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ORIGINAL ARTICLE

An experimental live chimeric porcine circovirus 1-2a vaccine decreases porcine circovirus 2b viremia when administered intramuscularly or orally in a porcine circovirus 2b and porcine reproductive and respiratory syndrome virus dual-challenge model

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ABSTRACT

Commercially available inactivated vaccines against porcine circovirus type 2 (PCV2) have been shown to be effective in reducing PCV2 viremia. Live-attenuated, orally administered vaccines are widely used in the swine industry for several pathogens because of their ease of use yet they are not currently available for PCV2 and efficacy. The aims of this study were to determine the efficacy of a live-attenuated chimeric PCV2 vaccine in a dual-challenge model using PCV2b and porcine reproductive and respiratory syndrome virus (PRRSV) and to compare intramuscular (IM) and oral (PO) routes of vaccination. Eighty-three 2-week-old pigs were randomized into 12 treatment groups: four vaccinated IM, four vaccinated PO and four non-vaccinated (control) groups. Vaccination was performed at 3 weeks of age using a PCV1-2a live-attenuated vaccine followed by no challenge, or challenge with PCV2b, PRRSV or a combination of PCV2b and PRRSV at 7 weeks of age. IM administration of the vaccine elicited an anti-PCV2 antibody response between 14 and 28 days post vaccination, 21/28 of the pigs being seropositive prior to challenge. In contrast, the anti-PCV2 antibody response in PO vaccinated pigs was delayed, only 1/27 of the pigs being seropositive at challenge. At 21 days post challenge, PCV2 DNA loads were reduced by 80.4% in the IM vaccinated groups and by 29.6% in the PO vaccinated groups. PCV1-2a (vaccine) viremia was not identified in any of the pigs. Under the conditions of this study, the live attenuated PCV1-2a vaccine was safe and provided immune protection resulting in reduction of viremia. The IM route provided the most effective protection.

Key words Coinfection, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, vaccine efficacy.

Introduction

Porcine circoviruses are divided into two main genotypes: PCV1 and PCV2 (1–3). PCV1 was initially identified as a cell culture contaminant of the porcine kidney cell line PK-15 (4) and is generally thought to be non-pathogenic in pigs (5, 6). In contrast, PCV2 is pathogenic and associated with a number of diseases in pigs, including

reproductive failure in breeding animals (7, 8) and post-weaning clinical manifestations such as systemic disease, respiratory disease, enteritis, and porcine dermatitis and nephropathy syndrome (PDNS) (9, 10). PCV2 is a small, non-enveloped, single-stranded DNA virus with a circular genome of 1767 to 1768 nt (11, 12). It belongs to the genus *Circovirus* in the family *Circoviridae* (13). The genome of PCV2 consists of two ORFs: ORF1 encodes proteins associated with viral replication (Rep and Rep') (14), and ORF2 encodes the immunogenic capsid protein (15). A third ORF, ORF3, is reportedly involved in apoptosis of lymphocytic and hepatic cells (16), although its role in PCV2 pathogenesis remains unclear (17). Several PCV2 subtypes have been described, including PCV2a and PCV2b which are prevalent worldwide (18).

Coinfection of pigs with PCV2 and PPV (19–21), PCV2 and *Mycoplasma hyopneumoniae* (22), and PCV2 and PRRSV (23–25) have been shown to increase PCV2 replication and the severity of clinical disease. Among the known co-infecting pathogens, PRRSV is the most commonly identified virus in field cases of PCVAD (26, 27). Accumulating evidence suggests that co-infection of pigs with two or more pathogens substantially increases the severity of disease in pig production systems (28, 29).

Prior to commercial PCV2 vaccines becoming widely available, PCVAD caused significant economic losses to swine producers (29); however, with the approval and extensive use of several inactivated or subunit PCV2 vaccines, these losses have been minimized in many pig production systems across the world (30–34). The currently available commercial PCV2 vaccines include two subunit vaccines based on the PCV2 capsid protein expressed in the baculovirus system and an inactivated vaccine based on a PCV2 virus (9). All of these vaccines are based on the PCV2a subtype, which several studies have shown to be cross-protective against PCV2b challenge (35, 36).

An experimental live chimeric vaccine was generated with the idea that it might provide more broad cross protection and better immunity, and could be adapted for use by the oral route. The experimental chimeric PCV2 vaccine was developed by replacing the ORF2 of PCV1 with the ORF2 of PCV2a in the genomic backbone of the non-pathogenic PCV1 (37). An inactivated version of the chimeric PCV2 vaccine, which was known under the trade name Suvaxyn PCV2 (Fort Dodge Animal Health, Overland Park, KS, USA) and developed and licensed for pigs 3 weeks of age and older, became commercially available in 2006 (9). It was later voluntarily removed from the market but was then reintroduced in August 2011 in a reformulated version under a new name: Fostera PCV (Pfizer Animal Health, Madison, NJ, USA). Previous studies using the experimental live attenuated PCV2 vaccine demonstrated no evidence of reversion of the live attenuated PCV1-2 to its parental wild-type viruses (PCV1 or PCV2) after 11 serial passages in PK-15 cells and the PCV1-2 was found to be genetically stable during three serial passages in pigs (38). In addition, the experimental live chimeric PCV2 vaccine was shown to be attenuated in pigs and to induce strong protective immunity in the PCV2a challenge model (39) and in a triple challenge model (40).

