Vaccine 37 (2019) 6688–6695

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A *Porcine circovirus* type 2b (PCV2b)-based experimental vaccine is effective in the PCV2b-*Mycoplasma hyopneumoniae* coinfection pig model

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ARTICLE INFO

Article history:
Received 11 July 2019
Received in revised form 3 September 2019
Accepted 4 September 2019
Available online 16 September 2019

Keywords:
Porcine circovirus (PCV)
PCV2b
Coinfection
Vaccination
Conventional pig model

ABSTRACT

Porcine circovirus type 2 (PCV2) is one of the major swine pathogens causing high economic losses due to PCV2-associated disease (PCVAD). PCV2 infection is not only immunosuppressive by damaging lymphoid tissues but is also exacerbated by co-infections with other pathogens including *Mycoplasma hyopneumoniae*. While PCV2 can be divided into several genotypes, currently only PCV2a, PCV2b and PCV2d are globally prevalent and considered of major importance. Most commercial PCV2 vaccines are based on PCV2a isolates; however, the high prevalence of PCV2b and PCV2d in the global pig population is raising concerns among pig veterinarians. The objective of this study was to evaluate the efficacy of an experimental PCV2b-based subunit vaccine in a combined PCV2b and *M. hyopneumoniae* coinfection model. Briefly, a total of 49 PCV2- and *M. hyopneumoniae*-free 3-week-old pigs were randomly divided into four groups: A non-vaccinated, non-infected NEG-CONTROL group, a non-vaccinated, PCV2b-infected, POS-CONTROL group, and two vaccinated and PCV2b-infected groups (SINGLE-VAC, DUAL-VAC). SINGLE-VAC and DUAL-VAC pigs were vaccinated at 3 weeks of age and DUAL-VAC pigs received a booster dose at 5 weeks of age. All pigs, except NEG-CONTROLs, were experimentally infected with *M. hyopneumoniae* 28 days after initial vaccination and challenged with PCV2b one week later. The pigs were necropsied 21 days after PCV2b challenge. Prior to PCV2b challenge, both vaccinated groups had detectable humoral and cell-medicated immune responses to PCV2. Vaccination significantly reduced PCV2b viremia and also reduced or eliminated PCV2-associated lymphoid lesions compared to the POS-CONTROL pigs. Under the study conditions, an experimental PCV2b vaccine protected conventional growing pigs against PCV2b viremia and associated lesions in a coinfection model with some advantages of the two-dose regimen versus the one dose regimen. Both protocols induced neutralizing antibodies against PCV2a and PCV2d prior to challenge.

1. Introduction

Porcine circovirus type 2 (PCV2), a small single-stranded circular DNA virus in the family of the *Circoviridae* [1], is difficult to be eliminated from pig herds and contributes to considerable economic losses associated with a variety of clinical manifestations collectively called PCV2-associated disease (PCVAD) when uncontrolled [2]. At least eight subtypes of PCV2 are recognized, PCV2a-h [3–5]. PCV2a was the most prevalent genotype during the 1990s, after which PCV2b became predominant resulting in a genotype shift around 2003 [6]. The emergence of PCV2b in North America and Europe was associated with severe clinical outbreaks causing mortality rates of up to 50% [7]. PCV2c was reported in Denmark from archived material obtained in 1980 (EU148503) and 1987 (EU148504) [8], and in Brazil in 2010 (KJ094599) [9]. Due to its limited prevalence today, PCV2c is considered of minor importance. Since 2012, PCV2d has been increasingly observed in the global pig population resulting in another genotype shift between 2010 and 2014 [10]. The PCV2e genotype was identified in several samples collected from the USA in 2006 [11] and in

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https://doi.org/10.1016/j.vaccine.2019.09.029
0264-410X/

2015 (KT870147; KT785288; KT785289; KT785290) [10,11], in 2014 from Mexico (KT795287) [12], and from China in 2002 (MH465483) [4], from 2012 to 2016 (MH465485, MH465490, MH465488, MH465401) [4] and in 2017 (MF589523, MF589524) [13]. Like PCV2c, PCV2e is currently considered of minor importance since its prevalence is low even though it has been circulating in the North American pig population for at least 10 years. PCV2f has been detected in samples from China in 1999 (MF139076, MF139077, MF139078) [3] and from India in 2014 (LC004750; LC008135, LC008137) [5]. The real impact of PCV2f on swine health is still unknown. Three sequences, from Vietnam in 2012 (JX099786), from China in 2009 (FJ998185), and from the Ukraine in 2015 (KP420197), were recently reclassified as PCV2g [5]. Finally, PCV2h has been identified from China in 2012 (MH465453, MH465473) [4] and from Vietnam in 2011 (JQ181592), 2012 (JX506730) and 2014 (KM042398) [5]. Despite the increasing diversity within PCV2, PCV2a and PCV2b and PCV2d remain the most prevalent genotypes reported around the world and are of major clinical significance.

It is well established that PCV2 infection is necessary but not always sufficient by itself to cause PCVAD [14,15]. To better mimic a real field situation, the presence of co-factors including concurrent infections with other swine pathogens is important for full manifestation of clinical PCVAD under experimental conditions [2,14]. *Mycoplasma hyopneumoniae* is frequently found as a co-infecting agent in PCVAD cases and has been shown to enhance PCV2-associated disease when given one week before PCV2 infection [16,17].

