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Novel chimeric porcine circovirus (PCV) with the capsid gene of the emerging PCV2b subtype cloned in the genomic backbone of the non-pathogenic PCV1 is attenuated *in vivo* and induces protective and cross-protective immunity against PCV2b and PCV2a subtypes in pigs

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ABSTRACT

Porcine circovirus type-2b (PCV2b) is the primary global causative agent of porcine circovirus-associated disease (PCVAD). In this study, we first constructed a novel chimeric virus (PCV1-2b) with the PCV2b capsid gene cloned into the backbone of non-pathogenic PCV1. A pathogenicity study conducted in caesarean-derived colostrum-deprived pigs showed that pigs inoculated with PCV1-2b ($n=10$) had decreased lymphoid lesions and significantly lower viral load at 21 dpi, and significantly lower viremia starting at 14 dpi compared to pigs inoculated with PCV2b ($n=10$). All PCV1-2b infected pigs remained clinically healthy, while four of ten PCV2b-infected pigs died or were euthanized early due to clinical PCVAD. In a subsequent challenge study, conventional pigs were first vaccinated with PCV1-2b ($n=20$) or left unvaccinated ($n=20$), and 10 pigs in each group were then challenged with PCV2a and PCV2b, respectively. Vaccinated pigs had no detectable viremia and significantly decreased overall lymphoid lesion scores and lower viral loads compared to unvaccinated controls. The results indicate the chimeric PCV1-2b virus is a good candidate for a live-attenuated vaccine against both PCV2b and PCV2a subtypes.

1. Introduction

Porcine circovirus (PCV) is a small, non-enveloped DNA virus which belongs to the family Circoviridae [1]. Type 1 PCV (PCV1) was discovered as a contaminant of the porcine kidney PK-15 cell line in the mid-seventies, and is non-pathogenic in pigs [2]. In 1997, a variant strain of PCV, designated PCV type 2 (PCV2), was discovered in piglets with postweaning multisystemic wasting syndrome (PMWS) in Canada [3–6]. As more and more clinical conditions are linked to PCV2 infection, the American Association of Swine Veterinarians approved the name porcine circovirus associated disease (PCVAD) in 2006 which includes clinical manifestations such as wasting, respiratory signs, enteritis, reproductive failure and porcine dermatitis and nephropathy syndrome (PDNS) [7]. PCV2 is currently considered to be one of the most economically important viral pathogens in pig populations globally [8]. Observation of severe clinical PCVAD in conventional pigs experimentally infected with PCV2 alone is uncommon, and coinfection with other swine pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV) or porcine parvovirus (PPV) is usually required to induce the full-spectrum of clinical PCVAD [7,9–12]. However, infection of caesarean-derived, colostrum-deprived (CD/CD) pigs with PCV2 alone has resulted in severe clinical PCVAD and mortality [13–17]. Several comprehensive reviews of the pathogenesis, immunology, and molecular biology of PCV2 are available [7,8,18–23].

Although the genomic organization of the pathogenic PCV2 and the non-pathogenic PCV1 is similar, they share only approximately 68–76% nucleotide sequence identity in the genome [24–26] and differences in transcriptional patterns and antigenic profile of the capsid protein have been reported [27–29]. The two major genes encoded by the viral genome include the 942 bp replicase (rep) gene [30] and the 702 bp capsid gene [31]. The rep gene is conserved between PCV1 and PCV2 with about 83% nucleotide sequence identity while the capsid gene shares only about 67–70% identity [18]. Currently, at least three subtypes have been identified worldwide:

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PCV2a, PCV2b, and PCV2c [32,33]. PCV2a and PCV2b have both been associated with clinical PCVAD of varying degrees of severity [34–39]. Prior to 2005, only PCV2a was found in the United States and Canada, while both PCV2a and PCV2b were present in Europe and China [33,40]. Since 2005, PCV2b strains were recognized in the United States and there has been a global shift in subtype resulting in a dominant prevalence of PCV2b in pig populations, concurrently with increased severity of clinical PCVAD [33,39–46]. The pathogenicity of PCV2c is unclear, as it has only been reported in non-diseased herds in Denmark [33].

The current available commercial vaccines are all killed or recombinant vaccines based upon the PCV2a subtype [7,23]. We have successfully developed an inactivated vaccine, Suvaxyn PCV2® One dose™, based upon the PCV1-2a chimeric virus (with the capsid gene of PCV2a in the backbone of PCV1) [47–49]. However, since the PCV2b has now become the globally dominant subtype, and since PCV2a and PCV2b differ by as much as 10% nucleotide sequence identity [50,51], it is unknown whether the current PCV2a subtype-based vaccines provide complete protection against the PCV2b subtype. Several studies have demonstrated effectiveness of current commercial vaccines against PCV2b challenge [52–54]. Our recent work has demonstrated that a PCV2a-based modified live-attenuated vaccine (MLV) induces as good protective immunity as the commercial inactivated or subunit vaccines [57]. However, since cell-mediated immunity also plays an important role in PCV2 protection [47,48,55,56], a MLV, which is not currently available, would thus be a more preferable vaccine.

In this study, we first generated an infectious DNA clone of PCV2b subtype, and then constructed a novel chimeric virus, PCV1-2b, containing the immunogenic capsid gene of the PCV2b subtype in the genomic backbone of the non-pathogenic PCV1. The pathogenicity and immunogenicity of the chimeric PCV1-2b virus were first evaluated in CD/CD pigs. Subsequently, a challenge and cross-challenge study was performed in conventional pigs to determine the vaccine efficacy of the PCV1-2b chimeric vaccine virus. We demonstrated that the chimeric PCV1-2b virus is attenuated in pigs and induces protective immunity against PCV2b and cross-protective immunity against PCV2a. Therefore, this new chimeric PCV1-2b virus would be an excellent candidate as a MLV against both PCV2b and PCV2a.

2. Materials and methods

2.1. Cells

A subclone of the PK-15 cell line that is free of PCV1 contamination was produced previously by end-point dilution of the PK-15 cells (ATCC CCL-33) [51,58], and used for the generation of infectious virus stocks and infectivity titration of the virus stocks in this study.

2.2. PCV1 and PCV2 virus isolates

The PCV1 infectious DNA clone was constructed in previous studies and shown to be non-pathogenic in pigs [47,48,58]. PCV2a isolate ISU-40895 (GenBank accession no. AF264042) was recovered from a pig with PCVAD in an Iowa farm in 1998 [51] and has been used extensively in PCV2 pathogenicity studies [47,48,58–63]. PCV2a-40895 is capable of causing PCVAD microscopic lesions and clinical disease in experimental conditions [59,60,62]. The PCV2b strain used in the study was confirmed to be an authentic PCV2b subtype by sequencing of the entire viral genome (GenBank accession no. GU799576). The genomic DNA of the PCV2b was used as the source for the construction of the infectious DNA clones of PCV2b and chimeric PCV1-2b. The pathogenicity of the PCV2b virus was not determined, prior to this study, in experimental infections.

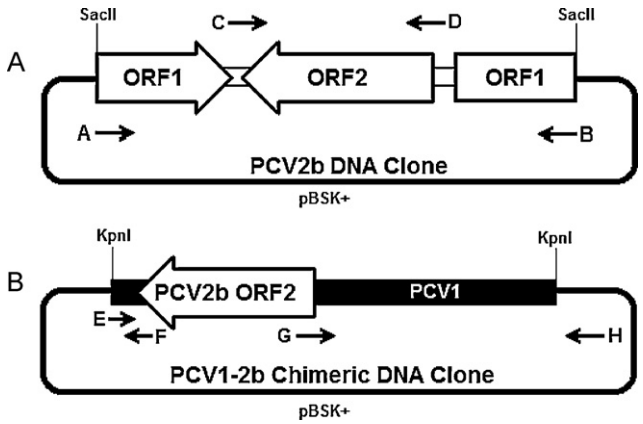

Fig. 1. Construction and genomic organization of full-length PCV2b and chimeric PCV1-2b DNA clones. (A) PCV2b monomeric DNA clone. Complete genome of wild-type PCV2b was cloned in pBSK+ vector using unique Sac II site after amplification with PCR primers A and B (Table 1). (B) Chimeric PCV1-2b monomeric DNA clone. Chimeric PCV1-2b DNA clone was constructed by overlap extension PCR using PCR primers C-H (Table 1).