Recently, the vaccine efficacy of IM administration of the live-attenuated chimeric PCV2 experimental vaccine based on subtype PCV2a was tested in a triple challenge model using PCV2b, PPV and PRRSV (41). In conventional pigs with variable amounts of anti-PCV2 antibodies and degrees of PCV2 viremia at the time of vaccination, the live-attenuated chimeric PCV2 vaccine was found to reduce the amount of PCV2 DNA in serum compared to non-vaccinated challenged pigs (41). In addition to the chimeric PCV2 vaccine based on PCV2a, a novel chimeric PCV2 virus with the PCV2b capsid gene cloned into the backbone of PCV1 was recently described (42). In a single challenge model in SPF pigs using a PCV2a or PCV2b challenge, IM administered attenuated live chimeric PCV2b vaccine was found to decrease lymphoid lesions and to prevent detectable PCV2 viremia (42). The efficacy of the live-attenuated chimeric PCV2b vaccine administered by combined IM and intranasal routes was also evaluated in a PCV2b-PRRSV-PPV triple challenge model and found to induce protective immunity in SPF pigs (40).

The potential advantages of using a live-attenuated vaccine include an overall better immune response due to activation of cellular immunity in addition to humoral immunity. Moreover, PO administration of live-attenuated vaccines could potentially result in activation of the mucosal immune system, which is important in first defense against pathogens transmitted predominately via the fecal-oral route such as PCV2. In addition, administration through drinking water reduces the risk (needle breakage, missed pigs) and cost (labor, needles) associated with IM administration. The primary objective of this study was to compare the efficacy of IM and PO routes of vaccination using a live-attenuated chimeric PCV2 vaccine in a PCV2b-PRRSV dual-challenge model.

MATERIALS AND METHODS

Animals and housing

Eighty-three, 14-day-old, colostrum-fed, crossbred SPF pigs were obtained from a herd confirmed to be free of PCV2, PRRSV, and SIV by routine serological testing. The pigs were weaned and transported to the Livestock Infectious Disease Isolation Facility at Iowa State University, Ames, Iowa, USA. On the day of arrival, the pigs were randomly assigned to one of 12 groups (as described in

Table 1. Experimental design

Group designation	n	Vaccination route	Challenge	
			PCV2	PRRSV
Negative controls	7	-	-	-
PCV2-I	7	-	Yes	-
PRRSV-I	7	-	-	Yes
PCV2-PRRSV-CoI	7	-	Yes	Yes
IM-non-challenged	7	IM	-	-
IM-PCV2-I	7	IM	Yes	-
IM-PRRSV-I	7	IM	-	Yes
IM-PCV2-PRRSV-CoI	7	IM	Yes	Yes
PO-non-challenged	6	PO	-	-
PO-PCV2-I	7	PO	Yes	-
PO-PRRSV-I	7	PO	-	Yes
PO-PCV2-PRRSV-CoI	7	PO	Yes	Yes

Vaccination with the experimental live-attenuated chimeric PCV2 vaccine was performed on dpc −28 and the dual-challenge using PCV2b and PRRSV on dpc 0.

CoI, co-inoculated; I, inoculated.

Table 1) and eight rooms. Non-vaccinated (four rooms) and vaccinated groups (four rooms) were separated according to treatment group (PRRSV, PCV2, PCV2 and PRRSV, non-challenged pigs). Within each room, the pigs were contained in one (non-vaccinated groups) or two (vaccinated groups) raised wire decks equipped with one nipple drinker and one self-feeder. In the case of the vaccinated groups, the pigs were separated into pens by vaccine administration route, the pens being located on different sides of the room. All staff entering pens were required to change their outerwear between pens. All groups were fed *ad libitum* with a balanced, pelleted feed ration free of animal proteins (excluding whey) and antibiotics (Nature's Made, Heartland Co-op, West des Moines, IA, USA).

Experimental design

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (Institutional Animal Care and Use Committee number 8-08-6618-S). The experimental design is summarized in Table 1. Single infection groups were included as controls to better assess the consequences of dual-infection and the vaccine type used. Prior to starting the animal experiments, all pigs were confirmed to be PCV2-seronegative by PCV2 ELISA (43) and to be PRRSV-seronegative by a commercially available PRRSV ELISA (HerdChek PRRS virus antibody test kit 2XR, IDEXX Laboratories, Westbrook, MA, USA). Twenty-eight days before challenge (−28 dpc), pigs in the vaccinated groups received a PCV1-2a live-attenuated vaccine PO (n = 27) or IM (n = 28). A portion

of the vaccinated and non-vaccinated pigs were then challenged with wildtype PCV2b, PRRSV, or both PCV2b and PRRSV (Table 1) on 0 dpc. Necropsy was conducted at 21 dpc. Between −28 dpc and 21 dpc, blood was collected from all pigs on a weekly basis in 8.5 mL serum separator tubes (Fisher Scientific, Austin, TX, USA). The blood was centrifuged at 2000 g for 10 min at 4°C and serum stored at −80°C until testing. Serum samples were tested for amounts of anti-PCV2-antibody, anti-PRRSV-antibody, PCV1-2a DNA, PCV2 DNA, and PRRSV RNA. Tissues collected during necropsy were analyzed by IHC for the presence of PCV2 antigen.