The first commercial PCV2 vaccines became available as early as 2000 in Europe and in 2006 in North America and since have received wide acceptance among pig farmers worldwide, being the single highest selling prophylactic agent in the porcine industry [18]. Current commercial PCV2 vaccines are typically inactivated whole virus or subunit vaccines based on the PCV2a genotype. The introduction of PCV2 vaccination on pig farms minimized the occurrence of clinical disease and had a measurable positive economic impact in clinically affected and unaffected herds [19]. However, despite the widespread PCV2 vaccine usage, PCV2d has slowly begun to replace PCV2b, becoming the predominant global PCV2 strain. Additionally, the emergence of PCV2d has been linked to PCVAD outbreaks in PCV2-vaccinated herds on several occasions [20–23]. There are concerns by some that currently available PCV2a-based vaccines may not be effective against circulating PCV2d genotypes, since previous studies showed that PCV2b is more closely related to PCV2d (average *p*-distance ± SE; 0.055 ± 0.008) compared to PCV2a and PCV2d sequences (0.085 ± 0.01) [10].

Under experimental conditions, the cross-protection between different PCV2 subtypes has been examined [18,24]. Commercial PCV2a-based vaccines reduced PCV2d viremia, viral loads in tissue and PCV2d shedding and transmission [25,26]. Additionally, there was also a reduction of PCV2b viremia and a reduced transmission in pigs vaccinated with PCV2a-based vaccines versus non-vaccinated pigs [27]. However, under experimental conditions, an experimental PCV2b-based vaccine provided better protection against PCV2b viremia compared to a PCV2a-based vaccine [28]. Furthermore, an experimental PCV2d-based vaccine resulted in absence of PCV2d viremia during the 28 days after PCV2d challenge, while PCV2d viremia was detectable in a PCV2a vaccinated pig [29]. Thus, the possibility exists that homologous vaccines based on the PCV2 genotype present in the pig population may provide better protection than heterologous vaccines based on a different genotype. The objective of this study was to evaluate the efficacy of an experimental PCV2b vaccine administered as one or as a two-dose regimen in the PCV2b/*M. hyopneumoniae* coinfection model.

2. Materials and methods

2.1. Ethical approval and study location

The experimental protocol of this study was approved by the Iowa State University Institutional Animal Care and Use Committee (approval number 11-16-8389-S) and the Iowa State University Institutional Biosafety Committee (approval number 16-D/I-0016-A). The pigs were regularly monitored by veterinarians unrelated to the project personnel. The study was conducted at the Livestock Infectious Disease Isolation Facility, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA under biosafety level 2 (BSL2) conditions. All laboratory analyses were performed at Iowa State University. The experimental work was carried out between December 2017 and March 2018.

2.2. Animal source, housing and experimental design

Fifty, 2.5-week-old, mixed-gender crossbred piglets were purchased from a commercial U.S. breeding herd with low PCV2 circulation as evidenced by low incidence and levels of antibodies in the breeding sows and no history of PCV2 vaccination or PCVAD. The pigs were transported to the research facility, were blocked by litter, and were then randomly distributed into one of four experimental groups: a non-vaccinated, non-infected negative control group (NEG-CONTROL; n = 10), a non-vaccinated PCV2b infected positive control group (POS-CONTROL; n = 20), a group vaccinated once with a PCV2b vaccine and infected with PCV2b (SINGLE-VAC; n = 9; originally there were 10 pigs in this group but one pig was found dead after *M. hyopneumoniae* inoculation due to an intestinal torsion unrelated to the project and therefore was removed from the study), and a group vaccinated twice with a PCV2b vaccine and infected with PCV2b (DUAL-VAC; n = 10) (Fig. 1). After a week of acclimation to the research facility, SINGLE-VAC and DUAL-VAC pigs were vaccinated at 3 weeks of age and DUAL-VAC pigs were given a booster dose 2 weeks later at 5 weeks of age. All pigs except the NEG-CONTROL pigs were inoculated with *M. hyopneumoniae* at 7 weeks of age and with PCV2b at 8 weeks of age (Fig. 1). While the NEG-CONTROL pigs were housed together in a single room, a complete randomized block design was used to assign equal proportions of POS-CONTROL, SINGLE-VAC and DUAL-VAC pigs into each of three additional rooms. In each room, the pigs from the different groups were comingled to remove any possible room effect. Each room contained one pen of approximately 10 m^2, which was equipped with a nipple waterer and two self-feeders. The piglets were fed an appropriate complete feed ration according to their age without addition of antimicrobials or animal proteins (Heartland Co-Op, Prairie city, IA, USA). Feed and water were supplied *ad libitum*.

2.3. Sample collection and clinical parameters evaluated

To monitor viremia and serologic responses, serum samples were collected using BD Vacutainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) followed by centrifugation at 0 days post vaccination (dpv 0) and again at dpv 7, 14, 21 and 28 (*M. hyopneumoniae* challenge), dpv 35 (PCV2d challenge; day post PCV2 challenge 0 or dpc 0), dpv 42 (dpc 7), dpv 49 (dpc 14) and dpv 56 (dpc 21). To study PCV2 specific interferon γ (IFN-γ) responses, blood samples on dpv 35/dpc 0 were also collected using BD Vacutainer® CPT™ cell preparation tubes with sodium citrate (Becton, Dickinson and Company) and peripheral blood monocytes (PBMCs) were obtained from the buffy coat layer within 2 h after blood collection. All pigs were necropsied at dpc 21 to evaluate lesions and to collect tissue samples for further analysis.

