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A PCV2 vaccine based on genotype 2b is more effective than a 2a-based vaccine to protect against PCV2b or combined PCV2a/2b viremia in pigs with concurrent PCV2, PRRSV and PPV infection

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ABSTRACT

The predominant genotype of porcine circovirus (PCV) in the pig population today is PCV2b yet PCV2a-based commercial vaccines are considered effective in protecting against porcine circovirus associated disease. The objective of this study was to compare the ability of PCV2a- and PCV2b-based vaccines to control PCV2b viremia in a challenge model that mimics the U.S. field situation. Sixty-three pigs were randomly assigned to one of eight groups. Sixteen pigs were vaccinated with an experimental live-attenuated chimeric PCV1-2a vaccine based on genotype 2a and another 16 pigs with a chimeric PCV1-2b vaccine based on genotype 2b. Challenge was done 28 days post vaccination (dpv) using PCV2b (or a combination of PCV2a and PCV2b), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine parvovirus (PPV) to mimic what commonly occurs in the field. The experiment was terminated 21 days post challenge (dpc) or 49 dpv. Pigs vaccinated with the chimeric PCV1-2b vaccine had significantly higher levels of PCV1-2b viremia and shedding of the PCV1-2b vaccine virus in feces and nasal secretions but also a more robust humoral immune response as evidenced by significantly higher ELISA S/P ratios compared to the PCV1-2a vaccination. Regardless of challenge, the PCV1-2b vaccination significantly reduced the prevalence and amount of PCV2 viremia compared to the PCV1-2a vaccination. Interestingly, in the non-vaccinated pigs concurrent PCV2a infection resulted in clinical disease and increased macroscopic lung lesions compared to pigs challenged with PCV2b alone, further supporting the idea that concurrent PCV2a/PCV2b infection is necessary for optimal PCV2 replication.

1. Introduction

Porcine circovirus (PCV) type 2 (PCV2) was first recognized as a pathogen in pigs in the late 1990s [1]. Porcine circovirus associated disease (PCVAD) may manifest as diarrhea, wasting, pneumonia, systemic disease or reproductive failure [2]. In 2005–2006, a newly recognized PCV2 variant, genotype PCV2b, spread rapidly across North America [3] and a similar shift from PCV2a to PCV2b was identified concurrently around the world [4,5].

The increase in prevalence and severity of PCVAD led to the rapid development of control strategies [2,5,6]. PCV2 vaccines reduce clinical disease, viremia and lesions associated with PCV2 in the field [7–12] and under experimental conditions [13–16]. All current commercial PCV2 vaccines in the USA are based on PCV2a. Two are based on the capsid protein encoded by open reading frame (ORF) 2 expressed in the baculovirus system [6]. The other vaccine is based on a chimeric PCV1-2 using the ORF1 replicase gene from the non-pathogenic PCV type 1 (PCV1) as the genomic backbone and the ORF2 capsid gene of PCV2 [17,18].

The commercially available chimeric PCV1-2a vaccine is inactivated. An experimental live-attenuated chimeric PCV2 vaccine has been demonstrated to be effective for protection against PCV2 in growing pigs [18–21]. Serial passage of the live chimeric PCV1-2a in cell culture and pigs showed its genetic stability [22]. Although it has been demonstrated that initial infection with PCV2a confers cross protection against subsequent PCV2b challenge [23,24],

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a live-attenuated chimeric PCV1-2b vaccine based on genotype 2b has also been developed and demonstrated to be effective against PCV2b or PCV2a challenge [13,25,26].

The objective of this study was to determine if there are differences between PCV2a- and PCV2b-based vaccines in protecting against challenge with PCV2b or PCV2a/PCV2b in a model that mimics the U.S. field situation with concurrent porcine reproductive and respiratory syndrome virus (PRRSV) and porcine parvovirus (PPV) infection.

2. Materials and methods

2.1. Animals, housing, and experimental design

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee. Sixty-three, 14–35-day-old pigs were obtained from a swine herd free of PCV2, PRRSV and swine influenza virus. Breeding animals were vaccinated against PPV. At arrival, the pigs were blocked by age and randomly divided into eight treatment groups and rooms of 7–8 pigs, 32/63 pigs were vaccinated, and 28 days post vaccination (dpv) all pigs except negative controls were challenged with PRRSV, PPV and PCV2b (or PCV2a and PCV2b) (Table 1), and 21 days post challenge (dpc) all pigs were euthanized. Serum samples, nasal swabs and fecal swabs were collected weekly and stored at −80 °C until testing.

2.2. Clinical evaluation

Following challenge the pigs were individually monitored daily. Pigs were weighed at vaccination, challenge and necropsy.

2.3. Vaccination

The construction and *in vitro* and *in vivo* characterizations of the two experimental live-attenuated chimeric PCV1-2a and PCV1-2b vaccines have been described [13,17] and both have been shown to be attenuated and efficacious against PCV2 infection [13,18]. The chimeric PCV1-2a and PCV1-2b vaccines utilized in the study had an infectious titer of $10^{3.5}$ 50% tissue culture infective dose ($TCID_{50}$) per ml. Each vaccinated pig received 1 ml intramuscularly in the right neck area.

2.4. Challenge

PCV2a isolate ISU-40895 [17] and PCV2b isolate NC-16845 [24] were produced by transfection of the PCV-free PK-15 cells, reaching a final titer of $10^{4.5}$ $TCID_{50}$ per ml. PRRSV isolate ATCC VR2385 was propagated on MARC-145 cells. The 7th passage of the virus, with a titer of $10^{5.0}$ $TCID_{50}$ per ml was used [27]. PPV isolate NADL-8 was isolated in fetal porcine kidney cells from a naturally infected pig in 1977 [28] and was subsequently passaged in fetuses in the pregnant sow model. In this study, passage 4 of the PPV challenge virus stock was used at an approximate titer of $10^{4.9}$ $TCID_{50}$ per ml [26]. Each pig received 5 ml PCV2b (or 5 ml PCV2a and 5 ml PCV2b), 2.5 ml PRRSV and 1.0 ml PPV intranasally by slowly dripping each inoculum into both nostrils.