Clinical observation and average daily weight gain

All pigs were weighed on the day of arrival, vaccination and challenge and at necropsy. The average daily weight gain was calculated before (−28 to 0 dpc), after challenge (0 to 21 dpc), and for the entire study period (−28 to 21 dpc). In addition, all animals were examined daily for signs of illness such as: lethargy, respiratory signs, inappetance and lameness.

Vaccination

The pigs were vaccinated at −28 dpc with 2 mL of an experimental live-attenuated chimeric PCV2 vaccine with an ORF2 based on the PCV2a subtype (PCV1-2a) as previously described (37, 39) at a titer of 1.6×10^3 TCID$_{50}$ per mL. This is the same titer as was used for the inactivated version of the chimeric PCV2 vaccine (Suvaxyn PCV, Fort Dodge Animal Health). For the IM route of vaccination, 2 mL of the experimental PCV1-2a vaccine was injected into the right side of the neck using a 0.7 mm × 25.4 mm needle and a 3 mL syringe. For the PO route of vaccination, each pig was held in an upright position and the experimental vaccine administered by slowly dripping 2 mL into their mouths using a 3 mL syringe. The volume of vaccine dose for both IM and PO routes (2 mL) was chosen on the basis of what is routinely used and convenient for vaccinating pigs in the field.

Porcine circovirus type 2b challenge

The PCV2b isolate NC-16845 was propagated on PK-15 cells to produce a virus stock at an infectious dose of $2.5 \times 10^{3.0}$ TCID$_{50}$ per mL, which was used to challenge the pigs. At dpc 0, each pig in the PCV2-challenged groups (Table 1) received 1 mL of the virus inoculum IM into the right neck area and 3 mL (1.5 mL per nostril) intranasally by holding the pig in the upright position and administering the inoculum by slowly dripping 1.5 mL into each nostril using a 3 mL syringe.

Porcine reproductive and respiratory syndrome virus challenge

Porcine reproductive and respiratory syndrome virus isolate ATCC VR2385 (44, 45) was propagated on MARC-145 cells to produce an infectious stock with a titer of $1 \times 10^{5.0}$ TCID$_{50}$/mL. At dpc 0, each pig in the PRRSV-challenged groups (Table 1) received 2.5 mL of the PRRSV challenge virus inoculum intranasally in a similar fashion to that described for PCV2 inoculation.

Serology

All serum samples from all groups were tested for anti-PCV2-antibodies using the SERELISA PCV2 Ab Mono Blocking kit (Synbiotics Europe, Lyon, France) according to the manufacturers' instructions. The results were expressed as a SNc ratio, samples being considered negative if the SNc ratio was > 0.50 and positive if it was ≤ 0.50. Serum samples collected at −28, 0 and 21 dpc were tested for the presence of anti-PRRSV antibodies by ELISA (HerdChek PRRS virus antibody test kit 2XR, IDEXX Laboratories).

RNA and DNA extraction

Total nucleic acids were extracted from serum samples using the MagMax Viral RNA and DNA Isolation Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and an automated DNA/RNA extraction system (Thermo Scientific Kingfisher Flex, Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the manufacturers' instructions. Samples were extracted and run in single well qPCR reactions due to the large sample numbers, high cost of testing, and previous work by the author's group showing that triplicate wells give almost identical results (46).

Detection of porcine circovirus type 2 and porcine circovirus type 1-2 DNA by quantitative real-time polymerase chain reaction

Serum samples collected at −7, −14, −21, 0, 7, 14, and 21 dpc were tested for the presence of PCV1-2 DNA and samples collected at 0, 7, 14, and 21 dpc were tested for the presence of PCV2 DNA by quantitative real-time PCR assays using primer-probe combinations as described previously (46) with the following modifications: a commercially available master mix (TaqMan Fast Universal PCR Master Mix, Applied Biosystems) was used, the reaction volume was 25 μL, only one aliquot was tested for each sample and the thermal cycler conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Samples were considered negative when no signal was observed within the 40 amplification cycles. Five serial dilutions of a PCV2 genomic DNA clone (10^5 to 10^9 copies/mL) were used to generate a standard curve with a correlation coefficient of > 0.99 (46).

Detection of porcine reproductive and respiratory syndrome virus RNA by quantitative reverse transcriptase real-time polymerase chain reaction

Serum samples collected at 7, 14 and 21 dpc were tested for the presence and amount of PRRSV RNA as described previously (41). Samples were considered negative when no signal was observed within the 40 amplification cycles.

Necropsy

All pigs were humanely euthanized by intravenous pentobarbital sodium overdose (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) and necropsied at 21 dpc. The extent of macroscopic lung lesions (ranging from 0 to 100%) was estimated and scored as described previously (44). The sizes of superficial inguinal lymph nodes were compared among groups as described previously (47). Sections of lymph nodes (superficial inguinal, external iliac, mediastinal, tracheobronchial, and mesenteric), tonsil, heart, thymus, kidney, colon, spleen, liver, small (ileum) and large intestine (spiral colon) were collected at necropsy, fixed in 10% neutral-buffered formalin, and routinely processed for histological examination.

