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# The impact of porcine circovirus associated diseases on live attenuated classical swine fever vaccine in field farm applications

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ABSTRACT

Porcine circovirus associated diseases (PCVADs) are among the most important diseases affecting the worldwide swine industry. Vaccination against porcine circovirus type 2 (PCV2) infection has been utilized for disease control and effectively reduces clinical signs of PCVADs. To evaluate the efficacy of the PCV2 vaccine in field farms, we conducted a trial using conventional pigs immunized with the subunit PCV2 vaccine followed by PCV2 challenge. Immunized pigs demonstrated lower serum viral loads, less viral antigen staining in lymph nodes, and higher average daily weight gain, confirming the protective efficacy of the vaccine. However, low levels of PCV2 infection were still detected in vaccinated pigs after challenge, suggesting that the PCV2 vaccine was unable to eradicate the virus, which could lead to asymptomatic PCV2 subclinical infection (PCV2-SI) in pig farms. Additionally, PCV2 infection is a risk factor for impaired pig immune response development during the weaning to growth stages, which is a crucial period to receive vaccines against classical swine fever (CSF). Therefore, the impact of PCV2-SI or PCV2-systemic disease (PCV2-SD) on live attenuated CSF vaccine was investigated. After PCV2 challenge, there was no difference in levels of classical swine fever virus (CSFV) neutralizing antibodies (NA) between pigs with PCV2-SD and PCV2-SI, suggesting that the efficacy of CSF vaccine was compromised. Moreover, results of long-term monitoring of CSFV NA titers in PCV2-SI pigs with minimized interference by maternally-derived antibodies suggested that serum PCV2 viral loads greater than $10^2$ copies/mL may compromise the efficacy of CSF vaccine. Overall, a conventional pig model was established to demonstrate the impaired efficacy of the subunit PCV2 vaccine and its impact on the CSF vaccine in vaccination-challenge trials. Additionally, the impaired efficacy of the PCV2 vaccine resulted in increased PCV2-SI, eventually leading to compromised the live attenuated CSF vaccine induced NA response in field farm applications.



## 1. Introduction

Concurrent infection with porcine circovirus type 2 (PCV2) and other infectious viruses and/or pathogenic bacteria that induce porcine circovirus associated diseases (PCVADs), is one of the most complicated and fastidious disease patterns affecting pigs of all ages [1]. Depending on the clinical symptoms they induce, PCVADs can be further classified as PCV2-systemic disease (PCV2-SD), PCV2-subclinical infection (PCV2-SI), PCV2-reproductive disease or porcine dermatitis and nephropathy syndrome (PDNS), all of which cause massive economic losses to the pig industry worldwide [2,3]. For control of PCV2 infection, several commercial vaccines based on the PCV2a strain, including the expressed subunit capsid protein vaccine, the inactivated whole virus vaccine and the inactivated chimeric virus vaccine, have been widely used over the past decade, leading to significant reductions in the number of outbreaks of severe PCVADs in pig farms [3,4]. However, recent research has revealed emerging variant PCV2 genotypes and suggested that escaped variants may be a result of incomplete vaccine immunity in the field [5–10]. Previous reports showed that the cross-protective efficacy of PCV2a-based commercial vaccines led to reduced viremia, shedding and transmission of PCV2 in experimental models of heterologous PCV2 (PCV2b or PCV2d) challenge

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[11,12]. However, reductions in heterologous viremia has caused variations in cross-protective efficacy, which could result in widespread PCV2-SI in pigs from the weaning stage to the growth stage [13–15]. This period is especially crucial for piglets to receive essential primary vaccinations against classical swine fever (CSF), which contribute to the prevention of disease outbreaks in most non-CSF free countries in Asia, Eastern Europe and South America [16].

Application of the live attenuated CSFV vaccine can induce both humoral and cellular immune responses in pigs and elicit protective immunity against CSF. The induction of neutralizing antibody (NA) can be detected as early as 5 days post vaccination, and seroconversion and protective responses are observed 12 days post vaccination [17,18]. Pigs with NA titers greater than 1:32 have been demonstrated adequate to reduce clinical signs and prevent the spread and transmission of CSFV in populations at epidemic regions [19,20]. However, interference by maternally-derived antibodies (MDA) delivered via the colostrum can cause delayed and diminished expression of specific acquired immune responses and a faster decline in induced NA titers after vaccination in immunized piglets. Moreover, concurrent infection with potential pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV) during the weaning stage may be an additional risk factor dramatically compromising the efficacy of the CSFV vaccine in piglets [21–24]. However, few studies focused on the impact of PCV2 infection associated with live attenuated CSFV vaccine in SPF animal models, and no study emphasized in field farms applications. Since PCV2 infection is highly endemic in pig farms, the risks associated with the combination of MDA interference and the potential impact of PCVADs on live attenuated CSFV vaccine require intensive evaluation in pig farms where PCVADs is present [25].

In this study, animal trials were carried out using an artificial PCV2 challenge model to mimic PCV2-SD or PCV2-SI while monitoring MDA interference to disentangle whether coexisting factors simultaneously impair live attenuated CSFV vaccine induced neutralizing antibody response in conventional pigs. Elicited NA titers against CSFV, the viral load of PCV2 in serum and lymphoid tissues, PCV2-specific antibodies and average daily weight gain (ADWG) were evaluated. Furthermore, a long-term observation trial with postponed CSFV vaccination strategy to minimize MDA interference was conducted to elucidate the impacts of PCVADs on the live attenuated CSFV vaccine in pigs.

## 2. Materials and methods

### 2.1. Farm and animal selection

A conventional continuous flow production pig farm located in Taichung City, Taiwan was selected. Sows in this farm are routinely immunized with CSF-E2 subunit vaccine 3–5 weeks before parturition. Piglets in this farm regularly receive one dose of subunit PCV2 vaccine at 3–4 weeks old and two doses of live attenuated CSFV vaccine (Lapinized Philippines Coronel [LPC] strain) at 6 and 9 weeks old as part of a prime-boost vaccination program. Staging was performed at 4 weeks of age and all post-weaned piglets were moved to fattening units at 12–13 weeks of age. Before the start of trials, a cross-sectional analysis including ten pigs at six different ages (3, 6, 12, 15, 18 and 21 weeks; a total of 60 serum samples) was performed to profile the PCV2 infection status of the farm. Serum samples were analyzed by quantitative PCR to detect PCV2 viral DNA and enzyme-linked immunosorbent assays (ELISA) to assess antibody titers in serum. PCV2-specific antibodies in serum were analyzed using an SLK105 kit (BioChek BV, Reeuwijk, The Netherlands) according to the manufacturer's protocol, and the antibody titer was expressed as the sample to positive (S/P) ratio. Serum samples with an S/P ratio greater than 0.500 were considered to be positive. An IDEXX CSFV Ab test kit (IDEXX Laboratories Inc., Liebefeld, Switzerland) was used to analyze CSFV-specific antibody titers in serum, and results were expressed as the blocking percentage. According to the manufacturer, serum samples with a blocking percentage greater than 40% are considered to be positive.