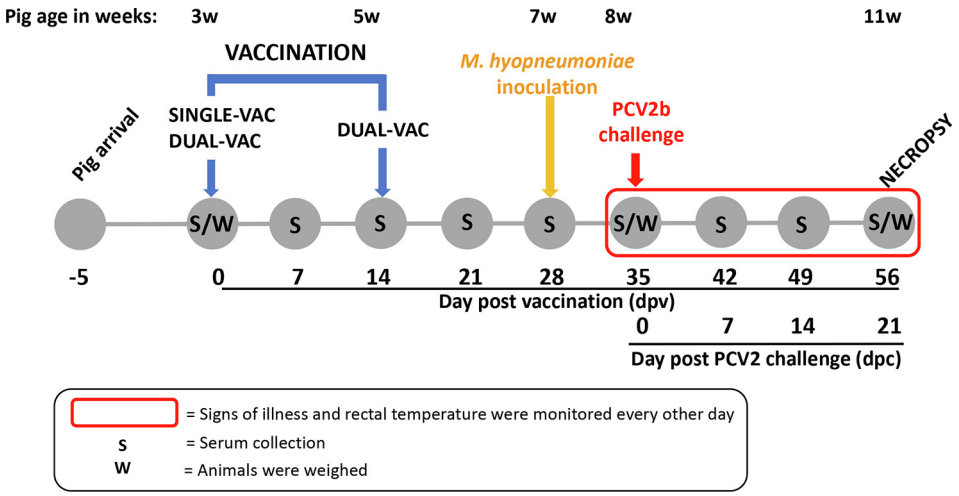

Fig. 1. Experimental design, time line of sample collections, and scoring of the clinical signs.

The pigs were weighed at the initial vaccination (dpv 0), at PCV2 challenge (dpv 35) and at necropsy (dpc 21) (Fig. 1), and the average daily weight gain (ADG) was calculated for the time from initial vaccination to PCV2b challenge (dpv 0–35) and from the PCV2b challenge to necropsy (dpv 35–56) (Table 1).

After each vaccination, all vaccinated pigs were observed for the occurrence of adverse reactions and reactions at the application site on a daily basis for three days. In addition, from PCV2b challenge until necropsy, all pigs were examined every other day for signs of illness such as lethargy, respiratory disease, inappetence and lameness and rectal temperatures were recorded.

2.4. Vaccine preparation and vaccination

The experimental vaccine used in this study (B058-003; Lot number 001/17, Ourofino Saúde Animal Ltda, Cravinho, SP, Brazil) was based on a PCV2b strain. The open reading frame 2 (ORF2) from the PCV2b was expressed in baculoviruses, purified and the resulting protein was formulated with a water-in-oil adjuvant suspension.

Pigs in the SINGLE-VAC and DUAL-VAC groups were vaccinated via the intramuscular route into the right neck area. Specifically, at dpv 0, pigs in the SINGLE-VAC group received 2 mL of the vaccine while pigs in the DUAL-VAC group received 1 mL. Two weeks later, the DUAL-VAC group received a booster dose of 1 mL.

2.5. Experimental inoculation

Pigs were challenged with *M. hyopneumoniae* at dpv 28. Propagation of *M. hyopneumoniae* is difficult *in vitro* and commonly lung homogenate from experimentally-infected pigs is used as challenge material [30,31]. Briefly, homogenate of frozen tissues of pigs infected with *M. hyopneumoniae*, strain 232, a derivative of *M. hyopneumoniae* strain 11 challenge inoculum (10^5 color changing units/mL) was diluted 1:100 in sterile Friis media on the day of inoculation and kept on ice until the challenge procedure as described [16]. All pigs were anesthetized and 10 mL of the diluted inoculum (all challenged groups) or sterile Friis media (NEG-CONTROL pigs) was administered by intratracheal intubation into the lower trachea. Following challenge, the pigs were monitored until they regained consciousness [16].

The PCV2b challenge was done at dpv 35, using a cell culture propagated PCV2b (NC-16845; GenBank accession number EU340258) virus stock [32] at approximately $10^{4.5}$ 50% tissue culture infectious dose ($TCID_{50}$) per mL. In brief, each pig, except the NEG-CONTROL animals, received 4 mL of the virus stock intranasally by slowly dripping 2 mL into each nostril [32]. The PCV2b used in the challenge was from a clinical field case submitted for diagnosis and confirmed to be PCV2 by the Iowa State University Veterinary Diagnostic Laboratory. The ORF2 amino acid sequence identity between the PCV2b-vaccine used in this study and the PCV2b challenge inoculum was 98.7%.

Table 1
Average daily weight gain in grams ± SEM from each group during two experimental periods, vaccination to PCV2b challenge (35 days) and PCV2b challenge to necropsy (21 days). There was no significant difference among groups as determined by one-way ANOVA.