2.5. Serology

All serum samples were tested by an ORF2-based anti-PCV2 IgG ELISA [29]. Samples were considered positive if the sample-to-positive (S/P) ratio was equal to or less than 0.2 [29]. All serum samples collected at 0 and 21 dpc were tested for the presence of antibodies to PRRSV by a commercial ELISA (HerdChek* PRRS 2XR; IDEXX Laboratories Inc., Westbrook, MA, USA) in which S/P

Table 1
Experimental design, average daily weight gain (ADWG) in grams (se) from challenge (28 days post vaccination or dpv) to necropsy (21 days post challenge or dpc and 49 dpv), macroscopic (score range from 0 to 100% of the lung surface affected by lesions) and microscopic (score range from 0 to 6) lung scores, prevalence and mean log_{10} amount of PRRSV RNA ± se, prevalence of PPV DNA in serum, and prevalence of PCV2 antigen in tissues collected at 21 days post challenge. PCV1-2a or PCV1-2b vaccination was done on trial day 0 and challenge with a combination of PCV2a, PCV2b, PRRSV and PPV was done 28 dpv.

Group designation	Pig No.	Vaccination[a]	Challenge[b]	ADWG	Lung lesion scores[c]		PRRS RNA	PPV DNA	PCV2 antigen
					Macroscopic	Microscopic			
VAC/2a-Ch/2b-PRRSV-PPV	8 (7)[d]	PCV1-2a	PCV2b, PRRSV, PPV	536.8 ± 35.8	$15.9 \pm 6.3^{A,B}$	$3.6 \pm 0.4^{A,B,C}$	7/7 (4.73 ± 0.74)[A]	1/7	0/7
VAC/2b-Ch/2b-PRRSV-PPV	8	PCV1-2b	PCV2b, PRRSV, PPV	481.6 ± 78.8	$12.9 \pm 3.3^{A,B}$	$3.5 \pm 0.3^{A,B,C}$	7/8 (5.15 ± 0.85)[A]	1/8	1/8
Ch/2b-PRRSV-PPV	8	None	PCV2b, PRRSV, PPV	511.6 ± 45.7	29.3 ± 5.5^{A}	4.8 ± 0.3^{A}	5/8 (3.16 ± 0.95)[A,B]	1/8	8/8
Negative controls	7	None	None	580.4 ± 64.5	0.0 ± 0.0^{B}	1.0 ± 0.3^{D}	0/7 (0.00 ± 0.00)[B]	0/7	0/7
VAC/2a-Ch/2a-2b-PRRSV-PPV	8	PCV1-2a	PCV2a, PCV2b,PRRSV,PPV	494.9 ± 52.6	$16.0 \pm 3.7^{A,B}$	$3.0 \pm 0.5^{B,C}$	7/8 (5.14 ± 0.76)	1/8	2/8
VAC/2b-Ch/2a-2b-PRRSV-PPV	8	PCV1-2b	PCV2a,PCV2b, PRRSV, PPV	424.0 ± 76.6	24.0 ± 3.6^{A}	$3.4 \pm 0.3^{A,B,C}$	8/8 (5.45 ± 0.34)[A]	1/8	3/8
Ch/2a-2b-PRRSV-PPV	8	None	PCV2a, PCV2b, PRRSV, PPV	451.9 ± 59.7	52.3 ± 9.5^{C}	$4.6 \pm 0.5^{A,B}$	7/8 (4.76 ± 0.74)[A]	1/8	8/8
Ch/PRRSV-PPV	8 (7)[d]	None	PRRSV, PPV	568.9 ± 63.5	$13.0 \pm 1.8^{A,B}$	$2.5 \pm 0.3^{C,D}$	6/7 (5.17 ± 0.81)[A]	0/7	0/7

[a] Intramuscularly with 1 ml live-attenuated chimeric vaccine virus.
[b] Intranasally with 5 ml of PCV2b or 5 ml of PCV2b and 5 ml of PCV2a, 1 ml PPV, and 2.5 ml PRRSV.
[c] Different superscripts (A, B, C, D) within a column indicate significant ($p < 0.001$) differences between groups.
[d] Data from the VAC/2a-Ch/2b-PRRSV-PPV pig which was euthanized at 34 dpv (6 dpc) and the Ch/PRRSV-PPV pig which died on 14 dpc were excluded. The VAC/2a-Ch/2b-PRRSV-PPV pig had no recognizable macroscopic lung lesions and an interstitial pneumonia score of 1 while the Ch/PRRSV-PPV pig had a macroscopic lung lesion score of 78 and microscopic interstitial pneumonia score of 3.

ratios equal to or less than 0.40 were considered negative, and a hemagglutination inhibition assay for PPV as described [30].

2.6. Detection and quantification of viral nucleic acids

Viral DNA and RNA was extracted from the serum samples and swab suspensions using the MagMax™ viral isolation kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) on the KingFisher Flex System (ThermoFisher Scientific, Pittsburgh, PA, USA). Serum samples and swabs collected on 0, 7, 14, 21, 28, 35, 42, and 49 dpv were tested for presence and amount of PCV1-2 and serum samples and swabs collected on 0, 7, 14, and 21 dpc were tested for presence and amount of PCV2 DNA, PRRSV RNA, and PPV DNA. The PCV2 DNA was detected by a quantitative real-time PCR assay as described [31] except that a 25 μl total reaction volume and 2.5 μl of DNA extract were used in this study. The PCV2 real-time PCR targets PCV2-ORF1 (PCV2 ORF1 PCR, present in the challenge virus), and the PCV1-2 PCR target an overlapping region of ORF1 from PCV1 and ORF2 from PCV2 (present in the vaccine virus). This allows for differentiating vaccine virus (ORF1 PCV2 negative, PCV1-2 positive) from challenge virus (ORF1 PCV2 positive, PCV1-2 negative). All serum samples that were positive for PCV2 DNA at 21 dpc were further tested with a PCV2a/2b differential real-time PCR assay [32] targeting ORF2 (PCV2a/b ORF2 PCR) to determine the PCV2 genotype(s) present. In addition, quantitative real-time PCR for PPV DNA and PRRSV RNA were performed on 21 dpc serum samples as described [26]. Viral concentrations were expressed as the mean viral DNA or RNA copy number per ml of serum or swab suspension. Samples with no signal by a cycle-threshold (C_T) of 40 were considered negative.