Histopathology

Microscopic lesions were evaluated by two veterinary pathologists (TO, PGH) who were blinded to the treatment groups. Lung sections were scored for the presence and severity of interstitial pneumonia, ranging from 0 (normal) to 6 (severe diffuse) (44). Sections of heart, liver, kidney, ileum, colon and thymus were evaluated for the presence of granulomatous inflammation and scored from 0 (none) to 3 (severe). Lymph nodes, spleen, and tonsil were evaluated based on LD and HR of follicles, ranging from 0 (normal) to 3 (severe) (22).

Immunohistochemistry

Immunohistochemistry for detection of PCV2-specific antigen was performed on formalin-fixed and paraffin-embedded sections of lymph nodes (superficial inguinal, external iliac, mediastinal, tracheobronchial, and mesenteric), tonsil, and spleen using a rabbit PCV2 polyclonal antiserum as described previously (48). PCV2 antigen scoring was done by a veterinary pathologist (TO) who was blinded to the animal group designations. Scores ranged from 0 (no signal) to 3 (more than 50% of lymphoid follicles contained cells with PCV2 antigen staining) (22).

Overall lymphoid lesions score

The overall lymphoid lesion score was calculated as previously described (22). 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. The scores (lesions and PCV2-IHC) of the seven lymphoid tissues ([lymph node pool] × 5, spleen, and tonsil) were added together and divided by 7. The lymph nodes examined and scored consisted of one section each of tracheobronchial, superficial inguinal, external iliac, mediastinal, and mesenteric lymph nodes.

Statistical analysis

For data analysis, JMP software version 8.0.1 (SAS Institute, Cary, NC, USA) was used. Summary statistics were calculated for all the groups to assess the overall quality of the data set including normality. Statistical analysis of the data was performed by one-way ANOVA for continuous data ($\log_{10}$ transformed PCR data, ELISA data, average daily weight gain and macroscopic lung scores). A P-value of < 0.05 was set as the statistically significant level. Pairwise tests using Tukey's adjustment were subsequently performed to determine which differences among groups were statistically significant. Real-time PCR results (copies per mL of serum) were $\log_{10}$ transformed prior to statistical analysis. Non-repeated nominal data (histopathology scores, IHC scores, and lymph nodes size) were assessed using a non-parametric Kruskal-Wallis one-way ANOVA, and if there was a significant difference, pairwise Wilcoxon tests were used to evaluate differences among groups. Differences in prevalence were determined by using χ^2 tests. Percent reduction for amount of PCV2 DNA was determined as follows: $100 - ([100 \times \text{mean } \log_{10} \text{ genomic copies/mL in the vaccinated group}] \div (\text{mean } \log_{10} \text{ genomic copies/mL in positive control animals}])$.

RESULTS

Clinical observation and average daily weight gain

No signs of illness were noted in any animals throughout the course of the study. There were no significant ($P > 0.05$) differences in body weight among the treatment groups at -28, 0 or 21 dpc. Mean group average daily weight gain from 0 to 21 dpc is summarized in Table 2. Vaccination did not impact the average daily weight gain from -28 to 0 dpc as there were no statistically significant differences between non-vaccinated pigs (n = 28; 14.4 ± 0.9 kg), pigs vaccinated PO (n = 27; 14.9 ± 0.7 kg), or pigs vaccinated intramuscularly (n = 28; 15.1 ±

0.7 kg). In addition, there were no significant differences in average daily weight gain in either of the two time frames from 0 to 21 dpc and from -28 to 21 dpc (data not shown).

Anti- porcine circovirus type 2 antibody concentrations

The antibody responses to PCV2 (prevalence and mean group SNc ratios) are summarized in Table 3. All non-vaccinated animals (negative controls, PCV2-I, PRRSV-I, PCV2-PRRSV-CoI) remained seronegative for PCV2 until 7 dpc. The groups not challenged with PCV2 (negative controls, PRRSV-I) remained seronegative throughout the study. After intramuscular vaccination, anti-PCV2 antibody was first detected at 2 weeks post vaccination (-14 dpc) at which time 2/28 of the pigs had seroconverted. By -7 dpc, 15/28 of the pigs were PCV2 seropositive, and by 0 dpc 21/28 of the pigs were seropositive. After PO vaccination, anti-PCV2 antibodies were first detected at 4 weeks post vaccination (0 dpc) in 1/27 of the pigs; non-PCV2 inoculated groups (PO-non-challenged, PO-PRRSV-I) had 5/13, 9/13, and 8/13 seropositive pigs at 7, 14, and 21 dpc, respectively (Table 3). From -14 dpc until the day of challenge, the mean group ELISA SNc ratios in all IM vaccinated groups were significantly ($P < 0.05$) lower than those of non-vaccinated pigs or pigs vaccinated PO. All pigs vaccinated IM continued to have the lowest mean ELISA SNc ratios after challenge. All groups that were vaccinated PO had significantly ($P < 0.05$) lower mean group SNc ratios than those of non-vaccinated pigs at -14 dpc.