Group	Pig No.	Vaccination to PCV2b challenge	PCV2b challenge to necropsy
NEG-CONTROL	10	499.9 ± 17.8	604.4 ± 47.7
DUAL-VAC	10	480.4 ± 24.1	719.7 ± 53.8
SINGLE-VAC	9	438.9 ± 26.9	725.0 ± 41.1
POS-CONTROL	20	421.6 ± 20.7	488.2 ± 66.3
P value		0.065	0.124

2.6. DNA extraction and detection and quantification of PCV2 by real time PCR

The presence and amount of PCV2 viral DNA in serum was determined by a quantitative real-time PCR assay. Total nucleic acids from serum samples collected on dpc 7, 14 and 21 were extracted using the MagMAX-96 nucleic acid isolation kit (ThermoFisher, Waltham, MA, USA) on the automated KingFisher Flex System (ThermoFisher Scientific) according to the instructions of the manufacturer. The copy numbers of PCV2 genome were estimated using a quantitative real-time PCR assay as described [33]. A cycle threshold (C_T) of 38 was considered negative. Appropriate negative and positive controls were included in each PCR run. For the positive control, a laboratory dilution of the challenge virus was prepared, and sterile water served as negative control.

2.7. Detection of Mycoplasma hyopneumoniae antibodies

Serum samples collected at dpv 0, dpv 28 and dpc 21 were tested for *M. hyopneumoniae* antibodies using the IDEXX *M. hyo* Ab Test (IDEXX, Westbrook, ME, USA). As per the manufacturer's directions, a sample was considered negative if the sample-to-positive (S/P) ratio was less than 0.30 and it was considered positive when the S/P ratio was greater than 0.40. A value between cutoffs was considered suspect.

2.8. Detection of anti-PCV2 IgG

Serum samples collected at dpv 0, dpv 28, dpv 35/dpc 0 and dpc 21 were tested for seroconversion to IgG anti-PCV2 using a commercial indirect ELISA PCV2 kit (INgezim CIRCO IgG R.11.PCV.K1; Ingenasa, Madrid, Spain), which contains ELISA plates coated with PCV2b ORF2 protein, as instructed by the manufacturer. The test was considered valid if the optical density $(OD)_{450}$ of the negative control was lower than 0.35 and if the OD_{450} of the positive control was higher than 0.7. A sample OD_{450} value of 0.25 more than the negative control was considered positive for PCV2 specific IgG antibodies, while an OD_{450} between 0.2 and 0.25 more than the negative control was considered as doubtful.

2.9. Detection and titration of PCV2a and PCV2d neutralizing antibodies

Neutralizing antibody levels were assessed as previously described [34] on serum samples collected at dpc 0 using PCV2a or PCV2d stock with the following modifications: The cells infected with the virus and test serum mixture were fixed with 2% paraformaldehyde (Thermo-Fisher) in phosphate buffered saline (PBS) for 15 min. This was followed by two washes of the cells with PBS. The cells were permeablized with 0.2% Triton X-100 (in PBS) for 30 min followed by two washes with PBS. Instead of a PCV2 positive control [34], a polyclonal anti-replicase PCV2 antibody produced in rabbits (Cat No. GTX133638; GenTex, Inc, Irvine, CA, USA) was used. The primary antibody was diluted 1:100, incubated for 1 h, followed by three PBS washes. An anti-rabbit FITC-conjugated secondary antibody (Alexa Fluor 488 conjugated; Invitrogen) was added to the samples, incubated for 1 h, and followed by three PBS washes to remove it. The cells were visualized with an inverted fluorescent microscope.

2.10. ELISpot assay

At the time of PCV2 challenge, PBMCs were isolated using BD Vacutainer® CPT™ tubes (Becton, Dickinson and Company) which were centrifuged at 1800*g* for 15 min. The obtained cells were washed twice in sterile calcium and magnesium free PBS by centrifugation at 300*g* for 10 min. The PCV2 specific IFN-γ secreting PBMC was estimated with a ready-to-use commercial ELISpot kit (Porcine IFN-gamma ELISpot Kit; R&D systems, Inc., Minneapolis, MN, USA). A total of 2.5×10^5 viable PBMC in 100 μL of RPMI 1640 medium supplemented with 10% heat-inactivated FBS were seeded into IFN-γ capture antibody coated microplates. The PBMCs were incubated for 36 h at 37 °C in a 5% CO_2 incubator, with 150 μL PCV2b virus per well. Then the ELISpot assay was performed according to the manufacturer's instructions. Briefly, cells were washed and incubated with a biotinylated detection antibody and any unbound antibody was washed off. Subsequently, alkaline-phosphatase conjugated to streptavidin was bound to the biotinylated detection antibody. Any unbound enzyme was removed by washing and a substrate solution (BCIP/NBT) was added. A blue-black colored precipitate was formed at the sites of cytokine localization and appeared as spots, with each individual spot representing an individual secreting cell. The IFN-γ spots were counted with an ELISpot reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

2.11. Necropsy and macroscopic lesions

At dpc 21, all pigs were euthanized by intravenous pentobarbital sodium overdose (Fatal Plus®, Vortech Pharmaceuticals, LTD, Dearborn, MI, USA) and necropsied. Macroscopic lesions were scored by a veterinary pathologist (PCG) blinded to the treatment status. The percentage of the lung surface affected with consolidation was subjectively estimated for each lung lobe and the total percentage of pneumonia was calculated based on weighted proportions of each lobe relative to the total lung volume as previously described [35]. The size of superficial inguinal lymph nodes was scored as described [16]. Any other lesions were recorded and sections of lymph nodes (superficial inguinal, external iliac, mediastinal, tracheobronchial, and mesenteric), tonsil, spleen, kidney, liver, and small intestines (ileum) were collected, fixed in 10% neutral-buffered formalin, and routinely processed for histological examination.