### 2.2. Experimental design and immunizations

Three trials were conducted to assess the potential effect of concurrent PCV2 infection on the live attenuated CSFV vaccine efficacy (Table 1). A baculovirus-expressed PCV2 capsid protein subunit emulsified with w/o/w adjuvant (PCV2 vaccine) and a live attenuated CSFV vaccine (LPC vaccine) were used to vaccinate pigs.

In trial I, a PCV2 challenge model was established to mimic PCVADs in conventional pig farms. Eight colostrum-fed pigs from the same litter were randomly assigned to two groups. Group A pigs (n = 4) were vaccinated with subunit PCV2 vaccine, whereas group B pigs (n = 4) were given a placebo (0.9% saline emulsified with adjuvant) at 3 weeks of age. At 7 weeks of age, all pigs were transferred to an isolated animal house and challenged with $2 \times 10^5$ 50% tissue culture infective dose ($TCID_{50}$) PCV2 via intranasal inoculation and intramuscular injection. The body weight of each pig was measured before immunization (3 weeks old), after PCV2 challenge (7 weeks old) and at the end of the trial (11 weeks old) to calculate ADWG. All pigs were sacrificed at 11 weeks of age. Conversion of serum PCV2-specific antibodies and PCV2 viral DNA load were also monitored to assess infection after artificial PCV2 challenge. ADWG, PCV2 specific antibody titer and PCV2 viral load were used as the criteria to define PCVADs in trial I [3].

In trial II, ten colostrum-fed piglets from the same litter were randomly assigned to two groups. Group C pigs (n = 6) were immunized with subunit PCV2 vaccine, whereas group D pigs (n = 4) were immunized with a placebo (0.9% saline emulsified with adjuvant) at 3 weeks of age. All piglets were immunized with LPC vaccine at 6 weeks of age and transferred to an isolated animal house. Pigs were then challenged with $2 \times 10^5$ $TCID_{50}$ PCV2 via intranasal inoculation and intramuscular injection at 7 weeks of age to mimic PCVAD outbreaks in the field. Two weeks after PCV2 inoculation, pigs were given one dose of LPC vaccine as part of the regular CSFV vaccination program in field farm. All pigs were sacrificed at 11 weeks of age. ADWG, serum PCV2 viral load, PCV2-specific antibody titer and histopathological changes in mesenteric lymph nodes were examined to evaluate differences in PCVAD between group C and group D. Conversion of CSFV-specific NAs was also assessed to investigate the interference of PCV2 infection on LPC vaccine efficacy.

In trial III, a late CSFV vaccination strategy was applied to minimize MDA interference. Twenty piglets from the same batch were randomly assigned to two groups. Group E pigs (n = 8) were immunized with subunit PCV2 vaccine, whereas group F pigs (n = 12) were given a placebo (0.9% saline emulsified with adjuvant) at 3 weeks of age. All pigs were immunized with LPC vaccine twice as prime and boost immunizations at 12 and 15 weeks of age. Serum samples were collected at 12, 15 and 18 weeks of age to monitor serum PCV2 viral load and conversion of CSFV-specific NAs.

All pigs in this study were fed *ad libitum* and raised in the same facility (trial I and II) or the same field farm (trial III) to minimize the bias of environmental variations. All animal trials and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University under IACUC approval number 103–45.

**Table 1**
Pig groups and immunization schedules in this study.

| Trial | Group (number of pigs) | Age at vaccination and challenge (weeks) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 7 | 9 | 12 | 15 |
| I* | A (4) | PCV2 vaccination[a] | | PCV2 challenge[b] | | – | – |
| | B (4) | Placebo | | PCV2 challenge | | – | – |
| II** | C (6) | PCV2 vaccination | LPC[c] | PCV2 challenge | LPC | – | – |
| | D (4) | Placebo | LPC | PCV2 challenge | LPC | – | – |
| III*** | E (8) | PCV2 vaccination | | | | LPC | LPC |
| | F (12) | Placebo | | | | LPC | LPC |

* Pigs in trial I were vaccinated with subunit PCV2 vaccine (group A) or a placebo (0.9% saline emulsified with adjuvant, group B) at 3 weeks of age and challenged with PCV2 virus at 7 weeks of age to mimic the induction of PCVADs (PCV2-SI or PCV2-SD) after PCV2 infection at field farms.
** Pigs in trial II were vaccinated with subunit PCV2 vaccine (group C) or a placebo (0.9% saline emulsified with adjuvant, group D) at 3 weeks of age and LPC vaccine twice at 6 and 9 weeks of age. Pigs were challenged with PCV2 virus at 7 weeks of age to mimic natural infection.
*** Pigs in trial III were vaccinated with subunit PCV2 vaccine (group E) or a placebo (0.9% saline emulsified with adjuvant, group F) at 3 weeks of age and fed normally at the conventional farm from farrowing to slaughter to naturally expose them to PCV2-SI. All pigs were vaccinated with LPC vaccine twice at 12 and 15 weeks of age to minimize the interference of MDA.
[a] Baculovirus-expressed subunit PCV2 capsid protein emulsified with w/o/w adjuvant.
[b] Intranasal and intramuscular inoculation of PCV2 ($2 \times 10^5$ $TCID_{50}$) at 7 weeks of age.
[c] Immunization with live attenuated CSFV vaccine (LPC strain).

### 2.3. Detection of PCV2-specific antibodies

PCV2-specific antibody titers were determined using indirect immunofluorescence assays (IFA) following the method described in previous studies with slight modifications [26,27]. Briefly, a monolayer of PK15 cells (ATCC CCL-33, passaged fewer than 30 times) in a 96-well cell culture plate was infected with PCV2 at a multiplicity of infection of 0.4 and incubated at 37 °C and 5% $CO_2$ for 72 h. Cells were fixed with 4% formaldehyde (SF93-4, Fisher Scientific, Hampton, NH, USA) for 20 min and the plate was then rinsed three times with 300 μL phosphate buffered saline containing 0.05% Tween 20 (PBST). For each inactivated serum sample, sets of four-fold dilutions (from 1:4 to 1:1,048,576) were prepared using 3% bovine serum albumin (BSA) in PBST. Each diluted serum sample was added to the plate and incubated at 4 °C overnight. The plate was then rinsed six times with 200 μL PBST and 50 μL fluorescein isothiocyanate (FITC) conjugated anti-pig IgG antibody (1:500, F1638, Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated at 25 °C in the dark for 1 h. Finally, the plate was rinsed four times with 200 μL PBST and analyzed using a fluorescence microscope (IX-70, Olympus, Tokyo, Japan).