2.3. Generation of infectious DNA clones of PCV2b and chimeric PCV1-2b

The method for the construction of the infectious DNA clone of PCV2a-40895 has been reported previously [58], and a similar approach was used in this study to produce an infectious DNA clone of PCV2b (Fig. 1A). Briefly, the full-length genome of PCV2b was amplified by PCR using a pair of primers A and B (Table 1) with an overlapping region containing the Sac II restriction enzyme site. The PCR product was then digested with Sac II (New England Biolabs) and ligated into pBluescript II SK(+) (pBSK+) (Stratagene) to produce an infectious DNA clone of PCV2b.

To produce the chimeric PCV1-2b infectious DNA clone, overlap-extension PCR was used to replace the PCV1 capsid gene in the backbone of a PCV1 clone with the capsid gene from PCV2b (Fig. 1B). The full-length chimeric PCV1-2b genome was assembled from three overlapping PCR fragments (Table 1). Fusion PCRs consisting of two steps were used to assemble the chimeric PCV1-2b DNA clone: an assembly reaction without primers using 50 ng of each fragment as template (20 cycles of 95 °C 30 s, 55 °C 30 s, 68 °C 1 min) followed by amplification using outer primers (40 cycles of 95 °C 30 s, 55 °C 30 s, 68 °C 1 min). The 1043 bp fragment amplified from a PCV1 clone with primers G and H (Table 1) was first fused with the 718 bp fragment containing the entire capsid gene amplified from PCV2b with primers C and D (Table 1). The 122 bp fragment

Table 1
Oligonucleotide primers used in the construction of infectious DNA clones of PCV2b and chimeric PCV1-2b.

Primer	Sequence (5′ → 3′)	Amplicon size (bp)
A	TTT CCG CGG GCT GGC TGA ACT TTT GAA AG	1779
B	AGC CCG CGG AAA TTT CTG ACA AAC GTT AC	
C	CGT AAT GGT TTT TAT TTT TAA GGG TTA AGT GG	718
D	CTT TCA CTT TTA TAG GAT GAC GTA TCC AAG GAG G	
E	TTC GGG TAC CCG AAG GCC GAT T	122
F	CAC TTA ACC CTT AAA AAT AAA AAC CAT TAC GAT	
G	CCT CCT TGG ATA CGT CAT CCT ATA AAA GTG AAA G	1043
H	CAG TGG ATC CCC CGG GCT GCA GGA	

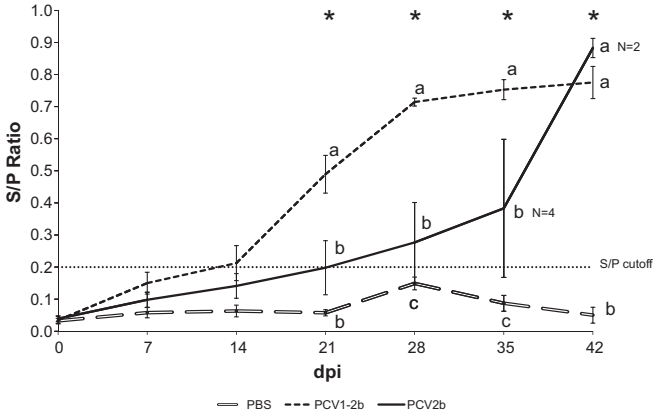

Fig. 2. PCV2 capsid-specific antibody response in caesarean-derived colostrum-deprived (CD/CD) pigs experimentally inoculated with the chimeric PCV1-2b virus and the wildtype PCV2b virus. The mean serum S/P ratio ± SEM is plotted for each treatment group throughout the experiment, with (*) indicating significant differences on that day. Due to death or early euthanasia of some PCV2b-infected pigs, fewer than five pigs were sampled in that group at 35–42 dpi. Groups with different letters are significantly different on that day.

amplified from PCV1 with primers E and F (Table 1) was then added, resulting in a complete chimeric PCV1-2b genome flanked by Kpn I restriction sites. The chimeric fusion product was subsequently digested with Kpn I (New England Biolabs), and cloned into pBSK+. Monomeric DNA clones were completely sequenced to confirm that no unwanted mutations had been introduced during PCR amplification steps. Previous studies showed that dimerized PCV2a clones with two copies of full-length PCV2a genome ligated head-to-tail in tandem are more efficient in generating infectious virus in both *in vitro* transfection in PK-15 cells and *in vivo* transfection in pigs [47,48,58]. Therefore, in this study both PCV2b and PCV1-2b clones were dimerized to produce more robust and efficient infectious clones.

2.4. Generation and titration of infectious PCV2a, PCV2b, and PCV1-2b virus stocks

To prepare inocula for the *in vivo* pig studies, infectious virus stocks were generated for PCV2a-40895, PCV2b, and chimeric PCV1-2b by transfection of PK-15 cells in T25 flasks with Lipofectamine LTX (Invitrogen) using dimerized infectious DNA clones [47,58]. The titration of these infectious virus stocks by IFA was performed in 48-well plates (BD-Falcon) essentially as described previously [58]. The 50% tissue culture infective dose ($TCID_{50}$) per ml was calculated according to the method of Reed and Muench.

2.5. Experimental design for the pathogenicity study of PCV2b and chimeric PCV1-2b in caesarean-derived colostrum-deprived (CD/CD) pigs

A total of 30 CD/CD pigs (Struve Labs, Manning, IA), approximately 9 weeks of age, were randomly assigned to three groups in rooms of 10 animals each. Prior to inoculation, each pig was weighed, bled, and confirmed to be negative for the presence of PCV2 antibodies (Fig. 2). Group 1 pigs were each mock-inoculated with 3 ml PBS buffer (2 ml intranasally and 1 ml intramuscularly) and served as uninfected controls. Pigs in group 2 were each inoculated with 3 ml of inoculum containing $10^{4.8}$ $TCID_{50}$ chimeric PCV1-2b virus (2 ml intranasally and 1 ml intramuscularly). Pigs in group 3 were each similarly inoculated with $10^{4.8}$ $TCID_{50}$ wildtype PCV2b virus. Blood samples were collected prior to inoculation, and weekly thereafter from each pig until necropsy at 21 or 42 days post-inoculation (dpi). At 21 dpi, five randomly assigned pigs from each group were weighed and necropsied. The remaining five pigs in each group were weighed and necropsied at 42 dpi.