2.12. Histopathology, immunohistochemical analysis and calculation of the overall lymphoid lesion score

Microscopic lesions were assessed by a veterinary pathologist (TO) blinded to the treatment status. Lymph nodes, spleen, and tonsil were evaluated for presence and degree of lymphoid depletion and granulomatous replacement of follicles ranging from 0 (normal) to 3 (severe) [16]. Lung sections were scored for presence and severity of interstitial pneumonia, ranging from 0 (normal) to 6 (severe diffuse) [36] and for presence and distribution of peribronchiolar lymphoid hyperplasia (0 = normal, 3 = severe, diffusely throughout the lung sections) [16]. Sections of ileum, liver and kidney were evaluated for the presence of granulomatous inflammation and scored from 0 (none) to 3 (severe).

Immunohistochemistry (IHC) for detection of PCV2 antigen was performed on formalin-fixed and paraffin-embedded sections of lungs, lymph nodes, tonsil, and spleen from all pigs using a rabbit PCV2 polyclonal antiserum [37]. PCV2 antigen scoring was done by a veterinary pathologist (TO) blinded to the treatment status. Scores ranged from 0 (no signal) to 3 (more than 50% of lymphoid follicles contained cells with PCV2 antigen staining) [16]. In addition, *M. hyopneumoniae* IHC was done on all lung tissues to confirm successful *M. hyopneumoniae* infection [16].

The overall lymphoid lesion score was calculated as described [16]. In brief, a combined scoring system for each lymphoid tissue that ranged from 0 to 9 (lymphoid depletion score 0–3; granulomatous inflammation score 0–3; PCV2 IHC score 0–3) was used. Pigs were classified as having no lesions (score of 0), mild (scores of 1, 2 or 3), moderate (scores of 4, 5 and 6) and severe (scores of 7, 8 and 9) lesions consistent with PCVAD.

2.13. Statistical analysis

The statistical analysis was performed using JMP software (JMP version Pro 13.1.0, SAS Institute Inc., Cary, North Carolina). Summary statistics were calculated to assess the overall quality of data. Analysis of variance (ANOVA) was used for cross-sectional assessment of the ADG data following a normal distribution and PCV2 viremia. Real-time PCR results (copies per mL serum) were $\log_{10}$ transformed prior to statistical analysis after which their distribution was normal. The significance level was $P < 0.05$, followed by pairwise testing using the Tukey-Kramer adjustments to identify the groups that were different. Non-repeated measures of necropsy and histopathology data were assessed using non-parametric Kruskal -Wallis ANOVA. If a non-parametric ANOVA test was significant ($P < 0.05$), then Wilcoxon tests were used to assess the differences of pairs of groups. Multiple groups were assessed using the Steel-Dwass test. The area under the curve (AUC) of viral PCV2 shedding of each animal and the total AUC for each group was calculated using the log transformed values of the viral loads over time.

3. Results

3.1. No detectable adverse reactions to the experimental vaccines

The vaccine was a water-in-oil adjuvant suspension and no problems were encountered during administration of the vaccine. No adverse reactions such as redness or swelling were observed at the vaccination site in any of the vaccinated SINGLE-VAC or DUAL-VAC pigs after vaccination.

3.2. Confirmation of successful M. hyopneumoniae infection

All pigs were negative for *M. hyopneumoniae* antibodies at arrival at the research facility and at the time of *M. hyopneumoniae* challenge (dpv 28). The ELISA results obtained at termination of the study are presented in Table 2. Pigs challenged with *M. hyopneumoniae* and PCV2b were observed coughing from 13 days after challenge with *M. hyopneumoniae* (6 days after challenge with PCV2) until study termination. The majority of the *M. hyopneumoniae* infected pigs had detectable amounts of *M. hyopneumoniae* antigen in lung tissues (Table 2).

3.3. Clinical signs and weight gain

No statistical differences were observed, in coughing or other clinical signs, among the challenged groups. No significant difference in ADG was observed among treatments during the two periods, i.e., from vaccination to PCV2b challenge day (0–35 dpv) and from PCV2b challenge to necropsy day (0–21 dpc) (Table 1).

3.4. Humoral immune responses against PCV2

The prevalence of pigs that had seroconverted to PCV2 in each group, and the mean group anti-PCV2 IgG S/P ratios (±SEM) are shown in Table 3. All pigs were PCV2 seronegative at the time of vaccination. Pigs from both vaccinated groups (8/9 SINGLE-VAC pigs and 10/10 DUAL-VAC pigs) had seroconverted by dpv 28 (*M. hyopneumoniae* challenge) differing significantly ($P < 0.05$) from the unvaccinated groups; DUAL-VAC pigs had significantly higher anti-PCV2 IgG levels compared to SINGLE-VAC pigs. By dpc 21, the PCV2 antibody levels were similar in all PCV2 challenged groups regardless of vaccination status.