### 2.4. PCR quantification of PCV2 viral load in serum

Total DNA was extracted from 200 μL serum samples at each sampling time point using a DNeasy Blood & Tissue kit (69506, Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. PCV2 viral DNA was assessed using a previously described TaqMan probe-based PCR quantification system with some modifications [28] and expressed as copies per milliliter of serum. Briefly, PCV2-specific primers (PCV2-Cap-F: 5′-GGGAGCAGGGCCAGAATT-3′ and PCV2-Cap-R: 5′- CGCTCTGTGCCCTTTGAATACT-3′, 100 nM) and a fluorescent TaqMan probe (PCV2-ORF2-p: 5′-FAM-ACCTTA ACCTTTCTTATTCTG-MGB-3′, 100 nM) were designed to detect PCV2 ORF2. For each 25 μL reaction, the primers and probe for the target gene were added to 2 × TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) to a final concentration of 0.1 μM along with 5 μL DNA template. The pGEM-PCV2/ORF2 plasmid containing the full length PCV2 ORF2 gene was ten-fold serially diluted to generate a standard curve for quantification. The reaction was carried out as follows: incubation at 50 °C for 2 min; AmpliTaq Gold activation at 95 °C for 10 min; and 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Real-time quantitative PCR was performed using a LightCycler®480 instrument (Roche diagnostic GmbH, Mannheim, Germany) and the crossing point (Cp) value of each reaction was calculated using LightCycler®480 software version 1.5 (Roche Life Science).

### 2.5. Detection of PCV2 antigen in lymphoid tissues

Mesenteric lymph nodes were collected from pigs in trial II (groups C and D), trimmed, fixed with 10% neutral formalin and subjected to immunohistochemical staining for PCV2 antigen as described in a previous study [29]. Paraffin-embedded tissue sections were dewaxed in xylene and rehydrated through graded alcohols (100% to 50%). Endogenous peroxidase activity was quenched by treating sections with 3% $H_2O_2$ for 10 min. After rinsing with distilled water, the sections were digested with proteinase K solution (20 μg/mL in Tris-ethylene diamine tetra acetic acid (EDTA) buffer, pH 8.0) at 37 °C for 20 min, cooled at room temperature for 20 min and rinsed with Tris buffer containing 0.05% Tween 20 (pH 7.4). After rinsing, sections were blocked with blocking buffer (PBS containing 2% normal goat serum, 1% BSA, 0.1% Triton X-100, 0.05% Tween 20 and 0.05% sodium azide) at room temperature for 30 min. The sections were then incubated with a 1:1000 dilution of PCV2-specific monoclonal antibody (M.11.PCV.I36A9, Ingenasa, Madrid, Spain) overnight at 4 °C. The sections were rinsed twice with Tris buffer, incubated with horseradish peroxidase (HRP) rabbit/mouse solution (K4605, Dako North America, Inc., Carpinteria, CA, USA) for 30 min and rinsed with Tris buffer. For color development, the sections were incubated with 1:300 DAB + chromogen (K4605, Dako North America, Inc.) at room temperature for 3 min. Finally, the sections were counterstained with Mayer's hematoxylin and assessed using a light microscope.

### 2.6. Detection of CSFV-specific neutralizing antibodies

Specific NAs against CSFV (LPC strain) were measured to evaluate the efficacy of the CSFV vaccine. For each inactivated serum sample, sets of two-fold dilutions (from 1:2 to 1:1024) were prepared using blank Dulbecco's Modified Eagle's Medium and incubated with 200 $TCID_{50}$ LPC virus at 37 °C for 1 h. After incubation, PK15 cells ($2 \times 10^4$ per well) were added to the cell culture plate and incubated at 37 °C and 5% $CO_2$ for 72 h. The cells were then fixed with 4% formaldehyde. The CSFV-E2-specific monoclonal antibody WH303 (PA0826, Veterinary laboratories agency,

Weybridge, UK) (1:1000) was used to recognize infected PK15 cells by IFA. The NA titer of each serum sample was the highest dilution able to inhibit PK15 cells infected with CSFV (LPC strain). Back titrations of the CSFV used in the NA test were conducted for each assay. The virus was ten-fold serially diluted, added to $3 \times 10^4$ cells and incubated at 37 °C and 5% $CO_2$ for 72 h. The virus was detected using the monoclonal antibody WH303 and titers were calculated using the Reed-Muench method [30]. Using this method, the back titer of the virus should be between 30 and 300 $TCID_{50}$.

### 2.7. Statistical analysis

ADWG (kg), PCV2-specific antibody titer ($log_2$), serum PCV2 viral load ($log_{10}$) and CSFV-specific NA titer ($log_2$) were expressed as mean ± standard error of the mean (SEM) for each group. Welch's two sample *t*-test was applied to assess differences between the two groups. Data analysis was performed using R software version 3.4.4 (The R Foundation, Vienna, Austria), and differences were considered statistically significant for P values less than 0.05.

## 3. Results

### 3.1. Seroprofiling and clinical findings at the field farm

A cross-sectional analysis was performed at the selected field farm to profile PCV2 infection status and assess the decline in CSFV-specific MDA in pigs of different ages. The serum PCV2 viral load decreased from 4.121 ± 0.105 $log_{10}$ copies/μL at 3 weeks of age to 2.502 ± 0.388 $log_{10}$ copies/μL at 12 weeks of age, then slightly increased to 3.050 ± 0.340 $log_{10}$ copies/μL at 15 weeks of age. Additionally, assessment of PCV2-specific antibody titers showed that seroconversion occurred between 6 and 15 weeks of age. No clinical signs of PCVADs were noted at the farm, confirming PCV2-SI status. Monitoring of CSFV-specific MDA using an IDEXX CSFV Ab test kit revealed that CSFV-specific MDA were high at 3 weeks of age then declined significantly (Supplementary Fig. 1).