2.6. Vaccination of specific-pathogen-free (SPF) pigs with chimeric PCV1-2b followed by challenge and cross-challenge of vaccinated pigs with wildtype PCV2b and PCV2a, respectively

A total of 40, 3-week-old, cross-breed SPF pigs were purchased from a commercial farm (Virginia Tech Swine Center, Blacksburg, VA) that is known to be free of PCV, PRRSV, PPV, *M. hyopneumonia*, and swine hepatitis E virus. The piglets were randomly assigned to two groups of 20 pigs each, and housed separately. Prior to inoculation, each pig was weighed, bled, and confirmed to be negative for PCV2 antibodies. Group 1 pigs were each vaccinated intramuscularly (IM) with 1 ml of the chimeric PCV1-2b virus ($10^{3.5}$ $TCID_{50}$ per pig). Group 2 pigs were each mock-vaccinated IM with 1 ml PBS buffer and served as unvaccinated controls. All animals were monitored daily for clinical signs, and blood samples were collected prior to inoculation, and weekly thereafter from each pig through 56 days post-vaccination (dpv). At 56 dpv, the 20 vaccinated pigs were weighed and further divided into two groups of 10 pigs each and housed in separate rooms: 10 vaccinated pigs were each challenged with $10^{4.8}$ $TCID_{50}$ wildtype PCV2b virus (2 ml intranasally and 1 ml IM), and the other 10 vaccinated pigs were each similarly cross-challenged with $10^{4.8}$ $TCID_{50}$ wildtype PCV2a virus. The 20 unvaccinated control pigs were also further divided into two groups of 10 pigs each, and similarly inoculated with PCV2b and PCV2a, respectively. Blood samples were collected weekly through 21 days post-challenge (dpc) (or 77 dpv), at which time all pigs were weighed and necropsied.

2.7. Serology

ELISA was used to detect anti-PCV2 IgG in each serum sample as previously described [64]. All pigs were confirmed to be PCV2 seronegative by ELISA prior to the start of the animal experiments.

2.8. Clinical evaluation

Following inoculation, vaccination or challenge, pigs were evaluated daily for clinical signs of PCVAD including wasting, respiratory distress, and behavioral changes such as lethargy and inappetence.

2.9. Gross pathology and histopathology

Necropsies were performed at the designated time for the pathogenicity (dpi 21 and 42) or challenge experiment (dpc 21 or dpv 77) on all pigs in a blinded fashion. Estimates of macroscopic lung lesions (ranging from 0 to 100% of the lung affected) and lymph node size (ranging from 0 [normal] to 3 [four times the normal size]) were made and scored for each pig [62,65].

Sections of lung, lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), tonsil, heart, thymus, ileum, kidney, colon, spleen, and liver were collected during each necropsy and processed routinely for histological examination and immunohistochemistry (IHC). Also, samples of tracheobronchial lymph node (TBLN) were collected from each pig for DNA extraction and quantification of viral genomes by real-time PCR. Microscopic lesions in the lungs, heart, liver, kidney, ileum, and colon were scored in a blinded manner, as described previously [62]. Lymph nodes, spleen, and tonsil were evaluated based on lymphoid depletion and histiocytic replacement of follicles, ranging from 0 (normal) to 3 (severe) [62].

2.10. Immunohistochemistry (IHC)

IHC for detection of PCV2-specific antigen was performed on formalin-fixed, paraffin-embedded sections of lung, lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), tonsil, heart, thymus, ileum, kidney, colon, spleen, and liver using a rabbit polyclonal antiserum [66]. The scores of PCV2 antigen in each tissue were estimated in a blinded fashion ranging from 0 (no antigen) to 3 (greater than 50% lymphoid follicles contained cells with positive PCV2 antigen staining in lymphoid tissues or high amount of PCV2 antigen in other tissue sections) [62].

2.11. Overall microscopic lymphoid lesion scores

The average scores of the overall microscopic lymphoid lesions were calculated for each pig as described previously [62]. These lesion scores are based on the combined lymphoid depletion (LD), histiocytic replacement (HR), and PCV2 antigen present in the lymphoid tissues as determined by IHC.

2.12. Quantitative real-time PCR to determine viral DNA loads

Total DNA was extracted from serum samples using the QIAamp DNA minikit (Qiagen Inc.) according to the "blood and body fluids" protocol supplied by the manufacturer. TBLN tissues collected during necropsies were homogenized to produce a 10% tissue homogenate suspension in sterile PBS buffer, and total DNA was extracted using the QIAamp DNA minikit with the "tissue" protocol supplied by the manufacturer (Qiagen Inc.). All TBLN DNA extracts were diluted at least 1:100 in sterile H_2O in order to eliminate background fluorescence from SYBR green binding to porcine genomic DNA. Two SYBR green I-based qPCR assays were modified for use in this study to quantify the PCV2 genomes present in TBLN and serum DNA extracts [67,68].

In the CD/CD pig pathogenicity study, we utilized a previously published qPCR assay that amplifies part of PCV2b capsid gene to quantify viral genomes present in serum and TBLN [68]. The protocol was modified to enable the use of the Sensimix SYBR and fluorescein kit (Quantace). Each 25 μl reaction contained 200 nM each primer (P1: 5′-ATAACCCAGCCCTTCTCCTACC-3′; P2: 5′-GGCCTACGTGGTCTACATTTCC-3′), 200 μM dNTP, 3 mM $MgCl_2$, and 5 μl DNA extract. Triplicate reactions were run for each sample in MyIQ qPCR thermocycler (BioRad) using a modified program (95 °C 10 min; 35 cycles of 95 °C 15 s, 59 °C 30 s, 72 °C 30 s). Viral genomes were quantified against duplicate standards of PCV2b infectious DNA clone using the relative C_t method, with a 6-log linear range of quantification (10^3 to 10^8 copies).

In the challenge and cross-challenge conventional SPF pig study, we used a previously published qPCR assay that amplifies a conserved region of PCV2 replicase gene region to quantify the amounts of viral genomes present in serum and TBLN tissues [67]. This assay does not detect the chimeric PCV1-2b vaccine virus used in the vaccination, and thus allowing for accurate quantification of the challenge PCV2a or PCV2b viruses only. This protocol was modified to enable the use of the Sensimix SYBR and fluorescein kit. Each 25 μl reaction contained 200 nM each primer (PCV2-83F: 5′-AAAAGCAAATGGGCTGCTAA-3′; PCV2-83R: 5′-TGGTAACCATCCCACCACTT-3′), 200 μM dNTP, 5 mM $MgCl_2$, and 5 μl DNA extract. Triplicate reactions for each sample were run in a MyIQ qPCR thermocycler using a modified program (95 °C 10 min; 35 cycles of 95 °C 15 s, 60 °C 15 s, 72 °C 15 s). Viral DNA genomes were quantified against duplicate standards of PCV2b infectious DNA clone using the relative C_t method, with a 5-log linear range of quantification (5×10^2 to 5×10^6 copies).

2.13. Sequence confirmation of virus detected by qPCR

DNA extracts of TBLN tissues from selected pigs in each group were tested to confirm the virus detected by qPCR was the same virus that was inoculated into pigs. PCV1-2b in the CD/CD pathogenicity study was confirmed by PCR amplification and partial sequencing using primers specific for PCV1-2b (PCV2F: 5′-TGTTGAAGATGCCATTTTTCC-3′; PCV1R: 5′-GAGGAGTTCTACCCTCTTCC-3′). PCV2a and PCV2b were amplified and sequenced using primers specific for PCV2 (PCV2F: 5′-TGTTGAAGATGCCATTTTTCC-3′; PCV2R: 5′-GAGGTGTTCGTCCTTCCTCA-3′).

2.14. Statistical analysis

Serology and serum qPCR were analyzed using repeated measures analysis of variance (ANOVA). TBLN qPCR from the CD/CD pathogenicity study was analyzed using simple ANOVA. For all the ANOVA models simple effects comparisons were investigated using the slice option of the Glimmix procedure followed by Tukey's procedure for multiple comparisons. TBLN qPCR results from the challenge study were analyzed using the exact Wilcoxon 2-sample test. Scores from lymphoid lesions, histopathology lesions, and IHC from both experiments were assessed with the exact Kruskal–Wallis one-way ANOVA, followed by the exact Wilcoxon 2-sample test for the two-way comparisons of interest. The two-way comparisons were adjusted for multiple comparisons using Bonferroni's procedure. Statistical significance was set to alpha = 0.05. All analyses were performed using commercially available software (SAS version 9.2, Cary, NC, USA).