2.7. Necropsy and macroscopic lesions

All pigs were euthanized by intravenous pentobarbital sodium (Fatal-Plus®; Vortech Pharmaceutical, Ltd., Dearborn, MI, USA) overdose on 49 dpv (21 dpc). The total amount of macroscopic lung lesions, ranging from 0% to 100%, was estimated and scored blinded to the treatment status as described [33]. Sections of the mediastinal, mesenteric, superficial inguinal, external iliac and tracheobronchial lymph nodes, spleen, tonsil and lungs were collected during necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histopathology.

2.8. Microscopic lesions and immunohistochemistry (IHC)

Lung tissue sections were scored for the presence and severity of interstitial pneumonia, with scores ranging from 0 (normal) to 6 (severe diffuse) [33]. Lymphoid tissues were evaluated for lymphoid depletion and histiocytic replacement of follicles with scores ranging from 0 (none) to 3 (severe) [34].

Detection of PCV2 antigen was performed by PCV2 IHC on formalin-fixed and paraffin-embedded sections of lymph nodes, spleen and tonsil using a rabbit polyclonal antiserum [35]. The PCV2 antigen scores ranged from 0 (negative) to 3 (more than 50% of the lymphoid follicles contain cells with PCV2 antigen staining). Scoring was done by a veterinary pathologist (TO) blinded to treatment status [34].

The overall microscopic lymphoid lesion score (lymphoid depletion, histiocytic inflammation, PCV2-antigen) was calculated as described [34], ranged from 0 to 9, and pigs were grouped into four categories: 0 (normal), 1–3 (mild), 4–6 (moderate), 7–9 (severe). The mean treatment group lymphoid score was compared between groups.

2.9. Statistical analysis

For data analysis, SAS® software version 9.2.0 (SAS Institute, Cary, NC, USA) was used. Summary statistics were calculated for groups to assess the distributional property and data that were not distributed normally (PCR data) were log transformed prior to analysis. The model to analyze continuous data collected over time (PCV2, PRRSV and PPV genomic copies and ELISA S/P ratios) was a repeated measures analysis of variance (ANOVA), where group, time and their interaction were fixed effects and pig was considered as random effect. Differences among the interacting groups in the repeated measures ANOVA were assessed using Tukey's *t*-test. A *p*-value of less than 0.05 was considered significant. Percent reduction for PCR data was calculated as described [20]. Non-parametric Kruskal–Wallis ANOVA was used on non-repeated data (histopathological and IHC scoring), and Wilcoxon tests were used to evaluate differences between pairs. Differences in prevalence were determined by using Fisher's exact test.

3. Results

3.1. Clinical disease and average daily weight gain

Clinical signs were not evident in any of the pigs until challenge. After challenge, most of the inoculated pigs developed mild respiratory disease characterized by occasional sneezing and increased respiratory rates. One VAC/2a-Ch/2b-PRRSV-PPV pig developed bloody diarrhea and was euthanized on 34 dpv (6 dpc). One Ch/PRRSV-PPV pig died after routine bleeding on 14 dpc due to massive hemorrhages in the neck area. One Ch/2a-2b-PRRSV-PPV pig developed severe respiratory distress, cyanosis, and inability to stand and was euthanized on 20 dpc.

From the time of vaccination to challenge between 0 dpv and 28 dpv, the average daily weight gain was not different ($p = 0.9039$) between non-vaccinated pigs (363.6 ± 27.9 g) or pigs vaccinated with PCV1-2a (342.2 ± 44.9 g) or PCV1-2b (347.0 ± 47.3 g). Similarly, there was no significant difference in average daily gain after challenge until necropsy between 28 dpv (0 dpc) and 49 dpv (21 dpc) (Table 1).

3.2. Serology

The prevalence of PCV2 seropositive pigs and mean group S/P ratios in the different groups throughout the trial period are summarized in Table 2. Pigs vaccinated with the PCV1-2b vaccine had a stronger and earlier anti-PCV2 IgG response compared to pigs vaccinated with the PCV1-2a vaccine. On 14 dpv, 81.3% (13/16) of the pigs vaccinated with PCV1-2b were seropositive whereas only 31.3% (5/16) of the pigs vaccinated with PCV1-2a had seroconverted to PCV2 (Table 2). PCV1-2b vaccinated pigs had significantly ($p < 0.05$) higher mean group S/P ratios compared to PCV1-2a vaccinated pigs at 14 dpv, 21 dpv, and 28 dpv. The Ch/2a-b-PRRSV-PPV pig that had to be euthanized had not seroconverted to PCV2.

All pigs were anti-PRRSV IgG negative at the day of PRRSV challenge and the majority (94.4%; 51/54) of the pigs challenged with PRRSV had seroconverted by 21 dpc (data not shown). The negative control pigs had no detectable anti-PRRSV antibodies at 21 dpc. The pigs were positive for PPV antibodies at the day of PPV challenge and remained seropositive until 21 dpc. Pigs challenged with PPV had higher titers at 21 dpc compared to the negative control pigs (data not shown).