### 3.2. An artificial PCV2 challenge model to induce PCVADs in conventional pigs

In trial I, a vaccination-challenge model using conventional pigs was established to assess the protective efficacy of a subunit PCV2 vaccine against PCVADs. Piglets were immunized with the subunit PCV2 vaccine at 3 weeks of age and challenged with $2 \times 10^5$ $TCID_{50}$ PCV2 at 7 weeks of age. No differences in PCV2-specific antibody titers or serum PCV2 viral loads were observed prior to vaccination at 3 weeks of age. At 7 weeks of age, prior to PCV2 challenge, PCV2-specific antibody titers were significantly higher in immunized pigs (group A, 11.000 ± 0.577 $log_2$) compared to control pigs (group B, 8.500 ± 0.500 $log_2$). At 8 weeks of age, after PCV2 challenge, a steep antibody response was observed in both groups A and B, but PCV2-specific antibody titers were significantly higher in pigs in group A (17.000 ± 0.577 $log_2$) compared to those in group B (13.500 ± 0.957 $log_2$) (Fig. 1a). Measurement of serum PCV2 viral loads revealed no significant differences at 7 weeks of age prior to PCV2 challenge. However, serum PCV2 viral loads were significantly lower in pigs in group A compared to those in group B at 9 weeks of age (2.000 ± 1.000 $log_{10}$ copies/μL versus 5.510 ± 0.450 $log_{10}$ copies/μL), 10 weeks of age (1.670 ± 1.200 $log_{10}$ copies/μL versus 4.780 ± 0.470 $log_{10}$ copies/μL) and 11 weeks of age (1.920 ± 1.060 $log_{10}$ copies/μL versus 4.580 ± 0.340 $log_{10}$ copies/μL) (Fig. 1b). Furthermore, ADWG was higher for pigs in group A (0.361 ± 0.072 kg) compared to those in group B (0.206 ± 0.076 kg) between 7 and 11 weeks of age (Fig. 1c). Therefore, after PCV2 challenge, PCV2-SI was present in conventional pigs administered the PCV2 vaccine, whereas PCV2-SD was present in unvaccinated pigs.

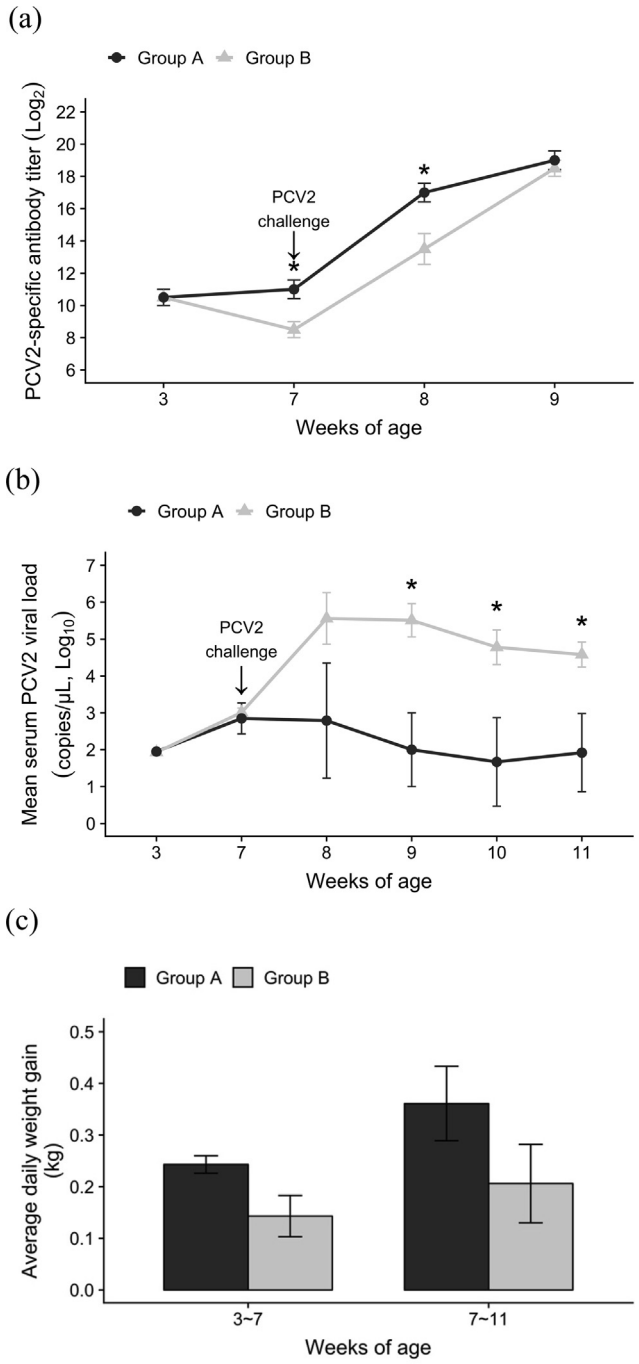


**Fig. 1.** PCV2 challenge model to mimic PCVADs in a conventional pig farm. In trial I, pigs at the field farm were vaccinated with subunit PCV2 vaccine (group A) or a placebo (0.9% saline emulsified with adjuvant, group B) at 3 weeks of age and challenged with $2 \times 10^5$ $TCID_{50}$ PCV2 at 7 weeks of age to mimic natural PCV2 infection. (a) PCV2-specific antibody titers were detected by IFA. Group A showed significantly higher antibody titers at 7 weeks of age prior to PCV2 challenge. Pigs in both group A and group B showed seroconversion after PCV2 challenge, but pigs in group A had significantly higher antibody titers at 8 weeks of age. (b) Serum PCV2 viral loads were detected by quantitative PCR. (c) ADWG analysis was conducted to monitor pig growth performance before and after PCV2 challenge. *P < 0.05.