Porcine circovirus type 1-2 viremia

The experimental PCV1-2 vaccine DNA was detected in serum samples from two, three, and two vaccinated pigs at -21, -14, -7 dpc, respectively which corresponds to 7, 14 and 21 days post vaccination. Among the PCV1-2 DNA positive pigs, PCV1-2 DNA was only observed at one time point, indicating that vaccine-induced viremia was of short duration. The distribution of PCV1-2 DNA positive pigs across groups was as follows: 2/5 IM-non-challenge, 1/5 IM-PCV2-I, 1/5 IM-PCV2-PRRSV-CoI and 1/5 PO-PRRSV-I. PCV1-2 DNA was not detected in serum samples from any of the pigs at 0, 7, 14, and 21 dpc (data not shown).

Prevalence and amount of porcine circovirus type 2 DNA in serum

Porcine circovirus type 2 DNA was not detected in any serum samples collected at 0 dpc or in any of non-PCV2 infected groups (negative controls, PRRSV-I, IM-non-challenged, IM-PRRSV-I, PO-non-challenged,

Table 2. Group mean values for average daily weight gain from 0 to 21 dpc, macroscopic lung lesions (score range: 0–100%), amount of PCV2 antigen in tissues as determined by immunohistochemistry (score range: 0–3), and the overall lymphoid lesion score (score range: 0–9)

Group designation	Average daily weight gain (kg)	Gross lung lesion scores	PCV2 antigen in tissues	Overall lymphoid lesions
Negative controls ($n = 7$)	14.9 ± 0.9	0.0 ± 0.0^A	0.0 ± 0.0	0.1 ± 0.1^A
PCV2-I ($n = 7$)	15.1 ± 1.0	0.0 ± 0.0^A	1.3 ± 0.6	$2.1 \pm 0.7^{B,C}$
PRRSV-I ($n = 7$)	13.3 ± 1.0	$10.0 \pm 2.4^{A,B}$	0.0 ± 0.0	0.4 ± 0.2^A
PCV2-PRRSV-CoI ($n = 7$)	11.8 ± 0.7	20.3 ± 6.8^B	1.7 ± 0.9	$3.0 \pm 0.9^{B,C}$
IM-non-challenged ($n = 7$)	15.2 ± 1.0	0.0 ± 0.0^A	0.0 ± 0.0	0.1 ± 0.1^A
IM-PCV2-I ($n = 7$)	14.6 ± 0.9	3.1 ± 2.5^A	0.4 ± 0.4	$0.8 \pm 0.4^{A,C}$
IM-PRRSV-I ($n = 7$)	14.3 ± 1.5	$10.3 \pm 3.8^{A,B}$	0.0 ± 0.0	0.6 ± 0.5^A
IM-PCV2-PRRSV-CoI ($n = 7$)	15.1 ± 1.6	0.3 ± 0.3^A	0.6 ± 0.2	$0.9 \pm 0.4^{B,C}$
PO-non-challenged ($n = 6$)	16.1 ± 0.8	0.0 ± 0.0^A	0.0 ± 0.0	0.0 ± 0.0^A
PO-PCV2-I ($n = 7$)	15.2 ± 1.4	0.9 ± 0.9^A	1.3 ± 0.5	3.1 ± 0.8^B
PO-PRRSV-I ($n = 7$)	15.1 ± 1.2	0.3 ± 0.3^A	0.0 ± 0.0	0.5 ± 0.3^A
PO-PCV2-PRRSV-CoI ($n = 7$)	13.5 ± 1.0	5.0 ± 3.6^A	0.7 ± 0.6	$0.9 \pm 0.5^{B,C}$

Different superscripts within a column (A, B, C) indicate significant ($P < 0.05$) differences in group means.

PO-PRRSV-I) at 7, 14 and 21 dpc (data not shown). The prevalence of PCV2 DNA positive pigs at 7, 14 and 21 dpc and the group means are summarized in Table 4. In non-vaccinated pigs (PCV2-I, PCV2-PRRSV-CoI), 12/14, 14/14, and 14/14 of the pigs were viremic at 7, 14, and 21 dpc, respectively. In pigs vaccinated IM, 3/14 pigs were viremic on 7, 14, and 21 dpc. In pigs vaccinated PO, 10/14, 11/14, and 10/14 of the pigs were viremic at 7, 14, and 21 dpc, respectively. Compared to the non-vaccinated groups, the PCV2 DNA load in the serum was reduced in the IM vaccinated groups by 79.2% (7 dpc), 84.6% (14 dpc) and 80.4% (21 dpc). For PO vaccinated groups, the PCV2 DNA load in the serum compared to the non-vaccinated pigs was reduced by 24.6% (7 dpc), 20.8% (14 dpc) and 29.6% (21 dpc), respectively.

Anti- porcine reproductive and respiratory syndrome virus antibody response

All pigs were negative for anti-PRRSV IgG at −28 and 0 dpc and non-PRRSV challenged pigs remained seronegative for PRRSV until 21 dpc. All pigs challenged with PRRSV had seroconverted by 21 dpc, there being no differences among groups in mean group S/P ratios.

Prevalence and amount of porcine reproductive and respiratory syndrome virus RNA in serum

Porcine reproductive and respiratory syndrome virus RNA was detected only in the groups inoculated with PRRSV and 100% (42/42) of the PRRSV inoculated animals were positive for PRRSV RNA at 7, 14, and 21 dpc. The group $\log_{10}$ PRRSV RNA means were not significantly different among the PRRSV-inoculated groups (data not shown).