At the time of PCV2b challenge, neutralizing PCV2 antibody titers were not different for PCV2a or PCV2d but were significantly higher in DUAL-VAC pigs compared to SINGLE-VAC pigs. Both vaccinated groups had significantly higher levels of neutralizing antibodies compared to POS-CONTROL and NEG-CONTROL pigs in which neutralizing antibodies were not detected ($P < 0.0001$).

3.5. PCV2-specific CMI response

Mean group PCV2b specific IFN-γ secreting cells (SC) in PBMCs, indicative of the level of cell-mediated immunity, are summarized in Fig. 2. Among the vaccinated groups, DUAL-VAC showed numerically higher levels of IFN-γ SCs compared to SINGLE-VAC, but this was not significant. However, DUAL-VAC pigs had significantly higher levels of PCV2b specific IFN-γ SC compared to NEG-CONTROL and POS-CONTROL groups (Fig. 2).

3.6. PCV2 viremia after PCV2b challenge

PCV2 DNA was not detected in any of the NEG-CONTROL pigs for the duration of the study. At 7 dpc, 30% (3/10) DUAL-VAC pigs, 22.2% (2/9) SINGLE-VAC pigs, and 90% (18/20) POS-CONTROL pigs were PCV2 viremic. Similarly, PCV2 viremia was detected at 14 dpc in 50% (5/10) of the DUAL-VAC pigs, in 55.6% (5/9) of the SINGLE-VAC pigs, and in 100% (20/20) of the POS-CONTROL pigs. At 21 dpc, PCV2 DNA was detected in serum samples from 50% (5/10) of the DUAL-VAC pigs, 66.7% (6/9) of the SINGLE-VAC pigs and in 100% (20/20) of the POS-CONTROL pigs. The average PCV2 viremia length in PCV2 infected pigs was 1.3 ± 0.3 weeks for DUAL-VAC pigs, 1.4 ± 0.3 weeks for SINGLE-VAC pigs and 2.9 ± 0.1 weeks for the POS-CONTROL pigs. The PCV2 viremia length was significantly ($P < 0.05$) longer in POS-CONTROL pigs

Table 2
Characteristics of the *M. hyopneumoniae* infection at 28 days post *M. hyopneumoniae* infection/21 days post PCV2b infection.

Group	Pig No.	Macroscopic lung lesions[a]	Peribronchiolar lymphoid hyperplasia score[b]	*M. hyopneumoniae* antigen score[c]	*M. hyopneumoniae* specific antibody response[d]
NEG-CONTROL	10	$0 \pm 0^{A,e}$	0.2 ± 0.1^{A}	$0/10\ (0 \pm 0)^{A}$	$0/10\ (0.0 \pm 0)^{A}$
DUAL-VAC	10	15.5 ± 3.1^{B}	2.1 ± 0.2^{B}	$9/10\ (1.9 \pm 0.3)^{B}$	$1[4]/10\ (0.3 \pm 0)^{B}$
SINGLE-VAC	9	13.3 ± 3.3^{B}	2.2 ± 0.4^{B}	$8/9\ (1.8 \pm 0.4)^{B}$	$1[2]/9\ (0.2 \pm 0)^{B}$
POS-CONTROL	20	21.6 ± 2.8^{B}	1.9 ± 0.2^{B}	$18/20\ (1.7 \pm 0.2)^{B}$	$4[5]/20\ (0.2 \pm 0.0)^{B}$

[a] Mean group percentage of the total lung surface affected by lesions ± SEM.
[b] Score range from 0 = normal to 3 = severe lymphoid hyperplasia around airways, diffuse distribution.
[c] Determined by immunohistochemistry. Data presented as number of IHC positive pigs/total number of pigs. Score range from 0 = no antigen to 3 = abundant antigen in several airways.
[d] Determined by ELISA. Data presented as number of positive pigs [number of suspect pigs]/total number of pigs (mean ELISA sample-to-positive ratios ± SEM).
[e] Different superscripts $^{(A, B)}$ within a column indicate signficantly ($P < 0.001$) different group mean differences.

Table 3
PCV2b specific IgG response over time. PCV2 vaccination was done on day post vaccination (dpv) 0 (DUAL-VAC, SINGLE-VAC groups) and again on dpv 14 (DUAL-VAC group). The pigs in the DUAL-VAC, SINGLE-VAC and POS-CONTROL groups were challenged with PCV2d on dpv 35 or day post challenge (dpc) 0. Data are presented as number of positive pigs/total number of pigs per group (PCV2 specific IgG sample to positive ratio ± SEM).

