnostic Laboratory (VDL) or submitted directly by participating veterinarians for the purpose of this study. The age of the pigs ranged from 1 to 26 weeks for 69.4% (1162/1675) of the serum samples in addition to 26.1% (438/1675) samples with no detailed age information; only 4.5% (75/1675) samples were collected from pigs of 1–9 or 27–30 weeks of age.

If PCV2 vaccination and product information was available, this was included in the investigation. The PCV2 vaccines utilized in the USA at and before the time of sample collections included Suvaxyn PCV (now reformulated and available as Fostera PCV; Pfizer Animal Health, Inc.), Ingelvac CircoFLEX (Boehringer Ingelheim Vetmedica, Inc.) and Circumvent PCV (Merck Animal Health, Inc.). While Suvaxyn PCV can be administered in a one (2 ml) or two dose (1 ml twice) fashion, Ingelvac CircoFLEX is licensed for one dose administration (1 ml) and Circumvent PCV is licensed for two dose administration (2 ml twice) in the USA. All of these vaccines are licensed for usage in healthy pigs 3 weeks and older and have been available in the USA since 2006. The age of vaccinated pigs from which serum samples were collected ranged from 12 to 25 weeks (mean \pm SEM, 18.1 \pm 0.2) for Ingelvac CircoFLEX and 10–23 weeks for both Suvaxyn PCV (mean \pm SEM, 17.2 \pm 0.2) and Circumvent PCV (mean \pm SEM, 11.9 \pm 0.2). In addition to the serum samples, 100 lung tissue samples from 75 sites (1–5 samples for each farm) located in 13 USA states (CO, IA, IL, IN, MD, MI, MN, MO, NC, ND, OK, UT and WI) were obtained through the ISU-VDL from pigs diagnosed with PCVAD based on histopathology using general requirements such as microscopic lesions associated with high amounts of PCV2 antigen as

determined by immunohistochemistry. The age of the pigs ranged from 5 to 23 weeks (11.9 \pm 0.6) for tissue samples, with 33.0% (33/100) of the pigs having no available age information. All the serum and tissue samples were collected between November 2010 and June 2011.

DNA extraction. DNA was extracted from the serum samples or tissue homogenates using an automated extraction machine (King Fisher Flex 96 Ambion; Thermo Scientific) and the MagMAX-96 isolation kit (Ambion) according to the manufacturer's instructions. Serum samples were subjected to extraction directly, whereas tissue samples were processed prior to extraction as follows: tissue samples were mechanically homogenized in sterile Earle's medium (Sigma-Aldrich) at a ratio of 1 g to 3 ml by using a stomacher (Seward Stomacher 80; Seward) for 120 s. The homogenates were centrifuged at 3200 g for 30 min at 4 °C and the supernatant was transferred into 5 ml tubes. The tubes were stored at –20 °C until the day of the extraction.

Quantitative differential real-time PCR for PCV2a and PCV2b.

Quantitative differential PCR for PCV2a and PCV2b was performed in a multiplex manner using the same primers and probe as described previously (Shen *et al.*, 2010a). Probes for PCV2a and PCV2b were synthesized and labelled with CAL Fluor Orange 560 and Quasar 670 (Biosearch Technologies), respectively. The multiplex real-time PCR consisted of a total volume of 25 μ l containing 12.5 μ l of the commercially available master mix (TaqMan Universal PCR master mix; PE Applied Biosystems), 2.5 μ l extracted sample DNA, 1 μ l (0.4 μ M) of each of the two primers, 0.5 μ l (0.2 μ M) of each of the two probes and 7 μ l of DNase/RNase-free water. The reactions were carried out in a 7500 Fast Real-Time PCR system (ABI) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The results were expressed as the number of PCV2 genomic copies ml⁻¹. Serial dilutions of PCV2a and PCV2b genomic DNA clones were used to generate standard curves that were demonstrated to have a correlation coefficient of more than 0.90. The sensitivity of the differential PCR for PCV2a and PCV2b was 1.6 \times 10³ PCV2 DNA copies ml⁻¹ (four copies per reaction) for both PCV2a and PCV2b. There were no cross-reactions with PCV1, PCV1–2a and between PCV2a and PCV2b.