3. Results

3.1. PCV2b and chimeric PCV1-2b DNA clones are infectious when transfected into PK-15 cells

Full-length single copy and tandemly dimerized DNA clones of PCV2b and chimeric PCV1-2b were constructed and verified by full-length sequencing. Transfection of PK-15 cells with dimers of both DNA clones resulted in the production of infectious progeny virions as detected by IFA with PCV2 capsid-specific monoclonal antibodies. The infectious titers of both PCV2b and chimeric PCV1-2b virus stocks were approximately $10^{4.5}$ $TCID_{50}$/ml.

3.2. Chimeric PCV1-2b virus is attenuated in CD/CD pigs whereas the wildtype PCV2b virus induces pathological lesions and clinical diseases characteristic of PCVAD

To definitively assess the pathogenic potential of the chimeric PCV1-2b virus, the pathogenicity study was conducted in a CD/CD pig model which has been shown to be the most reproducible and highly sensitive PCVAD disease model [14–17].

3.2.1. Clinical signs and gross lesions

CD/CD pigs experimentally inoculated with chimeric PCV1-2b virus or PBS buffer had no apparent clinical signs of PCVAD throughout the study, whereas inoculation of CD/CD pigs with wildtype PCV2b resulted in the PCVAD-associated death or early euthanasia in four of the 10 PCV2b-infected pigs: one pig died at 18 dpi, another died at 27 dpi, and two other pigs had to be euthanized at 34 dpi due to progressive weight loss.

Pigs from each of the three treatment groups had similar weight gain through 21 dpi, but the wildtype PCV2b-infected pigs had a decrease in weight gain in four of the five pigs after 42 dpi (three of which died or were euthanized early due to weight loss). Macroscopic lung lesions were not observed in pigs inoculated with the

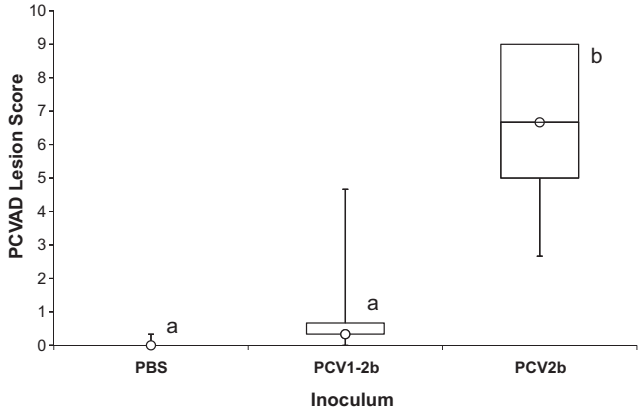

Fig. 3. Comparison of the overall lymphoid lesion scores at 21 dpi in the caesarean-derived colostrum-deprived (CD/CD) pigs inoculated with PBS buffer, chimeric PCV1-2b virus, and wildtype PCV2b virus. Combined lymphoid depletion, histiocytic replacement, and PCV2-specific antigen scores for the lymph nodes, spleen, and tonsil were compared between treatments. The PCV2b-inoculated pig that died at 18 dpi was included in the analysis. Circles indicate median, boxes are 1st and 3rd quartiles, and whiskers indicate total range of data. Treatments with different letters are significantly different.

chimeric PCV1-2b virus, whereas the three PCV2b-inoculated pigs necropsied at 27 dpi and 34 dpi displayed moderate lung lesions. Lymph nodes were enlarged in both PCV1-2b and PCV2b infected pigs compared to the PBS controls. However, the number of pigs with enlarged lymph nodes and the magnitude of enlargement was less in PCV1-2b-infected pigs than in wildtype PCV2b pigs. At 21 dpi, 3/5 PCV1-2b-infected pigs had enlarged lymph nodes (mean score = 1.2), compared to 4/5 wildtype PCV2b-infected pigs (mean score = 1.8). At 42 dpi, 2/5 PCV1-2b pigs had enlarged lymph nodes (mean score = 0.6), compared to 3/5 wildtype PCV2b pigs (mean score = 1.2).

3.2.2. Microscopic lesions

Microscopic lesions were analyzed by treatment in two groups: all pigs necropsied at 21 dpi or before (including the PCV2b-infected pig that died at 18 dpi), and all pigs necropsied at 42 dpi (including the three PCV2b-infected pigs that died or were euthanized at 27 dpi and 34 dpi).

Microscopic lesions in various tissues are summarized in Table 2. There were no remarkable microscopic lesions in pigs inoculated with PBS buffer. Characteristic PCV2-associated microscopic lesions in lymphoid tissues in pigs inoculated with chimeric PCV1-2b virus were decreased in incidence and severity compared to the pigs experimentally inoculated with the wildtype PCV2b at both 21 and 42 dpi (Table 2). Microscopic lesions in non-lymphoid tissues included mild to severe infiltration with lymphocytes and macrophages (Table 2). Lesions in the small and large intestine were found almost exclusively in wildtype PCV2b-infected pigs.

The mean group overall lymphoid lesion score in pigs inoculated with the chimeric PCV1-2b virus was significantly ($p = 0.045$) lower than that in pigs inoculated with the wildtype PCV2b virus and was not different from pigs inoculated with PBS buffer at 21 dpi (Fig. 3).

3.2.3. PCV2 serology

Serum samples taken from pigs that died or were euthanized early were analyzed along with those from the next scheduled necropsy (e.g. serum from the pig that died at 18 dpi was included in the analysis of samples from 21 dpi necropsy). PCV2 capsid-specific IgG antibodies were detected in the sera of some PCV1-2b-infected pigs as early as 7 dpi, with seroconversion in 9/10 pigs by 21 dpi (Fig. 2). In pigs experimentally inoculated with wildtype PCV2 virus,

Table 2
Distribution of histopathological lesions in tissues of caesarean-derived colostrum-deprived (CD/CD) pigs experimentally inoculated with PBS buffer, PCV1-2b chimeric virus, and wildtype PCV2b virus.

dpi	Group	Inoculum	No. of pigs with lesions/no. examined (mean score)											
			Lung	Liver	Thymus	Heart	Kidney	Ileum	Colon	Lymph nodes		Spleen		Tonsil	
										LD[a]	HR[b]	LD[a]	HR[b]	LD[a]	HR[b]
21/nec[c]	1	PBS	3/5 (0.8)	1/5 (0.4)	0/5	0/5	0/5	0/5	0/5	1/5 (0.2)[I]	0/5[I]	0/5	0/5	0/5[I]	0/5[I]
	2	PCV1-2b	4/5 (1.0)	3/5 (1.0)	1/5 (0.2)	0/5	2/5 (0.6)	1/5 (0.2)	0/5	2/5 (0.6)[I,II]	1/5 (0.4)[I,II]	1/5 (0.2)	1/5 (0.2)	1/5 (0.2)[I,II]	1/5 (0.4)[I,II]
	3	PCV2b	5/5 (2.8)	5/5 (2.4)	2/5 (0.6)	1/5 (0.2)	3/5 (0.8)	4/5 (2.0)	3/5 (1.0)	5/5 (2.6)[II]	5/5 (2.2)[II]	4/5 (2.0)	4/5 (1.8)	5/5 (2.4)[II]	5/5 (2.2)[II]
42/nec[c]	1	PBS	0/5	0/5[I]	0/5	0/5	0/5[I]	0/5	0/5	0/5[I]	0/5	0/5	0/5	0/5	0/5
	2	PCV1-2b	4/5 (1.0)	4/5 (0.8)[I,II]	0/5	3/5 (0.6)	3/5 (1.6)[I,II]	1/5 (0.2)	0/5	2/5 (0.4)[I,II]	1/5 (0.4)	0/5	0/5	1/5 (0.2)	1/5 (0.2)
	3	PCV2b	3/5 (2.0)	5/5 (1.4)[II]	2/4[d] (0.75)	1/5 (0.2)	5/5 (2.6)[II]	4/5 (1.6)	4/5 (1.0)	5/5 (2.2)[II]	4/5 (2.0)	3/5 (1.2)	3/5 (1.0)	4/5 (2.0)	3/5 (1.8)

[I, II]Groups that have statistically significant differences in group median score have different numerals.
[a] Lymphoid depletion.
[b] Histiocytic replacement.
[d] Thymus was not present in one of the pigs tested.
[c] Pigs that died or were euthanized early due to PCVAD were included in the analysis.

the seroconversion was less uniform: only 3/10 pigs were seropositive by 21 dpi, and 5/10 pigs had no detectable seroconversion in the study. However, only 2/5 PCV2b-infected pigs survived to the scheduled necropsy at dpi 42, and both of them did seroconvert at 28 dpi. Of the pigs that died or were euthanized early due to clinical PCVAD, only 1/4 had detectable PCV2-specific antibody at any time, and none had detectable serum anti-PCV2 antibody on the day they were necropsied. No PCV2 capsid-specific antibodies were detected in any of the PBS buffer-inoculated pigs throughout the study.