Gross lesions

Macroscopic lesions were characterized by lungs that failed to collapse, were a mottled tan color, and had variable amounts of cranioventral tan consolidation (particularly in pigs infected with PRRSV). The group mean gross lesion scores are summarized in Table 2. Interestingly, the IM-PCV2-PRRSV-CoI group had a lower mean group lung lesion score than the IM-PCV2-I and IM-PRRSV-I groups; however, this was not statistically significant. Lymph node sizes ranged from normal to double in size without differences among groups.

Microscopic lesions and porcine circovirus type 2 antigen in tissues

Microscopic lung lesions were characterized by mild-to-moderate, focal-to-multifocal interstitial pneumonia characterized by type 2 pneumocyte hypertrophy and hyperplasia and increased numbers of lymphocytes and macrophages in the alveolar septa. In general, the lesions appeared to be in the resolving stages. Lymphoid lesions were characterized by mild-to-severe lymphoid depletion of follicles and histiocytic replacement of primary or secondary follicular nodes in lymph nodes, tonsil, and spleen. PCV2 antigen was not detected in any of the non-PCV2 challenged pigs. The prevalence of PCV2 IHC positive animals was as follows: PCV2-I, 3/7; PRRSV-PCV2-CoI, 5/7; IM-PCV2-I, 1/7; IM-PCV2-PRRSV-CoI, 4/7; PO-PCV2-I, 5/7; and PO-PCV2-PRRSV-CoI, 1/7. Mean group PCV2 IHC scores are summarized in Table 2. In general, PCV2-associated lesions were mild (overall lymphoid score range 0 to 3) in IM-PCV2-I and the IM-PCV2-PRRSV-CoI groups, mild-to-moderate (overall lymphoid score range 0 to 6) in PO-PCV2-I and PO-PCV2-PRRSV-CoI and

Table 3. Prevalence and mean SNc ratio ± SE in each group of pigs on different dpc.

Group	−28 dpc	−21 dpc	−14 dpc	−7 dpc	0 dpc	7 dpc	14 dpc	21 dpc
Negative controls	0/7 (0.75 ± 0.01)	0/7 (1.10 ± 0.03)	0/7 (1.02 ± 0.05)	0/7 (0.87 ± 0.02)	0/7 (0.80 ± 0.05)	0/7 (0.80 ± 0.03)	0/7 (0.76 ± 0.02)	0/7 (0.71 ± 0.02)
PCV2-I	0/7 (0.77 ± 0.01)	0/7 (1.12 ± 0.02)	0/7 (1.03 ± 0.04)	0/7 (0.89 ± 0.02)	0/7 (0.86 ± 0.02)	0/7 (0.82 ± 0.02)	6/7 (0.30 ± 0.07)	7/7 (0.26 ± 0.05)
PRRSV-I	0/7 (0.85 ± 0.05)	0/7 (1.15 ± 0.02)	0/7 (1.00 ± 0.05)	0/7 (0.84 ± 0.03)	0/7 (0.83 ± 0.04)	0/7 (0.80 ± 0.02)	0/7 (0.73 ± 0.02)	0/7 (0.79 ± 0.02)
PCV2-PRRSV-CoI	0/7 (0.79 ± 0.01)	0/7 (1.12 ± 0.03)	0/7 (1.06 ± 0.04)	0/7 (0.89 ± 0.02)	0/7 (0.83 ± 0.03)	0/7 (0.81 ± 0.04)	6/7 (0.26 ± 0.07)	7/7 (0.23 ± 0.03)
IM-non-challenged	0/7 (0.79 ± 0.01)	0/7 (1.12 ± 0.03)	0/7 (0.85 ± 0.03)	4/7 (0.54 ± 0.05)	7/7 (0.29 ± 0.02)	7/7 (0.26 ± 0.06)	7/7 (0.17 ± 0.03)	7/7 (0.08 ± 0.03)
IM-PCV2-I	0/7 (0.76 ± 0.03)	0/7 (1.11 ± 0.04)	0/7 (0.89 ± 0.03)	3/7 (0.62 ± 0.10)	4/7 (0.48 ± 0.10)	6/7 (0.32 ± 0.06)	6/7 (0.17 ± 0.03)	7/7 (0.08 ± 0.03)
IM-PRRSV-I	0/7 (0.78 ± 0.02)	0/7 (1.15 ± 0.04)	2/7 (0.71 ± 0.10)	5/7 (0.38 ± 0.08)	6/7 (0.39 ± 0.10)	7/7 (0.20 ± 0.05)	7/7 (0.11 ± 0.03)	7/7 (0.08 ± 0.06)
IM-PCV2-PRRSV-CoI	0/7 (0.79 ± 0.02)	0/7 (1.14 ± 0.03)	0/7 (0.81 ± 0.04)	3/7 (0.57 ± 0.09)	4/7 (0.49 ± 0.10)	7/7 (0.20 ± 0.02)	7/7 (0.07 ± 0.02)	7/7 (0.04 ± 0.02)
PO-non-challenged	0/6 (0.84 ± 0.02)	0/6 (1.17 ± 0.03)	0/6 (0.92 ± 0.02)	0/6 (0.80 ± 0.02)	1/6 (0.69 ± 0.07)	2/6 (0.61 ± 0.07)	4/6 (0.41 ± 0.08)	4/6 (0.37 ± 0.09)
PO-PCV2-I	0/7 (0.82 ± 0.02)	0/7 (1.16 ± 0.03)	0/7 (0.88 ± 0.01)	0/7 (0.83 ± 0.01)	0/7 (0.89 ± 0.03)	1/7 (0.73 ± 0.04)	5/7 (0.41 ± 0.07)	5/7 (0.37 ± 0.08)
PO-PRRSV-I	0/7 (0.81 ± 0.02)	0/7 (1.16 ± 0.02)	0/7 (0.92 ± 0.02)	0/7 (0.79 ± 0.04)	0/7 (0.76 ± 0.04)	3/7 (0.57 ± 0.06)	5/7 (0.42 ± 0.07)	4/7 (0.44 ± 0.11)
PO-PCV2-PRRSV-CoI	0/7 (0.79 ± 0.03)	0/7 (1.11 ± 0.05)	0/7 (0.94 ± 0.02)	0/7 (0.84 ± 0.02)	0/7 (0.78 ± 0.04)	1/7 (0.63 ± 0.06)	6/7 (0.35 ± 0.06)	5/7 (0.23 ± 0.07)