3.3. Viremia and virus shedding

Chimeric vaccine viremia was detected in 10/16 pigs vaccinated with the PCV1-2a vaccine and 16/16 pigs vaccinated with

Table 2
Prevalence of anti-PCV2 IgG antibodies in sera and mean group sample-to-positive (S/P) ratios on different trial days. Vaccination was done on day 0, challenge was done 28 days after vaccination (dpv) and the pigs were necropsied at 49 dpv (21 days after challenge or dpc). Data presented as prevalence (mean S/P ratio ± se). Gray shaded areas indicate the presence of PCV2 seropositive pigs (S/P ratio > 0.02) within a treatment group. An S/P ratio of 0.2 was considered positive.

Group designation	Pig No	Trial Day[b]							
		0 dpv	7 dpv	14 dpv	21 dpv	28 dpv/0 dpc	35 dpv/7 dpc	42 dpv/14 dpc	49 dpv/21 dpc
VAC/2a-Ch/2b-PRRSV-PPV	8[a]	0/8 (0.11±0.01)	0/8 (0.05±0.00)	2/8 (0.15±0.02)^{A}	2/8 (0.15±0.02)^{A}	5/8 (0.26±0.06)^{A}	7/8 (0.57±0.08)^{A}	6/7 (0.58±0.09)^{A,B}	7/7 (0.80±0.08)^{A}
VAC/2b-Ch/2b-PRRSV-PPV	8	0/8 (0.13±0.01)	0/8 (0.06±0.00)	7/8 (0.29±0.04)^{B}	7/8 (0.40±0.06)^{B}	8/8 (0.58±0.04)^{B}	8/8 (0.71±0.05)^{A}	8/8 (0.64±0.07)^{A,B}	8/8 (0.79±0.06)^{A}
Ch/2b-PRRSV-PPV	8	0/8 (0.13±0.02)	0/8 (0.05±0.01)	0/8 (0.08±0.01)^{A}	0/8 (0.10±0.01)^{A}	0/8 (0.07±0.01)^{C}	2/8 (0.15±0.02)^{B}	6/8 (0.21±0.02)^{C,D}	8/8 (0.51±0.04^{B}
Negative controls	7	0/7(0.12±0.01)	0/7 (0.06±0.01)	0/7 (0.07±0.00)^{A}	0/7 (0.08±0.01)^{A}	0/7 (0.07±0.01)^{C}	0/7 (0.14±0.01)^{B}	0/7 (0.11±0.02)^{D}	0/7 (0.13±0.02)^{C}
VAC/2a-Ch/2a-2b-PRRSV-PPV	8	0/8 (0.12±0.01)	0/8 (0.06±0.01)	3/8 (0.17±0.03)^{A}	4/8 (0.19±0.06)^{A}	4/8 (0.26±0.05)^{A}	8/8 (0.61±0.09)^{A}	8/8 (0.76±0.09)^{A}	8/8 (0.91±0.06)^{A}
VAC/2b-Ch/2a-2b-PRRSV-PPV	8	0/8 (0.12±0.01)	0/8 (0.04±0.01)	6/8 (0.30±0.04)^{B}	7/8 (0.59±0.09)^{B}	8/8 (0.64±0.05)^{B}	8/8 (0.72±0.07)^{A}	8/8 (0.70±0.08)^{A,B}	8/8 (0.83±0.05)^{A}
Ch/2a-2b-PRRSV-PPV	8	0/8 (0.10±0.01)	0/8 (0.05±0.00)	0/8 (0.09±0.01)^{A}	0/8 (0.07±0.01)^{A}	0/8 (0.07±0.01)^{C}	6/8 (0.28±0.04)^{B}	7/8 (0.43±0.07)^{B,C}	7/8 (0.53±0.06)^{B}
Ch/PRRSV-PPV	8[a]	0/8 (0.13±0.01)	0/8 (0.05±0.01)	0/8 (0.08±0.01)^{A}	0/8 (0.07±0.02)^{A}	0/8 (0.06±0.01)^{C}	0/8 (0.13±0.01)^{B}	0/8 (0.14±0.01)^{C,D}	0/7 (0.13±0.02)^{C}

[a] One VAC/2a-Ch/2b-PRRSV-PPV pigs was euthanized at 34 dpv (6 dpc) and one Ch/PRRSV-PPV pigs died on 14 dpc.
[b] Different superscripts within a column (A, B, C, D) indicate significant ($p < 0.05$) different group mean S/P ratios.

PCV1-2b vaccine. Whereas PCV1-2b vaccinated pigs were viremic for 2 to 7 consecutive weeks, the PCV1-2a viremia was sporadic and often limited to one positive detection day (Fig. 1A). Pigs vaccinated with PCV1-2b had significantly ($p < 0.05$) higher concentrations of PCV1-2 DNA in serum on 7, 14, 21, 28, 35, and 49 dpv. PCV1-2 DNA shedding through nasal secretions and feces mimicked the PCV1-2 serum profiles for the two vaccinated groups (Fig. 1B and C).

ORF1-PCV2 DNA was not detected in any of the non-PCV2 infected control groups (Table 3). In contrast, all non-vaccinated PCV2 challenged pigs were viremic on 7 dpc, 14 dpc, and 21 dpc (Table 3 and Fig. 2). At 21 dpc, the percentage of PCV2 viremia reduction was 25% (VAC2a-Ch/2b-PRRSV-PPV), 44.1% (VAC2a-Ch/2a-2b-PRRSV-PPV), or 100% (VAC2b-Ch/2b-PRRSV-PPV and VAC2b-Ch/2a-b-PRRSV-PPV). At 21 dpc, PCV2 ORF1 and PCV2b ORF2 DNA were identified in groups challenged with PCV2b alone confirming the presence of challenge virus. PCV2b ORF2 DNA, PCV1-2 DNA, but not PCV2 ORF1 DNA was identified in VAC/2b-Ch/2a-2b-PRRSV-PPV pigs challenged with both PCV2a and PCV2b and corresponded to the vaccine virus. The PCV2a DNA (4/8 pigs) and PCV2b DNA (5/8 pigs) were identified in 5/8 VAC/2a-Ch/2a-2b-PRRSV-PPV pigs, indicating a mixed population of both challenge viruses in 4/8 pigs in this group. Finally, high concentrations of PCV2a and PCV2b DNA were detected in 8/8 Ch/2a-2b-PRRSV-PPV pigs indicating a mixed population of both challenge viruses in all

Table 3
Prevalence of PCV2 DNA positive pigs/total number of pigs in each group (mean amount of $\log_{10}$ PCV2 DNA ± se) in various sample types on 7, 14 and 21 days post challenge (dpc). PCV2 challenge occurred at 28 days post vaccination (dpv). Gray shaded areas indicate the presence of PCV2 DNA positive pigs within a treatment group.