Group	dpv 0	dpv 28	dpv 35/dpc 0	dpc 21
NEG-CONTROL	$0/10\ (0.24 \pm 0.04)^{A*}$	$0/10\ (0.22 \pm 0.02)^{A}$	$0/10\ (0.12 \pm 0.01)^{A}$	$0/10\ (0.21 \pm 0.02)^{A}$
DUAL-VAC	$0/10\ (0.2 \pm 0.01)^{A}$	$10/10\ (1.72 \pm 0.12)^{B}$	$10/10\ (1.51 \pm 0.09)^{B}$	$10/10\ (1.17 \pm 0.08)^{B}$
SINGLE-VAC	$0/9\ (0.19 \pm 0.01)^{A}$	$8/9\ (1.03 \pm 0.16)^{C}$	$9/9\ (1.12 \pm 0.22)^{B}$	$9/9\ (1.12 \pm 0.18)^{B}$
POS-CONTROL	$0/20\ (0.22 \pm 0.02)^{A}$	$0/20\ (0.27 \pm 0.02)^{A}$	$0/20\ (0.15 \pm 0.02)^{A}$	$15/20\ (0.72 \pm 0.08)^{A,B}$

[*] Different superscripts $^{(A, B, C)}$ indicate a significantly ($P < 0.05$) different group mean.

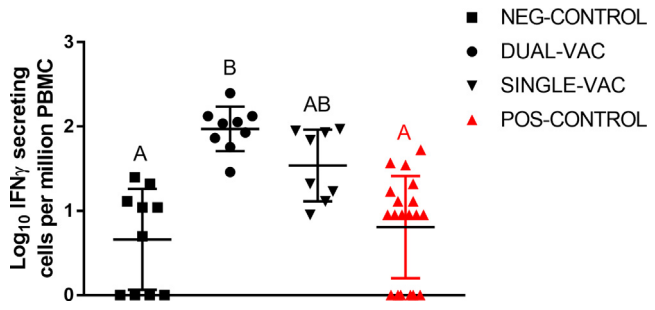

Fig. 2. Log_{10} IFNγ secreting cells per million PBMCs on the day of PCV2 challenge in the different treatment groups stimulated by using the challenge virus (PCV2b). Different superscripts $^{(A,B)}$ indicate significantly different group means.

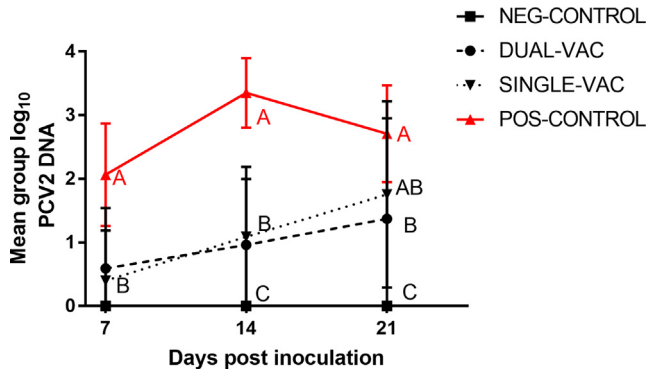

Fig. 3. Mean group PCV2 DNA levels in serum at 7, 14 and 21 days post PCV2b challenge. Pigs in the SINGLE-VAC and in the DUAL-VAC group were vaccinated 5 weeks prior to challenge and DUAL-VAC pigs were received a booster vaccination 3 weeks prior to challenge. The non-vaccinated PCV2b infected POS-CONTROL group is indicated by a red solid line. Different superscripts $^{(A,B,C)}$ on a certain day post PCV2b challenge indicate significantly different group means. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared to the two vaccinated groups. Mean log_{10} group PCV2 genomic copy numbers in serum are summarized in Fig. 3. The AUC was highest for the POS-CONTROL group (6.8) and significantly ($P < 0.0001$) lower for SINGLE-VAC (2.4) and DUAL-VAC (2.2) groups.

3.7. Macroscopic lesions

Lymph nodes were normal to mildly enlarged in all groups, without differences among the groups. In the POS-CONTROL group, 50% of the animals presented scores ≥ 2, while in the vaccinated groups more than 50% had a score below 2. Macroscopic lung lesions were limited to cranioventral consolidation typical of *M. hyopneumoniae* infection in the lungs of all challenged pigs (Table 2).

3.8. Microscopic lesions

Mild-to-severe lymphoid lesions characteristic of PCV2 infection were observed in the lymph nodes of all POS-CONTROL pigs (20/20) and detectable PCV2 antigen was present in lymphoid tissues of 14/20 pigs. It is noteworthy that none of the SINGLE-VAC pigs had evidence of PCV2-associated lymphoid lesions or PCV2 antigen in tissues while 2/10 DUAL-VAC pigs had mild lesions (lymphoid depletion score of 1 and a PCV2 IHC score of 1). The average group lymphoid score was 0 ± 0 for the NEG-CONTROL pigs, 0 ± 0 for the SINGLE-VAC pigs, 0.2 ± 0 for the DUAL-VAC pigs and 3.2 ± 0.3 for the POS-CONTROL pigs. The overall lymphoid score was significantly ($P < 0.001$) higher in the POS-CONTROL group compared to all other groups. Overall, the distribution of pigs in the different lesion score groups was as follows. Normal (score 0): 100% (10/10) of the NEG-CONTROL pigs, 100% (9/9) of the SINGLE-VAC pigs and 80% (8/10) of the DUAL-VAC pigs. Mild (score 1, 2 or 3): 20% (2/10) of the DUAL-VAC pigs and 60% (12/20) of the POS-CONTROL pigs. Moderate (score 4, 5 or 6): 35% (7/20) of the POS-CONTROL pigs. Severe (score 7, 8 or 9): 5% (1/20) of the POS-CONTROL pigs. The proportions of normal pigs among NEG-CONTROL, SINGLE-VAC and DUAL-VAC groups was not different and was significantly higher ($P < 0.001$) compared to the POS-CONTROL group.