### 3.3. The impact of PCVADs on the live attenuated CSFV vaccine

In trial II, piglets were immunized with the subunit PCV2 vaccine at 3 weeks of age and the LPC vaccine at 6 and 9 weeks of age according to the regular vaccination schedule of the field farm. Piglets were then challenged with $2 \times 10^5$ $TCID_{50}$ PCV2 at 7 weeks of age. At 3 weeks of age, there were no differences in PCV2-specific antibody titers or serum PCV2 viral loads between PCV2 immunized pigs (group C) and control pigs (group D). After immunization with the subunit PCV2 vaccine, PCV2-specific antibody titers were significantly higher in pigs in group C (12.000 ± 0.894 $log_2$) compared to those in group D (6.000 ± 0.000 $log_2$) at 7 weeks of age and gradually increased up to 9 weeks of age (group C: 18.800 ± 0.490 $log_2$). In contrast, late PCV2-specific antibody conversion was observed in pigs in group D at 9 weeks of age (16.667 ± 1.764 $log_2$), indicating that their induced immune responses were slower than those of vaccinated pigs (Fig. 2a). Additionally, after PCV2 challenge, serum PCV2 viral loads of pigs in group D increased significantly and were significantly higher than those of pigs in group C at 9 weeks of age (6.370 ± 0.530 $log_{10}$ copies/μL versus 3.690 ± 0.810 $log_{10}$ copies/μL), 10 weeks of age (6.400 ± 0.840 $log_{10}$ copies/μL versus 2.740 ± 0.740 $log_{10}$ copies/μL) and 11 weeks of age (4.760 ± 0.420 $log_{10}$ copies/μL versus 2.280 ± 0.440 $log_{10}$ copies/μL) (Fig. 2b). Furthermore, histopathological examination with hematoxylin and eosin staining showed more intact lymph follicle structures in pigs in Group C, whereas severe lymph depletion was observed in pigs in group D. Results of immunohistochemical staining of mesenteric lymph

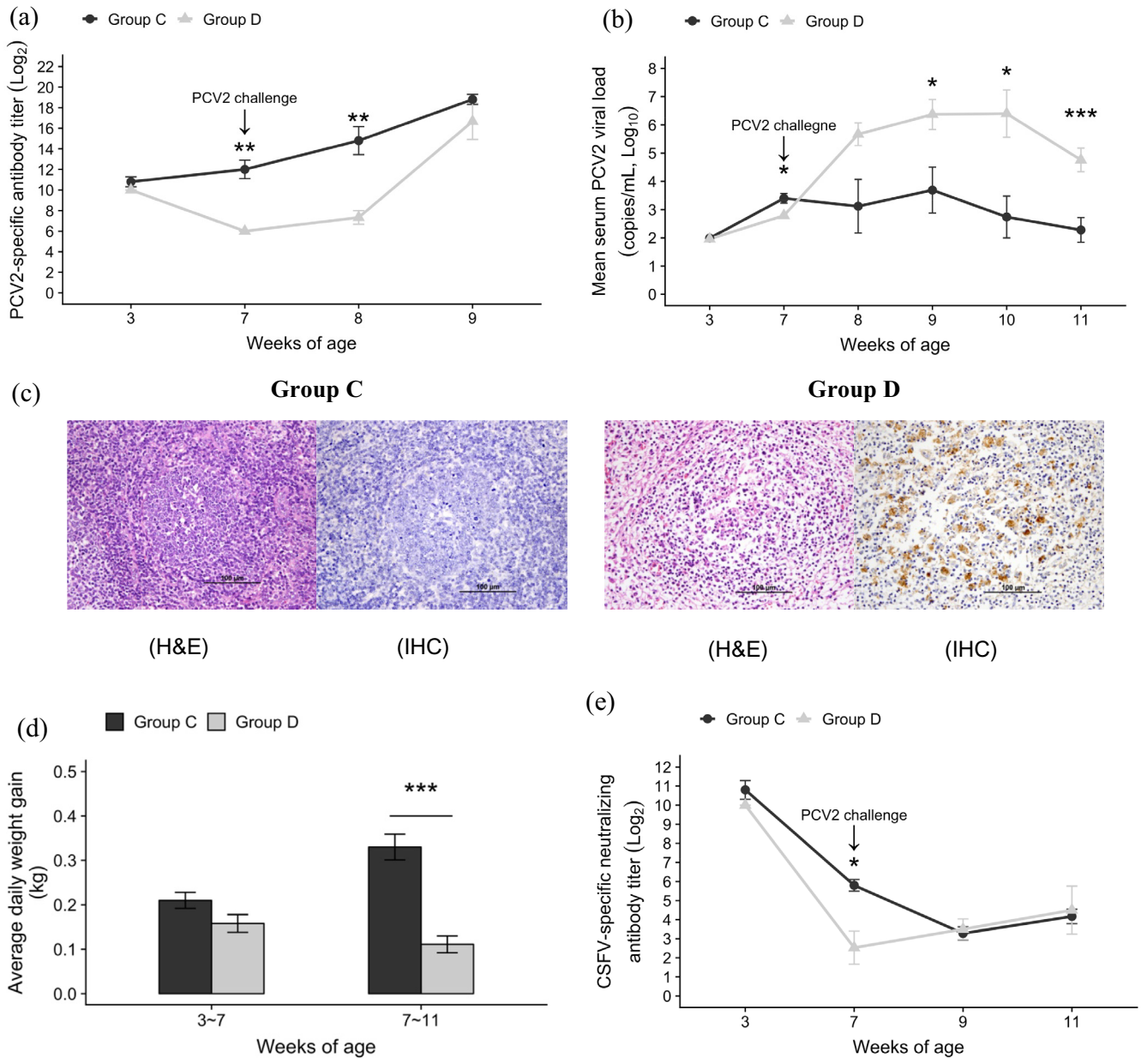


**Fig. 2.** Impact of PCV2-SD and PCV2-SI on live attenuated CSFV vaccine (LPC strain) efficacy. In trial II, PCV2-SD or PCV2-SI were induced by vaccination with subunit PCV2 vaccine (group C) or a placebo (group D) at 3 weeks of age, respectively, followed by challenge with PCV2 at 7 weeks of age. To evaluate the impact of PCV2 infection on CSFV vaccine efficacy, pigs in both group C and group D were vaccinated twice with the live attenuated CSFV vaccine (LPC strain) at 6 and 9 weeks of age according to the regular vaccination program. (a) Detection of PCV2-specific antibody titers by IFA. (b) Detection of serum PCV2 viral loads by quantitative PCR. (c) Histopathological changes and immunohistochemical (IHC) staining for PCV2 capsid protein (brown) in mesenteric lymph nodes. (d) Changes in ADWG before and after PCV2 challenge. (e) Changes in CSFV-specific NA titers. *P < 0.05, **P < 0.01, ***P < 0.001.

nodes showed more intense PCV2-specific antigen staining in lymphoid follicles of pigs in group D compared to those in group C (Fig. 2c). In addition, ADWG was significantly higher in pigs in group C (0.330 ± 0.030 kg) compared to those in group D (0.110 ± 0.020 kg) between 7 and 11 weeks of age after PCV2 challenge at 7 weeks of age (Fig. 2d). Therefore, this trial of PCV2-challenged conventional pigs revealed that PCV2-SD was present in unvaccinated pigs (group D), whereas PCV2-SI was present in pigs immunized with subunit PCV2 vaccine (Group C).