3.2.4. Prevalence and amount of PCV2-specific antigen in tissues by IHC

Prevalence and mean group amounts of PCV2 antigen in different tissues are summarized in Table 3. All pigs necropsied at 21 dpi or before (including the PCV2b-infected pig that died at 18 dpi) were analyzed together, and all pigs necropsied at 42 dpi (including the three PCV2b-infected pigs that died or were euthanized at 27 dpi and 34 dpi) were analyzed together. In general, the incidence and amount of PCV2 antigen were less in pigs inoculated with the chimeric PCV1-2b virus compared to the pigs inoculated with the wildtype PCV2b virus (Table 3). There was a single PCV1-2b-inoculated pig that accounted for the positive results in all 21 dpi tissues except the tonsil, where four of five pigs were positive. Tissues from PCV1-2b-inoculated pigs were mostly negative at 42 dpi, with the exception of the lymph nodes, in which low amounts of PCV2 antigen were detected in four of five pigs.

3.2.5. PCV2 viremia and serum viral loads

The PCV2 viral load in the sera of infected animals was determined using a modified qPCR assay that is known to work with both PCV2b and PCV1-2b [68]. All serum samples taken prior to inoculation at day 0 and from PBS buffer-inoculated pigs throughout the study were confirmed to have no detectable PCV2 DNA. Serum samples taken from pigs that died or were necropsied early due to PCVAD disease were analyzed along with those from the next scheduled necropsy.

The serum viral loads in pigs inoculated with the chimeric PCV1-2b virus were significantly ($p \leq 0.009$) lower than that in PCV2b-inoculated pigs from 14 dpi through the end of the study (Fig. 4A). The mean serum viral loads peaked at 10^7 genomic copies/ml at 14 dpi in PCV1-2b-infected pigs. The maximum value achieved for any individual PCV1-2b-infected pig was 10^8 genomic copies/ml at 14 dpi. In contrast, the mean serum viral loads in PCV2b-infected pigs remained above 10^8 genomic copies/ml from 14 dpi until after 28 dpi, with 10^{12} being the maximum value achieved by an individual pig. The four pigs that died early or were euthanized due to PCVAD had higher levels of serum viral loads than the other PCV2b pigs. After 7 dpi, PCV2b-infected pigs had at least ten-fold greater viral genomic copies per ml of serum than the PCV1-2b-infected pigs, and one hundred-fold greater at 21 dpi and 28 dpi.

3.2.6. PCV2 viral load in lymphoid tissues

All TBLN tissue samples from PBS buffer-inoculated pigs were negative for PCV2 DNA. In order to enable a meaningful comparison of group mean viral loads at 21 dpi and 42 dpi, pigs that died early or were euthanized due to PCVAD disease were tested but not included in the analysis. The viral load present in each mg of TBLN tissue was found to be significantly lower in PCV1-2b-inoculated pigs than in PCV2b-inoculated pigs at 21 dpi ($p = 0.005$) (Fig. 4B). Pigs that died or were euthanized early due to PCVAD had the highest viral loads in TBLN tissues among all that were tested.

Table 3
Immunohistochemical detection of PCV2 capsid-specific antigen in caesarean-derived colostrum-deprived (CD/CD) experimentally inoculated with PBS buffer, PCV1-2b chimeric virus, and PCV2b wildtype virus.

dpi	Group	Inoculum	No. of pigs positive/no. tested (mean score)									
			Lung	Liver	Thymus	Heart	Kidney	Ileum	Colon	Lymph nodes	Spleen	Tonsil
21/nec[a]	1	PBS	0/5[I]	0/5[I]	0/5	0/5	0/5	0/5	0/5	0/5[I]	0/5	0/5[I]
	2	PCV1-2b	0/5[I]	1/5 (0.2)[I,II]	1/5 (0.2)	0/5	1/5 (0.2)	1/5 (0.2)	1/5 (0.2)	1/5 (0.4)[I,II]	1/5 (0.2)	4/5 (1.0)[I,II]
	3	PCV2b	5/5 (1.6)[II]	5/5 (1.4)[II]	4/5 (1.6)	1/5 (0.2)	2/5 (0.4)	4/5 (2.0)	3/5 (1.6)	5/5 (2.2)[II]	4/5 (1.6)	5/5 (2.4)[II]
42/nec[a]	1	PBS	0/5	0/5	0/5	0/5	0/5	0/5	1/5 (0.2)	0/5[I]	0/5	0/5[I]
	2	PCV1-2b	0/5	0/5	0/5	0/5	1/5 (0.2)	0/5	0/5	4/5 (0.8)[I,II]	1/5 (0.2)	1/5 (0.2)[I,II]
	3	PCV2b	3/5 (1.6)	3/5 (1.4)	2/4[b] (1.5)	1/5 (0.2)	4/5 (1.8)	4/5 (1.8)	3/5 (1.2)	5/5 (2.2)[II]	3/5 (1.4)	5/5 (2.2)[II]

[I,II] Groups that have statistically significant differences in group median score have different numerals.
[a] Pigs that died or were euthanized early due to PCVAD were included in the analysis.
[b] Thymus was not present in one of the pigs tested.

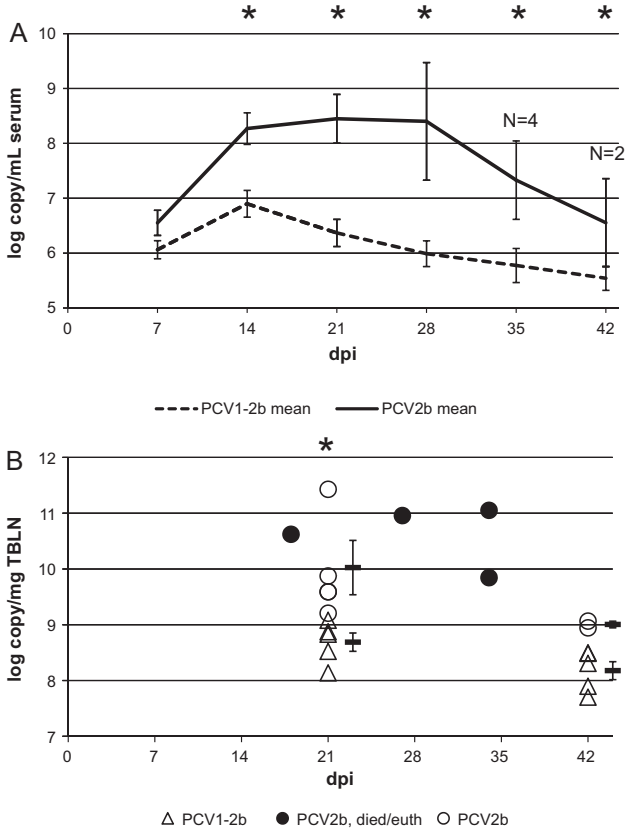

Fig. 4. Detection and quantification of viral DNA loads in serum and lymphoid tissues in caesarean-derived colostrum-deprived (CD/CD) pigs experimentally inoculated with the chimeric PCV1-2b virus and PCV2b virus. (A) Quantification of viral DNA loads in sera using qPCR. Group mean log viral genomic copies/ml of serum ± SEM is plotted for each treatment group, with (*) indicating significant differences on that day. All samples at 0 dpi as well as all samples from PBS-inoculated pigs were negative. Due to death or early euthanasia of some PCV2b-infected pigs, fewer than five pigs were sampled in that group at 35–42 dpi. (B) Quantification of viral DNA load in TBLN tissues using qPCR. Lymph node tissue viral DNA loads determined for each pig are plotted on the day they died or were euthanized, with group means ± SEM plotted at 21 and 42 dpi. All samples from PBS-inoculated pigs were negative, and PCV2b-infected pigs that died or were euthanized early due to PCVAD (filled circles) were not included in the analysis of group means. The (*) indicates significant differences on that day.