Groups that contain seropositive pigs are shaded in grey.
Data presented as number of positive pigs/total number of pigs per group (mean SNc ratio ± SE).

PCV2-I groups and mild-to-severe (overall lymphoid score range 0–8/) in the PCV2-PRRSV-CoI group. The mean group overall lymphoid scores are summarized in Table 2. Interestingly, the PO-PCV2-I group had a higher overall lymphoid score and a higher mean PCV2 IHC score compared to PO-PCV2-PRRSV-CoI group; however, this was not statistically significant.

DISCUSSION

An inactivated chimeric PCV2 vaccine (37) was one of the first products licensed for use in growing pigs (Suvaxyn PCV2, Pfizer Animal Health). All of the available commercial PCV2 vaccines to date are inactivated or subunit products and require one or two doses administered IM. While commercially available vaccines have been proven to be efficacious (31–34), the current products have some disadvantages, including the cost of the products and the labor required for administration. There is also increasing concern that currently available PCV2 vaccines may be becoming less effective over time in some herds. The primary objective of this study was to compare the efficacy of IM and PO routes of vaccination using an experimental live-attenuated chimeric PCV2 vaccine in a PCV2b-PRRSV dual-challenge model.

Because concurrent infection of pigs with PCV2 and PRRSV is common in swine herds (26, 49), it was important to evaluate the efficacy of the live-attenuated chimeric PCV2 vaccine in a dual-challenge model. Previously, an experimental live attenuated chimeric PCV2 vaccine based on subtype PCV2a and administered IM was tested in a triple challenge model utilizing PCV2b, PRRSV and PPV and compared to other commercially available inactivated or subunit vaccines (41). All of the PCV2 vaccines used in that study were effective at reducing PCV2 viremia during the growing period and after triple challenge with PCV2-PRRSV-PPV (41). However, in contrast to that study, which used conventional pigs that were seropositive and PCV2 viremic, in the current study we used PCV2 and PRRSV naïve pigs. In the current study, PRRSV viremia occurred in 100% of the animals in all groups infected with PRRSV and was detectable by 7 dpc. Concurrent PRRSV infection did not reduce vaccine efficacy as evidenced by the similar amounts of PCV2 DNA in all vaccinated groups regardless of challenge status (PCV2 versus PRRSV-PCV2). However, because it is not possible to differentiate between infectious and non-infectious virus particles by a PCR assay, we were not able to ascertain whether there were differences between groups in the amount of infectious PCV2.

Porcine circovirus type 1-2 DNA was identified in individual pigs (5/55) 7 to 21 days post vaccination and was not identified in any of the vaccinated pigs in the

Table 4. Prevalence and amount of porcine circovirus type 2 (PCV2) DNA positive pigs in each experimental group on different dpc

Group	7 dpc	14 dpc	21 dpc
PCV2-I	7/7 (5.3 ± 0.1)^A	7/7 (6.4 ± 0.2)^A	7/7 (6.0 ± 0.4)^A
PCV2-PRRSV-CoI	5/7 (3.7 ± 1.0)^A	7/7 (6.6 ± 0.3)^A	7/7 (6.1 ± 0.3)^A
IM-PCV2-I	3/7 (1.9 ± 0.9)^{A,B}	2/7 (1.3 ± 0.8)^B	2/7 (1.3 ± 0.8)^B
IM-PCV2-PRRSV-CoI	0/7 (0.0 ± 0.0)^B	1/7 (0.8 ± 0.8)^B	1/7 (1.1 ± 1.1)^B
PO-PCV2-I	4/7 (3.2 ± 1.1)^{A,B}	6/7 (5.5 ± 0.9)^A	6/7 (5.3 ± 0.9)^A
PO-PCV2-PRRSV-CoI	6/7 (3.5 ± 0.9)^A	5/7 (4.8 ± 0.9)^A	4/7 (3.2 ± 0.3)^{A,B}

Data presented as PCV2 DNA positive pigs/total number of pigs per group (mean group log$_{10}$ PCV2 DNA ± SE per mL serum).
Different superscripts within a column (A,B) indicate significant ($P<0.05$) differences between groups in mean group log$_{10}$ PCV2 DNA.