4. Discussion

PCV2 infection and the occurrence of PCVAD remains a challenge to the global pig industry requiring constant use of control regimens commonly in the form of vaccines [18]. Soon after the initiation of commercial sales, PCV2 vaccines became the highest selling prophylactic vaccine in porcine husbandry and have been responsible for decreased occurrence of PCVAD in pig herds worldwide [18]. However, the prevalence of PCV2 positive herds did not change substantially and it is well established that PCV2 vaccines decrease PCV2-associated lesions but do not eliminate virus replication and pig-to-pig transmission. Moreover, over the last two decades, the number of recognized PCV2 genotypes has changed from initially two (PCV2a and PCV2b) to eight (PCV2a to PCV2h) [3–5]. When clinical PCVAD was recognized in pigs for the first time in the mid-1990s [38], PCV2a was the most common genotype [39]. At the turn of the century, PCV2b started to be identified on a regular basis in cases of PCVAD outbreaks or epidemics that occurred initially in Europe [40] and during 2005–2006 in North America [7]. Once PCV2 vaccination was implemented, clinical PCVAD largely disappeared until cases of suspected "vaccine failures" were recognized for the first time as early as 2010–2013 [41] and associated with PCV2d. Currently, PCV2d is the most prevalent genotype worldwide [10]. During a 2016–2018 survey, the intra-PCV2b homology in Brazil ranged from 97–97.9%, while the intra-PCV2d homology ranged from 93.1–94.4% (personal communication AMMGC). All of these changes raise questions about the impact of large-scale PCV2a-based vaccination on efficacy against field exposure to PCV2b and PCV2d genotypes, including impact on viral evolution and possible selective advantages.

The vaccine tested in this study is based on a PCV2b strain. After selecting a PCV2 vaccine candidate, the subsequent validation and production steps including quality and safety validation may take several years. The vaccine candidate in this study was selected prior to the time when PCV2d emerged. Under experimental conditions, PCV2b-based vaccines have been shown to be more effective in reducing PCV2b [28] and PCV2d-based vaccines have been more effective reducing PCV2d [29] viremia compared to PCV2a-based vaccines. In further support of the concept to use PCV2b genotype antigen in vaccines, a commercial mixed PCV2a/2b vaccine based on chimeric PCV1-2a and PCV1-2b isolates (Fostera® Gold PCV2, Zoetis) was released on the US market in 2018 (https://www.zoetisus.com/news-and-media/zoetis-introduces-u.s.-pork-industry_s-first-vaccine-with-two-pcv2-genotypes.aspx; accessed on 26-Jun-2019). Under the circumstances, a PCV2b subunit vaccine was developed and tested in the PCV2b and *M. hyopneumoniae* combined challenge pig model. The challenge model was chosen as PCV2 alone is often not sufficient to result in significant differences among groups under experimental conditions. Previously it has been established that infection of pigs with *M. hyopneumoniae* followed by PCV2 infection 2 weeks later results in significantly more clinical signs and lesions compared to single

infection [16,30]. In the interest of reducing animal usage in research and to comply with 3R (replacement, reduction, refinement) guidelines, single infected groups were not included in this study. Successful experimental *M. hyopneumoniae* and PCV2b coinfection was achieved in the current study. Detection levels of *M. hyopneumoniae* antibodies are in general low using current serology assays [42]. As expected, and similar to our previous study [16], only a portion of the pigs seroconverted to *M. hyopneumoniae* while *M. hyopneumoniae* antigen in lung tissues was detectable in most pigs by termination of the study.

PCV2b vaccination stimulated development of neutralizing antibodies to PCV2a and PCV2d, resulted in higher levels of cellular immunity to PCV2b stimulation, significantly reduced PCV2b viremia compared to the non-vaccinated POS-CONTROL group and also significantly ($P < 0.001$) increased the percentages of pigs classified as normal/healthy with no evidence of PCV2-associated lesions (overall lesions score = 0). In this study, a subunit PCV2 vaccine candidate was tested. While it is suggestive that the obtained results may also be applicable to other PCV2 vaccine types, including inactivated vaccines, this has not been tested. A potential negative impact of *M. hyopneumoniae* infection on PCV2 vaccine efficacy was not evaluated; however, based on the obtained vaccine efficacy results it seems unlikely. When comparing the vaccination protocols, there was a slight advantage of giving the vaccine twice as a split dose (1 mL and 1 mL) two weeks apart compared to a single dose of 2 mL. Though not statistically significant, the two-dose regimen resulted in numerically higher levels of cellular immunity and higher levels of humoral immunity. Overall, the results of this study demonstrate that the PCV2b based vaccine candidate was efficient in a PCV2b and *M. hyopneumoniae* challenge model and no adverse reactions were observed after vaccination.

Funding

This work was supported by a grant through Ourofino Saúde Animal Ltda, Cravinhos, SP, Brazil. We would also like to acknowledge Biotechnology and Biological Sciences Research Council (BBSRC) support of the Roslin Institute Strategic Programme Control of Infectious Diseases (BBS/E/D/20002173 and BBS/E/D/20002174).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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