3.3. Vaccination with chimeric PCV1-2b virus confers protective immunity in conventional pigs against challenge with PCV2b subtype and cross-challenge with PCV2a subtype

Since the chimeric PCV1-2b vaccine is intended for usage in conventional pigs in the field, we thus conducted this vaccine efficacy and challenge study using conventional SPF pigs.

3.3.1. Clinical signs and gross lesions

None of the pigs developed clinical signs consistent with PCVAD throughout the study, and no difference in weight gain was observed between treatment groups. A single pig assigned to the unvaccinated/PCV2b challenge group died at 2 dpc from bacterial septicemia unrelated to PCVAD, and was not included in the analysis. Upon necropsy, macroscopic lung lesions were not observed in any of the treatment groups. Mild to moderately enlarged lymph nodes were seen in all treatment groups, with no significant differences observed between treatments.

3.3.2. Serology

All pigs were determined to be negative for antibodies against PCV2, PRRSV, PPV, and swine influenza virus (SIV) prior to the study.

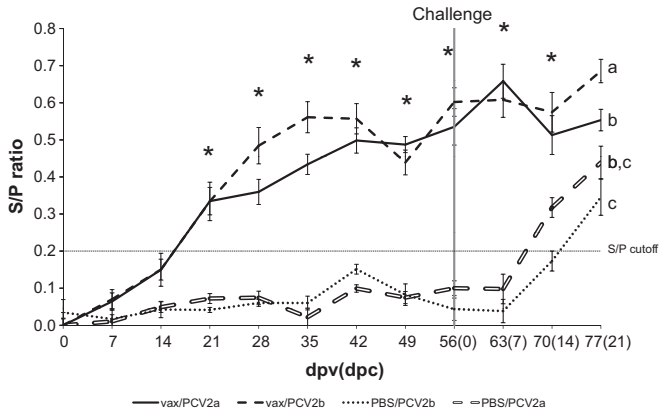

Fig. 5. PCV2-specific antibody response in conventional specific-pathogen-free pigs vaccinated with the chimeric PCV1-2b virus and subsequently challenged with PCV2a or PCV2b wildtype virus. The mean serum S/P ratio ± SEM is plotted for each treatment group throughout the experiment, with (*) indicating significant differences between vaccinated (vax) and unvaccinated (PBS) groups. All pigs were challenged with PCV2a or PCV2b at 56 dpv. Groups with different letters at 77 dpv (or 21 dpc) are significantly different on that day.

PCV2 capsid-specific antibodies were detected in the sera of PCV1-2b-vaccinated pigs as early as 14 dpv, with seroconversion occurred in 15/20 pigs at 21 dpv and 20/20 by 28 dpv (Fig. 5). The PCV2 IgG antibody titers peaked by 35 dpv and remained high at the time of challenge at 56 dpv. Antibodies to PCV2 were not detected in unvaccinated control pigs until after challenge at 14 dpc (Fig. 5): 10/10 PCV2a-challenged pigs were seropositive, compared to only 2/9 seropositive PCV2b-challenged pigs at 14 dpc. By 21 dpc, 7/9 unvaccinated PCV2b-challenged pigs had seroconverted.

3.3.3. Microscopic lesions

In general, the characteristic lymphoid depletion and histiocytic replacement scores were lower in vaccinated pigs compared to the unvaccinated controls (Table 4). The overall lymphoid lesion score in vaccinated pigs was significantly lower than that in unvaccinated pigs for both PCV2a ($p = 0.0001$) and PCV2b ($p = 0.04$) challenge groups (Fig. 6).

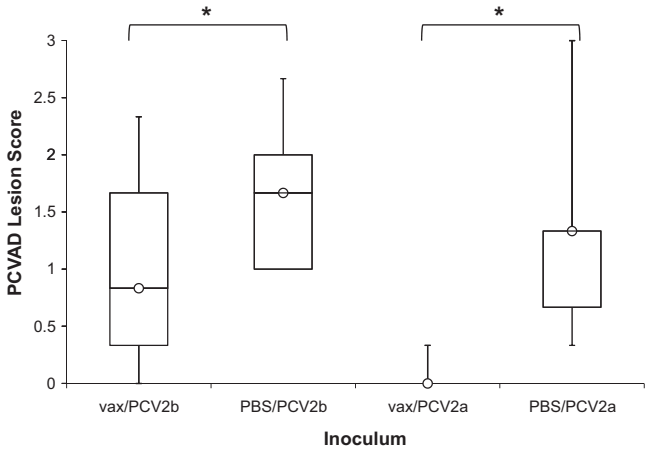

Fig. 6. Comparison of overall lymphoid lesion scores in conventional SPF pigs vaccinated with PCV1-2b or PBS buffer and subsequently challenged with PCV2a or PCV2b. Combined lymphoid depletion, histiocytic replacement, and PCV2-specific antigen scores for the lymph nodes, spleen, and tonsil were compared between vaccinated and unvaccinated pigs by challenge PCV2 virus subtype. Circles indicate median, boxes are 1st and 3rd quartiles, and whiskers indicate the range of data. Pairs of treatments with (*) are significantly different.

Table 4
Microscopic lesions and PCV2-specific antigen in lymphoid tissues during necropsy in conventional pigs vaccinated with PBS buffer or chimeric PCV1-2b candidate vaccine and subsequently challenged with PCV2a or PCV2b wildtype viruses.

Group	Inoculum (0 dpv)	Challenge (56 dpv)	No. of pigs with lesions/no. examined (mean score)								
			Lymph nodes			Spleen			Tonsil		
			LD[a]	HR[b]	IHC[c]	LD[a]	HR[b]	IHC[c]	LD[a]	HR[b]	IHC[c]
1	PCV1-2b	PCV2b	8/10 (0.9)[I]	3/10 (0.3)[I]	2/10 (0.2)	7/10 (0.7)	5/10 (0.5)	1/10 (0.1)	2/10 (0.2)	0/10	0/10
2	PBS	PCV2b	9/9 (1.7)[II]	9/9 (1.6)[II]	6/9 (0.8)	5/9 (0.6)	2/9 (0.3)	0/9	0/9	0/9	1/9 (0.1)
3	PCV1-2b	PCV2a-40895	0/10[I]	0/10	0/10[I]	0/10[I]	0/10	0/10	0/10	1/10 (0.1)	0/10
4	PBS	PCV2a-40895	9/10 (1.1)[II]	3/10 (0.4)	7/10 (1.1)[II]	6/10 (0.6)[II]	0/10	0/10	2/10 (0.2)	0/10	4/10 (0.4)

[I,II] Pairs of treatments that have statistically significant differences in group median score have different numerals.
[a] Lymphoid depletion.
[b] Histiocytic replacement.
[c] Detection of PCV2-specific antigen by immunohistochemistry (IHC); no. of pigs with positive IHC/no. examined (mean IHC antigen score).