No significant difference in CSFV NA titers was observed between groups C and D, indicating consistent distribution of MDA in pigs from the same litter at 3 weeks of age. At 6 weeks of age, CSFV-specific NA titers declined more slowly and were significantly higher in pigs in group C (5.800 ± 0.300 $\log_2$) compared to those in group D (2.530 ± 0.870 $\log_2$). However, after immunization with the live attenuated CSFV vaccine at 6 and 9 weeks of age, NA titers slowly increased in both groups C and D at 9 weeks of age (3.28 ± 0.340 $\log_2$ versus 3.500 ± 0.540 $\log_2$) and 11 weeks of age (4.170 ± 0.380 $\log_2$ versus 4.500 ± 1.260 $\log_2$), and no significant difference was observed between the two groups after PCV2 challenge at 7 weeks of age (Fig. 2e).

### *3.4. Low PCV2 viral loads impact the live attenuated CSFV vaccine efficacy*

Pigs in trial III were immunized with subunit PCV2 vaccine at 3 weeks of age and LPC vaccine at 12 and 15 weeks of age to minimize the interference of MDA on LPC vaccine efficacy. At 12 weeks of age, CSFV NA titers were similar between PCV2-immunized pigs (group E, 5.360 ± 0.530 $\log_2$) and non-immunized pigs (group F, 5.240 ± 0.260 $\log_2$). Additionally, no clinical signs of PCV2 were noted prior to LPC immunization. After administration of two doses of LPC vaccine, CSFV NA converted and were significantly higher in pigs in group E (7.440 ± 0.220 $\log_2$) compared to those in group F (5.080 ± 0.470 $\log_2$) at 18 weeks of age (Fig. 3a). Additionally, serum PCV2 viral loads increased slightly in both groups at 15 weeks of age (group E: 2.880 ± 0.130 $\log_{10}$ copies/μL; group F: 2.830 ± 0.090 $\log_{10}$ copies/μL). At 18 weeks of age, serum viral loads rapidly decreased and were lower, but not significantly, in pigs in group E (0.430 ± 0.280 $\log_{10}$ copies/μL) compared to those in group F (1.310 ± 0.420 $\log_{10}$ copies/μL) (Fig. 3b). Although PCV2 viral loads were low in all pigs and no obvious clinical signs were observed, polarized CSFV NA titers were noted in the two groups. Therefore, the relationship between serum PCV2 viral loads and CSFV NA titers of 18-week-old pigs were further investigated to assess the effect of PCV2-SI on LPC vaccine efficacy (Fig. 3c). In group E, CSFV NA titers were greater than 1:32 in all pigs, and serum PCV2 DNA were detected in only two pigs (25%) which were less than $\log_{10}$ 2 (mean: $\log_{10}$ 1.71). In contrast, 50% (6/12) of pigs in group F showed serum PCV2 DNA positive of which 83.33% (5/6) were greater than $\log_{10}$ 2 (mean $\log_{10}$ 2.84), and among these only two pigs developed protective CSFV-specific NA titers (>1:32).

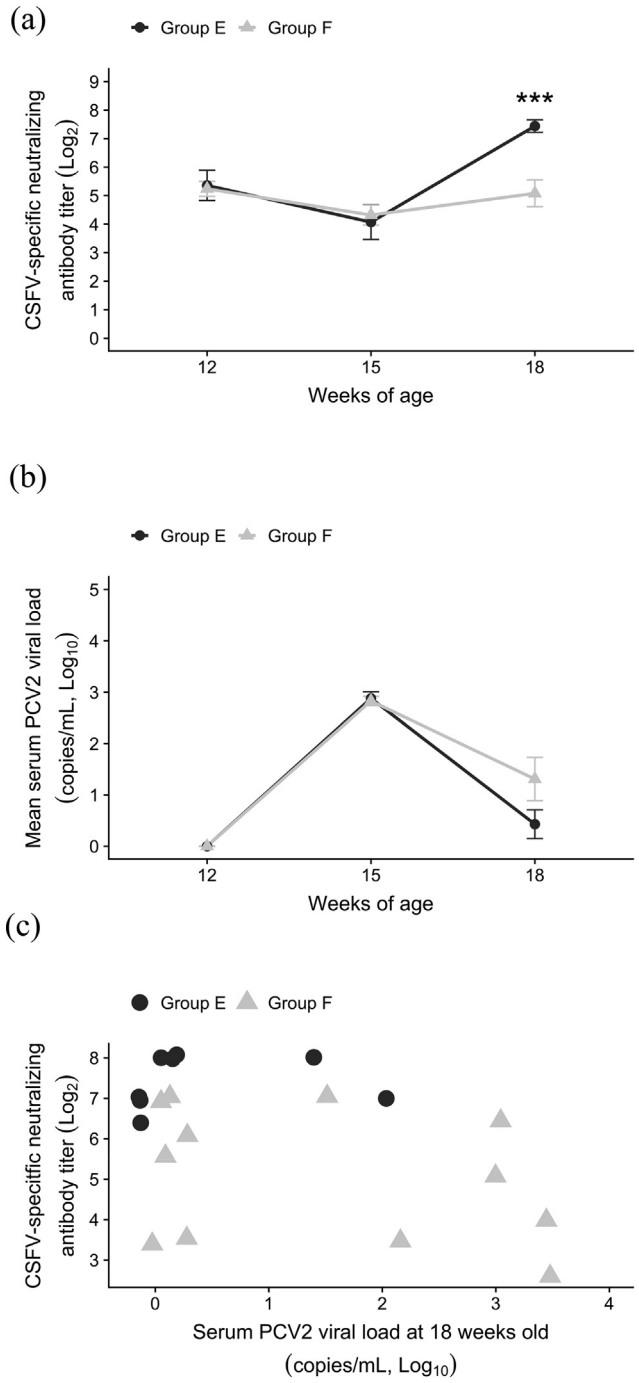


**Fig. 3.** Long-term observation of the impact of PCV2-SI at a field farm. In trial III, pigs at the field farm were vaccinated with subunit PCV2 vaccine (group E) or a placebo (group F) at 3 weeks of age and fed normally. Pigs in both groups were vaccinated twice with live attenuated CSFV vaccine (LPC strain) at 12 and 15 weeks of age to minimize the interference of MDA. All pigs at the farm were naturally infected with PCV2. Serum PCV2 viral loads were measured before and after CSFV vaccination to monitor clinical infection. The development of CSFV-specific NAs was monitored as an indicator of the protective efficacy of the live attenuated CSFV vaccine. (a) CSFV-specific NA titers after LPC vaccine immunization. (b) Serum PCV2 viral loads at 12, 15 and 18 weeks of age. (c) Dot plot of CSFV-specific NA titers and serum PCV2 viral loads at 18 weeks of age. *P < 0.05, **P < 0.01, ***P < 0.001.