Sample type	Treatment group	n	7 dpc[b]	14 dpc[b]	21 dpc[b]
Serum	VAC/2a-Ch/2b-PRRSV-PPV	8[a]	2/8 (1.76±1.16)^{A,B}	6/8 (5.40±1.23)^{A}	6/7 (6.41±1.09)^{A,B}
	VAC/2b-Ch/2b-PRRSV-PPV	8	0/8 (0.00±0.00)^{A}	0/8 (0.00±0.00)^{B}	0/8 (0.00±0.00)^{C}
	Ch/2b-PRRSV-PPV	8	8/8 (7.10±0.16)^{C,D}	8/8 (9.01±0.26)^{C,D}	8/8 (8.54±0.13)^{B}
	Negative controls	7	0/7 (0.00±0.00)^{A}	0/7 (0.00±0.00)^{B}	0/7 (0.00±0.00)^{C}
	VAC/2a-Ch/2a-2b-PRRSV-PPV	8	5/8 (4.15±1.23)^{B,C}	8/8 (6.97±0.32)^{A,C}	5/8 (4.96±1.47)^{A}
	VAC/2b-Ch/2a-2b-PRRSV-PPV	8	1/8 (0.92±0.92)^{A}	0/8 (0.00±0.00)^{B}	0/8 (0.00±0.00)^{C}
	Ch/2a-2b-PRRSV-PPV	8	8/8 (7.96±0.22)^{D}	8/8 (9.81±0.30)^{D}	8/8 (8.88±0.48)^{B}
	Ch/PRRSV-PPV	8[a]	0/8 (0.00±0.00)^{A}	0/8 (0.00±0.00)^{B}	0/8 (0.00±0.00)^{C}
Nasal swab	VAC/2a-Ch/2b-PRRSV-PPV	8[a]	2/8 (1.57±1.03)^{A,B}	2/8 (1.52±1.00)^{A}	3/7 (2.66±1.33)^{A,B,C}
	VAC/2b-Ch/2b-PRRSV-PPV	8	1/8 (0.86±0.86)^{B}	2/8 (1.57±1.03)^{A}	6/8 (4.97±1.10)^{A,C}
	Ch/2b-PRRSV-PPV	8	5/8 (4.34±1.29)^{A,C}	8/8 (8.54±0.15)^{B}	4/8 (3.79±1.47)^{A,B,C}
	Negative controls	7	0/7 (0.00±0.00)^{B}	0/7 (0.00±0.00)^{A}	0/7 (0.00±0.00)^{B,C}
	VAC/2a-Ch/2a-2b-PRRSV-PPV	8	8/8 (6.70±0.58)^{C}	7/8 (5.82±0.85)^{B}	3/8 (2.80±1.37)^{A,B,C}
	VAC/2b-Ch/2a-2b-PRRSV-PPV	8	2/8 (1.53±1.00)^{A,B}	1/8 (0.72±0.72)^{A}	3/8 (2.44±1.20)^{A,B,C}
	Ch/2a-2b-PRRSV-PPV	8	8/8 (7.03±0.15)^{C}	7/8 (8.18±1.18)^{B}	7/8 (7.25±1.07)^{A}
	Ch/PRRSV-PPV	8[a]	0/8 (0.00±0.00)^{B}	0/8 (0.00±0.00)^{A}	0/8 (0.00±0.00)^{B}
Rectal swab	VAC/2a-Ch/2b-PRRSV-PPV	8[a]	3/8 (2.47±1.21)^{A,B,C}	7/8 (5.74±0.95)^{A}	2/7 (2.26±1.50)^{A,B}
	VAC/2b-Ch/2b-PRRSV-PPV	8	1/8 (1.41±1.41)^{B,C}	0/8 (0.00±0.00)^{B}	4/8 (2.92±1.10)^{A,B}
	Ch/2b-PRRSV-PPV	8	6/8 (6.17±1.53)^{A,B}	7/8 (7.43±1.10)^{A}	6/8 (5.77±1.33)^{A}
	Negative controls	7	0/7 (0.00±0.00)^{C}	0/7 (0.00±0.00)^{B}	0/7 (0.00±0.00)^{B}
	VAC/2a-Ch/2a-2b-PRRSV-PPV	8	4/8 (3.44±1.31)^{A,B,C}	7/8 (5.32±0.78)^{A}	6/8 (6.17±1.45)^{A}
	VAC/2b-Ch/2a-2b-PRRSV-PPV	8	1/8 (0.92±0.92)^{C}	1/8 (1.12±1.12)^{B}	5/8 (4.33±1.37)^{A,B}
	Ch/2a-2b-PRRSV-PPV	8	7/8 (6.96±1.00)^{A}	8/8 (8.57±0.08)^{A}	6/8 (5.15±1.18)^{A,B}
	Ch/PRRSV-PPV	8[a]	0/8 (0.00±0.00)^{C}	0/8 (0.00±0.00)^{B}	0/8 (0.00±0.00)^{B}

[a] One VAC/2a-Ch/2b-PRRSV-PPV pigs was euthanized on 34 dpv (6 dpc) and one Ch/PRRSV-PPV pigs died on 14 dpc.
[b] Different superscripts within a column (A, B, C, D) indicate significant ($p < 0.05$) different group mean S/P ratios.