3.3.4. Amount of PCV2 antigen by IHC in tissues

Similarly to the histological lesions, in general the incidence and the amount of PCV2 antigen in tissues were reduced in vaccinated pigs compared to unvaccinated controls (Table 4).

3.3.5. Viremia and serum viral load

The amounts of PCV2a or PCV2b viral DNA in the serum after challenge were quantified using a modified qPCR assay that is specific for the detection of PCV2a or PCV2b rep gene but is not capable of amplifying the vaccine virus PCV1-2b [67]. Serum samples collected prior to challenge at 56 dpv had no detectable PCV2 DNA. There was no detectable PCV2a or PCV2b viremia in vaccinated pigs at any time point post-challenge. In contrast, PCV2a or PCV2b viremia was detected in all unvaccinated pigs at every time point post-challenge (Fig. 7A). There was no statistically significant difference in serum viral load between unvaccinated pigs challenged with PCV2a or PCV2b virus.

3.3.6. PCV2a or PCV2b viral load in lymphoid tissues

The viral loads in each mg of TBLN tissues were found to be significantly lower in vaccinated pigs compared to unvaccinated ones in both PCV2a ($p = 0.0001$) and PCV2b ($p < 0.0001$) challenge groups (Fig. 7B). Only 1/10 of the vaccinated and PCV2b challenged pigs had detectable PCV2b viral DNA in the TBLN tissues, with a viral load of 10^5 genomic copies/mg. Five of ten vaccinated and PCV2a challenged pigs had detectable PCV2a viral DNA in the TBLN tissues, each with a viral load of 10^5 to 10^6 genomic copies/mg. The viral load in the TBLN tissues of the unvaccinated pigs ranged from 10^8 to 10^{10} genomic copies/mg, regardless of PCV2a or PCV2b challenge.

4. Discussion

PCV2 infection and PCVAD continue to pose a major threat to swine industry worldwide. PCVAD arguably is the most economically important disease facing the swine industry today. Wasting, microscopic lesions of lymphoid depletion with histiocytic infiltration, and the presence of PCV2 antigen or DNA in the lesions are three characteristic criteria to diagnose PCVAD in a pig [21]. It is known, however, that not all pigs infected with PCV2 will develop clinical PCVAD and coinfecting viral and bacterial pathogens are usually necessary to induce the full-spectrum of clinical PCVAD [69,70]. Prior to 2005, the virus detected from PCVAD cases in the United States and Canada was almost exclusively of the PCV2a subtype. Consequently, four commercial vaccines all based on the PCV2a subtype were developed and are currently used worldwide. The PCV2a strains isolated worldwide are closely related and share 95–99% nucleotide sequence identities, and thus these commercial vaccines have been effective against PCV2a and PCVAD.

Recently, the outbreaks of more severe PCVAD cases in certain areas in the United States and Canada have been attributed to the emergence of a new PCV2b subtype [45]. The nucleotide sequences between PCV2a and PCV2b subtypes differ by as much as 10% and distinct amino acid sequence motifs distinguishing the two subtypes have been identified [41], thus raising the question whether

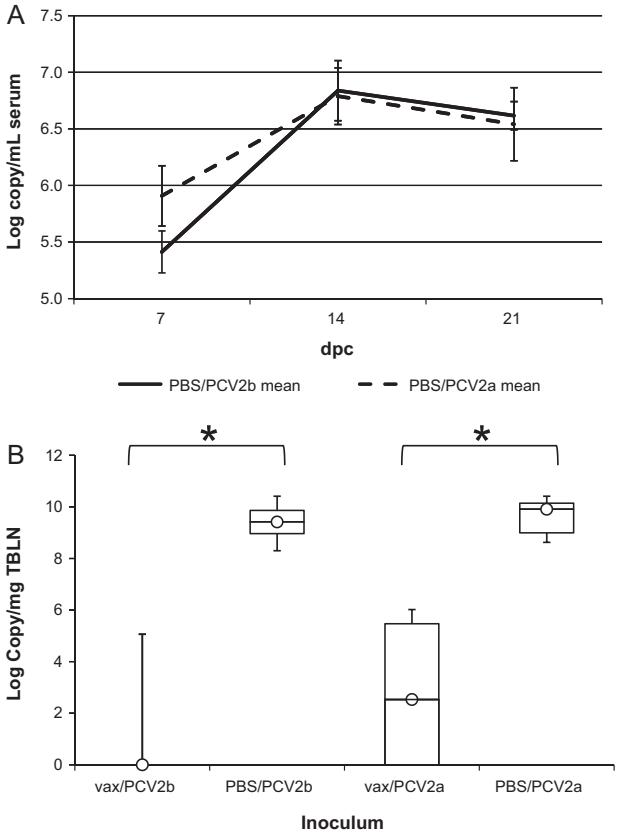

Fig. 7. Detection and quantification of PCV2 viral DNA loads in serum and lymphoid tissue samples in conventional pigs vaccinated with PBS buffer or PCV1-2b and subsequently challenged with PCV2a or PCV2b. (A) Quantification of viral DNA loads in sera using qPCR. Group mean log viral genomic copies/ml of serum ± SEM is plotted for each treatment group. All samples at 56 dpv (or 0 dpc) as well as all samples from vaccinated pigs were negative for PCV2a or PCV2b DNA. (B) Quantification of viral DNA loads in TBLN tissues using qPCR. Median viral DNA loads in lymphoid tissues determined for each pig are plotted. Circles indicate median, boxes are 1st and 3rd quartiles, and whiskers indicate the total range of data. Upon challenge with PCV2a or PCV2b, viral DNA was detected in the lymph nodes of all unvaccinated pigs, while only 1/10 vax/PCV2b pigs and 5/10 vax/PCV2a pigs had detectable viral DNA. The (*) indicates significant differences between vaccinated and unvaccinated pairs, based on challenge virus subtype.

or not the current commercial vaccines based exclusively on PCV2a subtype can fully protect against the new PCV2b subtype infection. In the past few years, the PCV2 prevalence in swine herds has shifted to predominantly PCV2b subtype, and in fact, the majority of recent PCVAD cases in the United States are associated with the PCV2b subtype [41,71]. The PCV2a subtype-based vaccines are still in use worldwide since the PCV2a vaccines have been shown to provide cross-protection [52–54,72]. However, a MLV will likely offer better protection than the current subunit or inactivated vaccines. Also, it makes sense for any new vaccine to be based on the currently circulating predominant PCV2b subtype. Therefore, the objective of this study was to develop a new generation modified live-attenuated vaccine based on the new PCV2b subtype, and to evaluate the efficacy of the PCV2b-based vaccine against both PCV2a and PCV2b challenges.

For use of the chimeric PCV1-2b virus as a live-attenuated vaccine, it is important to ascertain that the vaccine virus is attenuated. Though PCV2 alone rarely causes full-spectrum clinical disease in experimental models, the use of CD/CD pigs has resulted in successful reproduction of clinical PCVAD [9,14–17]. Therefore, in this study in order to definitively determine the pathogenicity of the chimeric PCV1-2b virus and compare it to its parental wildtype PCV2b virus, we chose the CD/CD pig model for the pathogenicity study. As expected, the wildtype PCV2b alone caused severe clinical PCVAD in CD/CD pigs, resulting in the death or early euthanasia due to clinical manifestation of PCVAD in 4/10 PCV2b-infected pigs. This is consistent with previous studies that have shown 25% mortality and significant clinical PCVAD in PCV2-infected CD/CD pigs [14,16,17]. The overall lymphoid lesion scores for PCV2b-infected pigs were consistent with moderate-to-severe systemic PCVAD [7]. The generally accepted threshold for viremia in severe PCVAD cases is 10^7 viral genomic copies per ml of serum, though host variation prevents a single definitive diagnostic cut-off [73–75]. By 21 dpi, 9/10 PCV2b-infected pigs were above this threshold, compared to only 1/10 chimeric PCV1-2b virus-infected pigs. The 4 pigs that died or were euthanized earlier due to PCVAD all had higher PCV2 DNA viral loads in serum and lymphoid tissues but had no detectable PCV2 IgG antibodies, indicating that these PCV2b-infected pigs died because of insufficient immune responses and higher level of virus replication. This result is consistent with a previously reported correlation between low antibody response and increased severity of PCVAD [76]. The data clearly demonstrated that the PCV2b subtype used in this study is highly virulent.