## 4. Discussion

PCVADs are some of the most important swine diseases worldwide and have seriously impacted the pig industry for decades. Since 2006, effective commercial PCV2 vaccines have been widely utilized in field farms to minimize losses due to PCVADs [3,14]. In this study, serum PCV2 DNA levels and antibody titers were assessed in pigs at a conventional pig farm where regular PCV2 vaccinations are administered. A cross-sectional analysis was used to determine PCV2 infection levels at the field farm prior to experimental trials. Based on results of monitoring, levels of serum PCV2 DNA decreased between 3 and 12 weeks of age, then increased slightly between 15 and 18 weeks of age before returning to low levels at 21 weeks of age. Serum PCV2-specific antibodies converted between 6 and 15 weeks of age (Supplementary Fig. 1). The dynamics of serum PCV2 DNA levels and PCV2-specific antibody titers indicated that pigs at this farm were repeatedly

infected with PCV2 between 6 and 15 weeks of age. Because most vaccines against important swine diseases should be administered during this period, repeated PCV2 infection may impact the development of immune responses, leading to polymicrobial infections and the induction of PCVADs. Moreover, impaired immune responses due to PCV2 infection may also affect the efficacy of other important swine vaccines.

Previous studies demonstrated that immunization with PCV2 vaccine significantly reduced the severity of clinical symptoms induced by PCV2 infection in pigs and resulted in higher ADWG, which is considered a clinical criterion to evaluate PCV2 vaccine efficacy [31,32]. In trial I, pigs immunized with the PCV2 vaccine had higher PCV2-specific antibody titers, lower serum PCV2 viral loads and higher ADWG after PCV2 challenge than non-vaccinated pigs. The conversion of PCV2-specific antibodies and the reduced PCV2 viral loads in vaccinated pigs confirm the efficacy of the subunit PCV2 vaccine [33]. However, the serum PCV2 viral loads of pigs in group A revealed that low levels of PCV2 infection were present in vaccinated pigs after PCV2 challenge despite the absence of obvious clinical symptoms during the experimental period. This suggests that conventional pigs in field farms may develop PCV2-SI after PCV2 vaccination. Feng et al. (2014) conducted a 1-year study of mass vaccination with the subunit PCV2 vaccine at a field farm and similarly found reduced incidence of PCVADs and lower PCV2 DNA levels during the experimental period. However, PCV2 infections recurred 4 months after stopping the vaccination program [34]. Furthermore, recent studies have demonstrated that current PCV2 vaccine efficacy levels cannot completely eradicate PCV2 infections at pig farms [14,15,35]. This means that pigs are repeatedly exposed to PCV2 infection, which may cause widespread asymptomatic PCV2-SI in pig farms. These PCV2 subclinical infected pigs (PCV2-SI) usually showed asymptomatic and were recognized as healthy pigs in farms.

In our previous study, six specific-pathogen-free (SPF) pigs immunized with LPC vaccine at 4 and 6 weeks of age showed seroconversion at 9 weeks of age (three weeks after completing the vaccination schedule, Supplementary Fig. 2). Huang et al. (2011) demonstrated that NA induction is delayed in PCV2-infected SPF pigs after immunization with live attenuated CSFV vaccine (LPC strain) and that clinical symptoms were severe after challenge with CSFV (ALD strain), demonstrating that PCV2 infection impacts LPC vaccine efficacy [36]. However, exposure to concurrent viral or bacterial infections and stress due to environmental changes or management factors complicate the situation at pig farms. In trial II, PCV2 vaccine efficacy was higher in pigs in group C, who showed more rapid seroconversion and had higher PCV2 antibody titers and ADWG and lower serum PCV2 viral loads after vaccination and challenge compared to those in group D. Additionally, immunohistochemical analysis of pigs in group C revealed mild staining of mesenteric lymph nodes. These results demonstrate that the protective efficacy of the subunit PCV2 vaccine minimizes clinical signs of PCVADs in field farms. However, serum PCV2 viral loads of pigs in group C indicated low levels of PCV2 infection at the end of the trial (4 weeks after PCV2 challenge), suggesting that PCV2-SI was present in pigs in this group. Moreover, no significant conversion of CSFV NAs was observed after LPC vaccination in either group during the trial period. Therefore, although the efficacy of the PCV2 subunit vaccine was confirmed, pigs that received the PCV2 subunit vaccine still had low levels of PCV2 virus in serum, which led to PCV2-SI and interfered with the CSFV NA response induced by the live attenuated CSFV vaccine (LPC strain).

MDAs in colostrum also interfere with the efficacy of live attenuated CSFV vaccines [20,23]. In trial III, a delayed LPC vaccine was administered at 12 and 15 weeks of age to minimize MDA interference and pigs were fed normally to assess the impact of natural PCV2 infection on live attenuated CSFV vaccine efficacy. CSFV NA titers were significantly different between pigs in group E and F. Although conditions were the same for pigs in both groups, pigs in group E had higher CSFV NA titers than those in group F at 18 weeks of age. Meanwhile, serum PCV2 viral loads increased slightly between 12 and 15 weeks of age after administration of the first LPC vaccine. Serum PCV2 viral loads then decreased rapidly in pigs in group E at 18 weeks of age, confirming the protective efficacy of the PCV2 vaccine when administered at 3 weeks of age. Recent studies have indicated that PCV2-SD may be induced in PCV2-infected pigs with serum PCV2 viral loads greater than $\log_{10}$ 6 copies/mL, whereas pigs with serum PCV2 viral loads below $\log_{10}$ 5 copies/mL may develop PCV2-SI [37–42]. In trial III, quantification of serum PCV2 viral loads in both groups showed that levels were lower than $\log_{10}$ 3 copies/mL, which clearly indicates that pigs were naturally infected with PCV2-SI in this field farm. However, the development of CSFV NA titers was impaired even in pigs with very low PCV2 viral loads. Moreover, comparisons of PCV2 viral loads and CSFV NA titers in 18-week-old pigs in groups E and F revealed that pigs with serum PCV2 viral loads less than $\log_{10}$ 2 copies/mL may develop sufficient protective immune responses against CSFV.