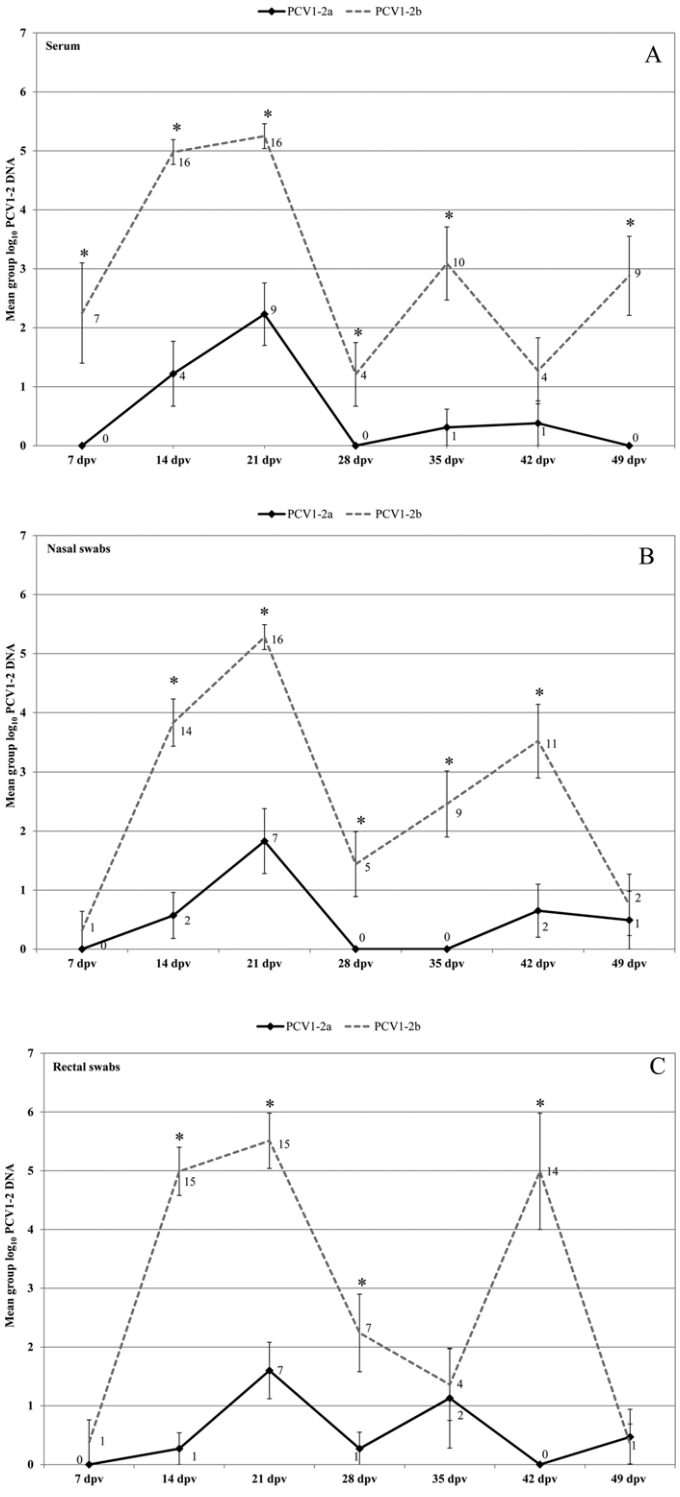

Fig. 1. Mean group amount of $\log_{10}$ PCV1-2 DNA in serum samples (A), nasal swabs (B) or fecal swabs (C) obtained from pigs vaccinated with PCV1-2a ($n = 16$) or PCV1-2b ($n = 16$) on 7, 14, 21, 28, 35, 42, and 49 day after vaccination (dpv). Numbers next to a group mean indicate the number of PCR positive pigs within a group at this time point. Asterisks indicate significant ($p < 0.05$) different group mean loads of $\log_{10}$ PCV1-2 DNA on a certain dpv. Error bars represent standard errors.

pigs in this group replicating at similar high levels. The prevalence and amount of PCV2 DNA in nasal secretions and feces was reduced in vaccinated pigs compared to non-vaccinated groups (Table 3). The amount of PCV2 DNA was significantly lower for pigs vaccinated with PCV1-2b compared to pigs vaccinated with PCV1-2a at

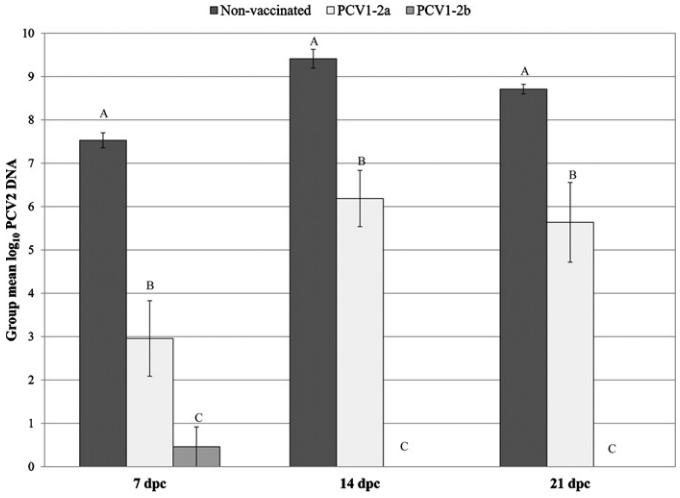

Fig. 2. Mean group amount of $\log_{10}$ PCV2 DNA in serum obtained from pigs vaccinated with PCV1-2a ($n = 16$) or PCV1-2b ($n = 16$) on 7, 14 and 21 days after challenge (dpc). Different superscripts (A, B, C) indicate significant ($p < 0.05$) different group mean loads of $\log_{10}$ PCV2 DNA on a certain dpc. Error bars represent standard errors.

7 and 14 dpc for nasal swabs (pigs concurrently infected with PCV2a and PCV2b) and 14 dpc for rectal swabs (Table 3).