The chimeric PCV1-2b virus was found to be significantly attenuated in the CD/CD pig model, despite the fact that the CD/CD pigs were inoculated with a dose that is at least 20-fold higher than the normal vaccination dose [48]. Attenuation of chimeric PCV1-2b virus was clearly demonstrated by quantitative and qualitative parameters that were used to compare the chimeric PCV1-2b and the wildtype PCV2b viruses. There was no mortality or morbidity seen in pigs infected with the chimeric PCV1-2b virus, compared to death and wasting seen in about half of the PCV2b-infected pigs. Histopathologic lesion scores and the amounts of PCV2-specific antigen in lymphoid tissues were consistently less in the chimeric PCV1-2b-infected pigs than in pigs infected with the wildtype PCV2b, indicating that the chimeric PCV1-2b virus causes only subclinical infection. Overall lymphoid lesion scores in chimeric PCV1-2b-infected pigs were not significantly different from the control pigs inoculated with PBS buffer. Additionally, lower PCV2b viral load in the serum and lymphoid tissues directly correlate to the significantly less characteristic lesions or disease severity in the chimeric PCV1-2b-infected pigs, as observed in previous studies [48,67,68,74,75,77–79]. Interestingly, the serum antibody levels in chimeric PCV1-2b virus-inoculated pigs were significantly higher at 28, 31, and 35 dpi when compared to wt PCV2b-inoculated pigs. The exact reason for this difference is unclear. However, since PCV2 replicates in lymphocytes and other immune cells, it is possible that the wt PCV2b may cause more damages to immune cells than the attenuated chimeric PCV1-2b virus, and thus resulting in a decreased level of immune response.

After demonstrating that the chimeric PCV1-2b virus is attenuated, we then conducted a combined immunogenicity and challenge study in conventional SPF pigs. Three-week-old conventional cross-breed SPF pigs were chosen for the immunogenicity/challenge experiment in order to more closely mimic field vaccination conditions since such a live-attenuated vaccine will be eventually used in conventional pigs. Though clinical PCVAD was not expected in this conventional SPF model based on our earlier published studies [26,35,47,48,58,80,81], it was anticipated that the level of viremia, viral loads and the characteristic histological lesions in lymphoid tissues induced by PCV2 in the conventional pig model would be sufficient parameters for evaluating vaccine efficacy [48]. As a live-attenuated vaccine, it is important to determine if chimeric PCV1-2b virus can induce sufficient level of protective antibody response upon vaccination of pigs. Two of the four currently available vaccines are based on recombinant PCV2a capsid proteins, and thus PCV2 capsid-specific humoral immune response is known to be important for protection. The results from this study showed that PCV2 capsid-specific antibodies were detected in the sera of PCV1-2b-vaccinated pigs as early as 14 dpv, and by 28 dpv all of the 20 vaccinated pigs had seroconverted to anti-PCV2 capsid-specific antibody. The antibody titers peaked by 35 dpv and remained high at the time of challenge at 56 dpv, indicating that the chimeric PCV1-2b vaccine virus is capable of eliciting a strong humoral immune response.

Upon challenge with either wildtype PCV2a or PCV2b subtype, pigs vaccinated with the attenuated chimeric PCV1-2b virus had significantly lower viral DNA loads in the serum and TBLN tissues, significantly decreased level of severity and incidence of characteristic microscopic lesions, and lower amounts of PCV2-specific antigen in lymphoid tissues compared to the unvaccinated controls, indicating that the chimeric PCV1-2b virus induced protective immunity against wildtype virus challenge. The results also showed that pigs vaccinated with the chimeric PCV1-2b virus were equally protected against homologous challenge with PCV2b subtype and heterologous challenge with the PCV2a subtype, as evidenced by the complete lack of PCV2a or PCV2b viremia, significant reduction in viral loads in lymphoid tissues, and significantly lower overall lymphoid lesion scores in vaccinated pigs compared to unvaccinated controls, regardless of challenge virus subtype. Therefore, it appears that the live-attenuated chimeric PCV1-2b vaccine candidate induces both protective and cross-protective immunity against both PCV2 subtypes.

Some recent studies have reported that, in general, the PCV2b subtype is associated with more severe clinical disease when compared to the PCV2a subtype [40,44,82]. However, it remains debatable whether or not the PCV2b subtype is more virulent than the PCV2a subtype, since other studies could not definitively show a significant difference in virulence between PCV2a and PCV2b [34–38]. Because of the sequence divergence between PCV2a and PCV2b, it is possible that the two subtypes of PCV2 may differ in pathogenicity since it has been shown that only two amino acid changes in the capsid gene were sufficient to alter the pathogenicity of PCV2a [80]. In the unvaccinated control group in the current study, where half of the pigs were challenged with PCV2a or PCV2b, we did not observe any significant difference in virulence between groups. The challenge doses for PCV2a and PCV2b were equivalent, but there was no significant difference in PCV2 viral loads in serum and TBLN tissues, the characteristic microscopic lesion scores, or the amounts of PCV2 antigen in lymphoid tissues between the PCV2a and PCV2b challenge groups. There were some minor differences in the antibody response, including a slightly delayed

seroconversion to PCV2b compared to PCV2a in the unvaccinated pigs (Fig. 5). In the vaccinated pigs, PCV2b challenge resulted in slightly greater overall lymphoid lesion scores while the PCV2a challenge resulted in a higher number of TBLN tissues positive by qPCR. Overall, the results are consistent with other studies that have found no significant difference in PCV2a and PCV2b pathogenicity under experimental conditions [34–38].

Differences in the antigenic profiles between PCV2a and PCV2b subtypes have been reported, and it has been speculated that a lack of sufficient level of cross-protection by the current PCV2a-based commercial vaccines may have contributed to an increase in PCVAD severity associated with the PCV2b subtype in pig populations worldwide [29,33,41,83]. Most of the sequence variations between PCV2a and PCV2b appear in the capsid gene, including a signature distinctive amino acid motif [41,50]. Antibodies raised against this motif are capable of differentiating between PCV2a and PCV2b *in vitro*, indicating possible differences in antigenicity (Beach et al., unpublished data). The results from this study showed that a live-attenuated vaccine, PCV1-2b, based on the capsid of the new PCV2b subtype does protect against heterologous challenge by PCV2a, thus supporting previous evidence of cross-protection of PCV2b subtype conferred by the PCV2a-based commercial vaccines [35,52–54].

In summary, the data from this study demonstrate that the chimeric PCV1-2b virus based on the new PCV2b subtype is attenuated in CD/CD pigs and induces protective and cross-protective immunity in vaccinated conventional pigs against both PCV2b and PCV2a challenge. The results will set the stage for further development of this chimeric PCV1-2b virus as the first live-attenuated vaccine against PCV2 infection and PCVAD. Although the PCV1-2b vaccine virus alone offered cross-protection against both PCV2a and PCV2b subtypes, it may be more advantageous in the future to combine the PCV1-2b vaccine from this study with the current PCV2a-based commercial vaccines for a more complete protection.

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