Real-time PCR for PCV1, chimeric PCV1–2a and PPV.

PCV1 (Shen *et al.*, 2011), PCV1–2a (Shen *et al.*, 2010b) and PPV (Shen *et al.*, 2010b) real-time PCR assays were performed as described previously. PCV1 real-time PCR was performed in a non-quantitative manner with a correlation coefficient of more than 0.90 in serial dilutions of PCV1 viral genomic DNA; there were no cross-reactions with PCV2a, PCV2b and PCV1–2a. Five progressive 10-fold dilutions of a PCV1–2a chimeric DNA clone were used to generate a standard curve, which had a correlation coefficient of more than 0.99. The sensitivity of the PCV1–2a real-time PCR is 8.13 \times 10⁴ copies ml⁻¹ (204 copies per reaction); the specificity test revealed that this assay had no cross-reactions with PCV1, PCV2a, PCV2b or PPV. Serial dilutions of a recombinant plasmid containing the PPV VP2 gene were used for obtaining a standard curve that had a correlation coefficient of more than 0.99. The specificity of the PPV PCR was evaluated and there were no cross-reactions with PCV1 and PCV2, and the detection limit of this assay was found to be 10³ copies ml⁻¹ (2.5 copies per reaction).

PCV2 sequencing. Part (667 bp) of the PCV2 ORF2 was amplified with P2ORF2F (5'-CTCTGAATTGTACATACATGGTTACACGGA-3') and P2ORF2R (5'-ATGACGTATCCAAGGAGCGGTTA-3') as forward and reverse primers, respectively (Gagnon *et al.*, 2010). The PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems) in 25 μ l mixtures containing 1.25 U (0.25 μ l) *Taq* DNA

polymerase (Invitrogen), 0.2 mM dNTP, 0.4 μ M each of the primers, 1.5 mM MgCl₂ and 4 μ l DNA extract. The cycling conditions were 5 min at 95 °C, followed by 35 cycles of 40 s at 95 °C, 40 s at 55 °C and 1 min at 72 °C and finally extension at 72 °C for 7 min. The amplified PCR products were separated by gel electrophoresis on 1% agarose gel (Amresco) and visualized by UV irradiation after ethidium bromide staining of the gel. The specific products were sequenced at the ISU DNA facility, Ames, Iowa.

Phylogenetic analysis. Sequences of 555 nt (9–563 nt) from PCV2 ORF2 were aligned by the CLUSTAL W method available in the MEGA 5.05 software (<http://www.megasoftware.net/>). Distance computation was expressed as the number of base substitutions per site between sequences by the Maximum Composite Likelihood model in MEGA 5.05. Nucleotide sequences with distances of less than 0.005 in pairwise comparisons (which means nearly identical or identical sequences, and limited to a maximum of 3 nt differences and one putative amino acid difference) were represented as one sequence and used in phylogenetic analysis. Sequences were aligned with the PCV2a, 2b and 2c genotype references (Segalés *et al.*, 2008) and 1A to 1C (in PCV2b genotype) and 2A to 2E (in PCV2a genotype) clade references (Olvera *et al.*, 2007). The nucleotide distance of sequences was evaluated by a neighbour-joining (NJ) tree using MEGA 5.05 (Kumar *et al.*, 2001). Confidence in the NJ tree was estimated by 1000 bootstrap replicates.

Statistical analysis. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) using the SAS software version 9.2.0 (SAS Institute). Real-time PCR results (copies ml⁻¹ of serum) were log₁₀ transformed prior to statistical analysis. For log-transformed real-time PCR results, linear mixed models were used for analysis. Site and barn (nested within site) were used as random effects and sample type (serum and tissue), vaccine status (vaccinated and non-vaccinated) or vaccine product (Ingelvac CircoFLEX, Suvaxyn PCV and Circumvent PCV) were used as fixed effect. For binary prevalence analyses, mixed effect logistic regression models were used. A *P*-value of less than 0.05 was set as a statistically significant level throughout this study.

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