The prevalence rates and concentrations of PRRSV RNA and PPV DNA at 21 dpc were not different among the groups (Table 1).

3.4. Macroscopic lesions

The Ch/2a-2b-PRRSV-PPV pig euthanized at 20 dpc due to severe respiratory distress had a lung lesion score of 93% and the lungs were characterized by severe diffuse interlobular edema, failure to collapse, and dark-red purple consolidation. At 49 dpv (21 dpc), macroscopic lung lesions were characterized by multifocal to diffuse discoloration (mottle-tan), failure to collapse, and cranioventral purple-tan consolidations (Table 1). When group mean lung lesion scores of non-vaccinated and PCV2-challenged pigs were compared to vaccinated and PCV2-challenged pigs, the scores were significantly ($p = 0.0003$) higher in non-vaccinated pigs ($n = 16$; 40.8 ± 6.1) compared to pigs vaccinated with PCV1-2a ($n = 15$; 15.9 ± 3.4) or pigs vaccinated with PCV1-2b ($n = 16$; 18.4 ± 11.0).

3.5. Microscopic lesions and amount of PCV2 antigen

Microscopic lung lesions for all other pigs are summarized in Table 1. Two pigs in each of the Ch/2b-PRRSV-PPV and the Ch/2a-2b-PRRSV-PPV groups had severe diffuse interstitial pneumonia (score = 6) which was multifocally granulomatous (including the pig euthanized on 20 dpc).

The mean group lymphoid scores for all pigs are summarized in Fig. 3. While the majority of the pigs had normal lymphoid tissues or developed only mild lesions, moderate lesions (score range from 4 to 6) were observed in 7/8 Ch/2b-PRRSV-PPVpigs, 2/8 VAC/2a-Ch/2a-2b-PRRSV-PPV, 1/8 VAC/2b-Ch/2a-2b-PRRSV-PPV, and 3/8 Ch/2a-2b-PRRSV-PPV pigs. Overall, 4 pigs developed systemic lymphoid lesions associated with abundant PCV2 antigen consistent with PCVAD (overall lymphoid lesions scores between 7 and 9). All 4 pigs belonged to the Ch/2a-2b-PRRSV-PPV group. Prevalence of PCV2 antigen (at least one positive lymphoid tissue) is summarized in Table 1.

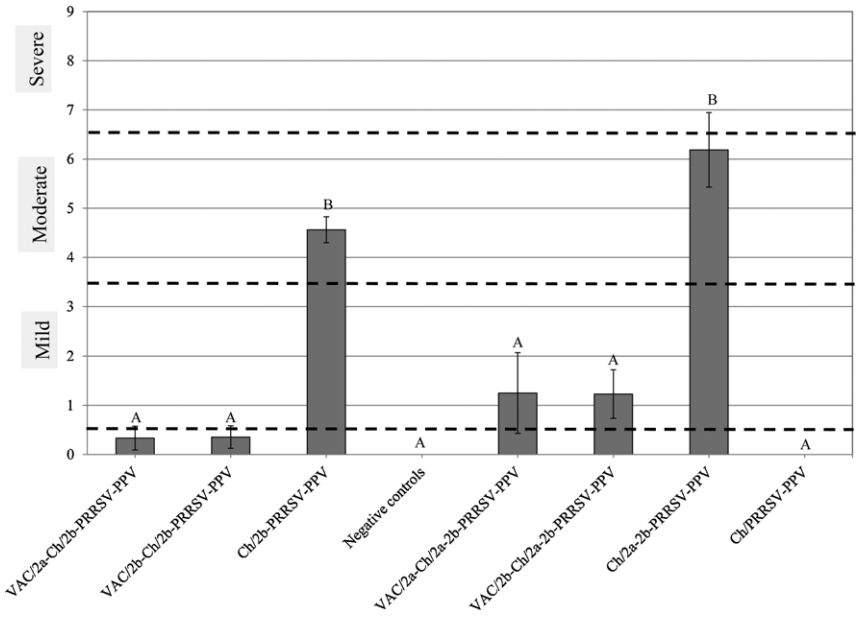

Fig. 3. Overall group lymphoid lesion score on 21 days after challenge (dpc) or 49 days after vaccination (dpv). The overall lymphoid lesion score ranges from 0 to 9. Severity ranges are indicated by dotted lines. Different superscripts (A, B) indicate significant ($p < 0.05$) different group means. The two pigs that were euthanized at 6 dpc (34 dpv) or died at 14 dpc had no microscopic lesions in lymphoid tissues (data not included). Error bars represent standard errors.

4. Discussion

Cross-protection between PCV2 genotypes has been demonstrated experimentally [23,24] and is further supported by the efficacy of PCV2a-based vaccines under field conditions [7–12]; however, PCVAD outbreaks in vaccinated herds do occur. Recently, PCV2b was identified in serum from vaccinated healthy growing pigs in the USA [36]. Moreover, during 2010, PCV2b DNA was found in a high percentage of plasma samples obtained from regional Midwest slaughter houses [37]. Similarly, in a field study analyzing PCV2 genotypes in five breeding herds across North America, PCV2b was highly prevalent [38]. These findings indicate that although PCV2a vaccines generally prevent clinical disease and reduce PCV2a prevalence, they may not significantly reduce the prevalence of PCV2b. The objective of this study was to do a side-by-side comparison of two experimental PCV2 vaccines based on 2a or 2b in growing pigs.