later stages of the experiment (0, 7, 14 and 21 dpc). Among the five PCR positive pigs, PCV1-2 DNA was only present at one point in time, indicating a short duration of viremia. This finding confirms the previous findings of Fenaux *et al.* (39), who did not identify PCV1-2 viremia in any vaccinated pigs. In addition, because co-infecting pathogens such as PRRSV are known to enhance PCV2 replication (23, 24, 50, 51), the absence of PCV1-2 viremia after challenge in PRRSV-infected pigs (IM-PRRSV-I, IM-PCV2-PRRSV-CoI, PO-PRRSV-I, PO-PCV2-PRRSV-CoI), as well as the absence of PCV2 specific staining in tissues of vaccinated non-challenged pigs (IM-non-challenged, IM-PRRSV-I, PO-non-challenged, PO-PRRSV-I) further emphasizes the attenuation and safety of this experimental PCV1-2 live vaccine. However, it needs to be emphasized that in the current study PRRSV was given 4 weeks after vaccination. Because PRRSV can be circulating continuously or at any time in relation to vaccination under field conditions, the results in the field could be different because of varying intervals between PRRSV infection and vaccination.

A novel aspect of the current study was evaluation of the PO route of administration of the experimental live-attenuated chimeric PCV2 vaccine. Previously, intra-lymphoid and IM routes of vaccination have been utilized for attenuated live PCV1-2 vaccines (37–39). When live PCV1-2 was administered via the IM and intra-lymphoid routes, pigs developed protective immunity against PCV2a (39). Most of the piglets seroconverted to PCV2 between 28 and 35 days post vaccination and, although not all the animals had seroconverted by the time of challenge, they were all protected against subsequent PCV2a challenge, suggesting that strong PCV2 antibody responses are not entirely necessary for protection (39). IM administration of a live PCV1-2 vaccine has also been demonstrated to be effective in conventional (41) and in SPF pigs (42). Similarly, combined IM and intranasal administration of live PCV2 vaccine reduced PCV2 viremia and associated lesions after challenge in SPF pigs (40). In our study, the majority of IM vaccinated pigs (21/28) had seroconverted four weeks after vaccination, which is in agreement with

previous studies (39, 40, 42). In contrast, among all the PO vaccinated pigs, only 1/28 pigs had seroconverted by four weeks post vaccination. The limited ability of the experimental live-attenuated PCV1-2 vaccine to induce a measurable systemic antibody response may be due to limited absorption and replication. Nevertheless, as evident from the PO-non-challenged group, PCV2 antibodies continued to increase beyond 4 weeks, indicating a delayed antibody response with the PO route of vaccination. Development of mucosal immunity by assessing presence of locally secreted PCV2 specific antibodies (for example in fecal supernatants) was not investigated, but may have given further insights into the effectiveness of this route.

In this study, PCV2 DNA in sera was detectable in all treatment groups challenged with PCV2b. This is in contrast to previous studies where PCV2 DNA was not detectable in vaccinated animals after challenge (39, 42). These conflicting results may be due to differences between studies in the detection methods for PCV2 DNA. For instance, the real-time PCR assay used in the current study is more sensitive than the gel-based PCR assay used previously (39). Other differences between studies include the utilization of a heterologous PCV2b challenge strain in the current study in contrast to a homologous PCV2a challenge strain used in a previous study (39).

Significant differences in prevalence and amount of PCV2 DNA, with a reduction of the amount of PCV2 DNA in sera ranging from 79.2% to 84.6%, were found in pigs vaccinated IM compared to non-vaccinated pigs. Moreover, only 21.4% of pigs vaccinated by the IM route were PCV2 viremic after PCV2 challenge. Among the IM vaccinated pigs that had no detectable seroconversion prior to challenge, subsequent PCV2 viremia was not observed in 1/3 IM-PCV2-I pigs and in 3/3 IM-PCV2-PRRSV-CoI pigs, indicating evidence of protection and strengthening the importance of cellular immune response. The amount of PCV2 DNA in sera was also reduced in pigs vaccinated PO; however vaccine efficacy in the PO vaccinated groups as measured by decreased incidence and degree of viremia was not as impressive as that of the IM vaccinated

groups. Specifically, PO vaccination reduced the amount of PCV2 DNA in sera by 20.8% to 29.6%, the prevalence of PCV2 viremia ranging from 71.4% to 78.6% between 7 and 21 dpc. In addition, except for the PO-PCV2-I group, the mean group PCV2 antigen amount in tissues was reduced by PCV2 vaccination. The differences in vaccine efficacy between the two different administration routes may be attributable to the interval between vaccination and challenge (4 weeks). The PO vaccination route appeared to induce a delayed antibody response suggesting that a longer interval is needed between vaccination and challenge. Alternatively, a higher dose may be required for induction of greater protective immunity with this route.

In conclusion, under the conditions of this study, an experimental live-attenuated PCV2 vaccine was safe and efficacious when used IM in a PCV2b-PRRSV dual-challenge model. Administration of the same product PO resulted in a lower level and delayed onset of protective immunity compared to IM administration. More studies are needed to improve the immunogenicity of the oral vaccine.

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DISCLOSURE

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