Under the study conditions, PCV2 vaccination using a live-attenuated chimeric vaccine resulted in circulation of vaccine virus as evidenced by PCV1-2 viremia in a portion of the vaccinated pigs. Vaccine-induced clinical signs were not observed and the average daily gain was comparable to that of non-vaccinated pigs. Viremia levels of vaccine virus peaked at 21 dpv and declined thereafter. Seroconversion to PCV2 in vaccinated pigs was detectable by 14 dpv. Vaccination with PCV1-2b resulted in significantly longer duration and higher levels of PCV1-2 viremia and nasal and fecal shedding compared to pigs vaccinated with PCV1-2a. Despite (or because of) higher PCV1-2 DNA levels in serum and feces and nasal secretions, PCV1-2b vaccinated pigs had a more robust humoral immune response as evidenced by significantly higher ELISA S/P ratios. Both vaccines had equivalent $TCID_{50}$ levels at vaccination time. Titration of both live-attenuated vaccines was performed in PK-15 cells, though the actual viral fitness was not compared between the two viruses. Anecdotal evidence suggests that chimeric PCV1-2b may be capable of higher levels of replication for reasons that have not been determined. This hypothesis is supported by ORF2-based differences between PCV2a and PCV2b [3], since this region encodes the capsid protein [39] involved in the binding and internalization of virus in cell [40]. Our previous work has shown that chimeric PCV1-2a replicates even more poorly than PCV2a in PK-15 cells [17]. This would help to explain why the same initial vaccine dose ($10^{3.5}$ $TCID_{50}$) would lead to a stronger PCV1-2b viremia compared to PCV1-2a in infected pigs. Our previous published work characterizing the pathogenicity of the chimeric PCV1-2b in a CD-CD pig model [13] supports its complete attenuation (lack of lymphoid lesions, lower tissue and serum viral load compared with PCV2b). In that study, apathogenic attenuated PCV1-2b was shown to have steady viremia levels above 10^6 genome copies/ml serum after infection with a dose approximately 20 times higher than in the present study. Thus, it is our opinion that higher numbers of viral genomes present in the serum is not itself indicative of decreased attenuation, and is rather an indication of a more replication-competent virus. Rather than decreasing the efficacy of the vaccine, it is likely that the increased strength of the immune response to PCV1-2b and improved protection was directly due to this increased ability of the vaccine virus to replicate in the pigs without causing disease.

Previously [19] a live-attenuated PCV1-2a vaccine appeared to have reduced PCV2b viremia to a greater level than what was seen with the PCV1-2a vaccine in the present study. Differences between the studies include a different stock of the PCV1-2a vaccine utilized (produced in 2009 *versus* 2011), differences in challenge viruses (a single challenge with PCV2b or a PCV2b-PRRSV challenge previously in contrast to a PCV2b-PPV-PRRSV or PCV2a-PCV2b-PPV-PRRSV challenge in the present study) and different pigs (same source but almost 2 years later). These factors could all have influenced the results.

While both vaccines significantly reduced PCV2 viremia, there was a significant difference between the vaccine types (2a or 2b based). Pigs vaccinated with PCV1-2b had significantly lower concentrations of PCV2 DNA in serum compared to pigs vaccinated with PCV1-2a. Furthermore, PCV2 viremia was not detectable in PCV2b vaccinated pigs at 14 dpc or 21 dpc. The percentage reduction in viremia on 21 dpc (49 dpv) compared to non-vaccinated positive control pigs ranged between 25 and 44.1% for PCV1-2a vaccinated pigs to 100% for pigs vaccinated with PCV1-2b. While it would appear that PCV2 challenge virus viremia was prevented entirely by one of the vaccination protocols utilized in this study, it

needs to be noted that PCV2 viremia was only measured at weekly intervals (a short viremia would have remained undetected) and all groups were positive for PCV2 DNA in nasal swabs and rectal swabs further indicating that viremia was likely not prevented entirely. Moreover, viremia detected by PCR only indicates presence of DNA and the exact effect of viremia on production parameters of pigs is still unclear. A side-by-side comparison of live-attenuated and inactivated vaccinates was not conducted in this study but it can be speculated that live-attenuated vaccines may induce a better cellular immune response which may explain the low levels of challenge virus in vaccinated pigs in the present study. Explanations why the efficacy of the live-attenuated PCV1-2a vaccine in PCV2a–PCV2b coinfected pigs was apparently reduced could include the greater humoral immune response induced by the PCV1-2b vaccine or a greater level of cross protection of PCV2b ORF2 compared to PCV2a ORF2. Follow-up studies perhaps including inactivated versions of the current live-attenuated vaccines would help further clarifying this question.

Recent epidemiologic investigations revealed a high frequency of concurrent PCV2a and PCV2b under field conditions in certain countries [37,38,41], and a possible role of combined PCV2a/PCV2b infection in triggering PCVAD [42]. Concurrent PCV2a and PCV2b infection was identified in 31.6% (12/38) of affected pigs, but not in healthy pigs [42]. Furthermore, high frequencies of PCV2a–PCV2b coinfection were identified in tissues from subclinical or clinically-affected pigs [43]. In infected cells with replicating virus, both genotypes were present, suggesting that simultaneous co-replication of PCV2a and PCV2b may be required for high viral loads and subsequent disease expression [43]. In this study, one Ch/2a-2b-PRRSV-PPV pig developed severe respiratory distress and had to be euthanized at 20 dpc. Macroscopic and microscopic examinations showed that PCV2a–PCV2b coinfected pigs had significantly more severe lesions compatible with PCVAD compared to pigs infected with PCV2b alone. In contrast, most of the Ch/2b-PRRSV-PPV pigs had moderate microscopic lesions indicating an effect (additive or potentiating) of concurrent PCV2a infection on development of lesions. In further support of this, three day-old gnotobiotic pigs inoculated with PCV2a and subsequently infected with PCV2b developed severe disease [44].

5. Conclusions

Under the study conditions, vaccination with an experimental attenuated-live chimeric PCV2 vaccine based on the PCV2b genotype was more effective in protecting pigs against the effects of PCV2b or concurrent PCV2a–PCV2b challenge compared to a vaccine based on the PCV2a genotype. In non-vaccinated pigs, coinfection with PCV2a and PCV2b resulted in clinical manifestation of PCVAD and enhanced systemic lymphoid lesions in conventional pigs compared to challenge with PCV2b alone.

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