Although several experimental animal trials have been established to evaluate the efficacy of PCV2 vaccines, differences in induced responses between vaccinated and non-vaccinated pigs may lead to incomplete results. Pigs in conventional farms have longer feeding periods, facing more environmental stresses, and encountering more unpredictable influences than experimental SPF pigs within a well-isolated facility model. These unaddressed factors in studies of PCV2 vaccine efficacy in field farm applications may explain the impaired efficacy of PCV2 vaccines, which may result in widespread PCV2-SI and induce further complication of polymicrobial diseases. The impact of asymptomatic PCV2 infection requires further study to better understand these issues.

In conclusion, this study mimicked repeated PCV2 infection at a field farm. Pigs immunized with the subunit PCV2 vaccine developed PCV2-SI after PCV2 challenge, which clearly demonstrates the impaired efficacy of the PCV2 vaccine in experimental models. Additionally, vaccination-challenge experiments revealed that PCV2-SI compromised NA responses induced by the live attenuated CSFV vaccine. Moreover, field trials involving long-term observation of conventional pigs from the post-weaning to finishing stages also illustrated the impact of PCV2-SI on the live attenuated CSFV vaccine efficacy. This study provides useful models for future studies on clinical PCV2 vaccine efficacy and the interference of PCV2-SI on the live attenuated CSFV vaccine.

## 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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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.08.039.

## References


[1] Opriessnig T, Halbur PG. Concurrent infections are important for expression of porcine circovirus associated disease. Virus Res 2012;164:20–32. https://doi.org/10.1016/j.virusres.2011.09.014.

[2] Alarcon P, Rushton J, Wieland B. Cost of post-weaning multi-systemic wasting syndrome and porcine circovirus type-2 subclinical infection in England – an economic disease model. Prev Vet Med 2013;110:88–102. https://doi.org/10.1016/j.prevetmed.2013.02.010.

[3] Segalés J. Best practice and future challenges for vaccination against porcine circovirus type 2. Expert Rev Vaccines 2014;14:1–15. https://doi.org/10.1586/14760584.2015.983084.

[4] Chae C. Commercial porcine circovirus type 2 vaccines: efficacy and clinical application. Vet J 2012;194:151–7. https://doi.org/10.1016/j.tvjl.2012.06.031.

[5] Xiao C-T, Halbur PG, Opriessnig T. Complete genome sequence of a novel porcine circovirus type 2b variant present in cases of vaccine failures in the United States. J Virol 2012;86:12469. https://doi.org/10.1128/JVI.02345-12.

[6] Seo HW, Park C, Kang I, Choi K, Jeong J, Park S-J, et al. Genetic and antigenic characterization of a newly emerging porcine circovirus type 2b mutant first isolated in cases of vaccine failure in Korea. Arch Virol 2014;159:3107–11. https://doi.org/10.1007/s00705-014-2164-6.

[7] Eddicks M, Fux R, Szikora F, Eddicks L, Majzoub-Altweck M, Hermanns W, et al. Detection of a new cluster of porcine circovirus type 2b strains in domestic pigs in Germany. Vet Microbiol 2015;176:337–43. https://doi.org/10.1016/j.vetmic.2015.01.013.

[8] Franzo G, Tucciarone CM, Cecchinato M, Drigo M. Porcine circovirus type 2 (PCV2) evolution before and after the vaccination introduction: a large scale epidemiological study. Sci Rep 2016;6:39458. https://doi.org/10.1038/srep39458.

[9] Xiao C-T, Harmon KM, Halbur PG, Opriessnig T. PCV2d-2 is the predominant type of PCV2 DNA in pig samples collected in the U.S. during 2014–2016. Vet Microbiol 2016;197:72–7. https://doi.org/10.1016/j.vetmic.2016.11.009.

[10] Kwon T, Lee D-U, Yoo SJ, Je SH, Shin JY, Lyoo YS. Genotypic diversity of porcine circovirus type 2 (PCV2) and genotype shift to PCV2d in Korean pig population. Virus Res 2017;228:24–9. https://doi.org/10.1016/j.virusres.2016.11.015.

[11] Oh Y, Seo HW, Park C, Chae C. Comparison of sow and/or piglet vaccination of 3 commercial porcine circovirus type 2 (PCV2) single-dose vaccines on pigs under experimental PCV2 challenge. Vet Microbiol 2014;172:371–80. https://doi.org/10.1016/j.vetmic.2014.05.028.

[12] Opriessnig T, Xiao C-T, Halbur PG, Gerber PF, Matzinger SR, Meng X-J. A commercial porcine circovirus (PCV) type 2a-based vaccine reduces PCV2d viremia and shedding and prevents PCV2d transmission to naïve pigs under experimental conditions. Vaccine 2017;35:248–54. https://doi.org/10.1016/j.vaccine.2016.11.085.

[13] Yang S, Yin S, Shang Y, Liu B, Yuan L, Zafar Khan MU, et al. Phylogenetic and genetic variation analyses of porcine circovirus type 2 isolated from China. Transbound Emerg Dis 2018;65:e383–92. https://doi.org/10.1111/tbed.12768.

[14] Afghah Z, Webb B, Meng X-J, Ramamoorthy S. Ten years of PCV2 vaccines and vaccination: iss eradication a possibility? Vet Microbiol 2017;206:21–8. https://doi.org/10.1016/j.vetmic.2016.10.002.

[15] Karuppannan AK, Opriessnig T. Porcine circovirus type 2 (PCV2) vaccines in the context of current molecular epidemiology. Viruses 2017;9. https://doi.org/10.3390/v9050099.

[16] Blome S, Staubach C, Henke J, Carlson J, Beer M. Classical swine fever – an updated review. Viruses 2017;9. https://doi.org/10.3390/v9040086.

[17] Xu L, Fan X-Z, Zhao Q-Z, Zhang Z-X, Chen K, Ning Y-B, et al. Effects of vaccination with the C-strain vaccine on immune cells and cytokines of pigs against classical swine fever virus. Viral Immunol 2017. https://doi.org/10.1089/vim.2017.0010.

[18] Graham SP, Everett HE, Haines FJ, Johns HL, Sosan OA, Salguero FJ, et al. Challenge of pigs with classical swine fever viruses after C-strain vaccination reveals remarkably rapid protection and insights into early immunity. PLoS ONE 2012;7:e29310. https://doi.org/10.1371/journal.pone.0029310.

[19] Terpstra C, Wensvoort G. The protective value of vaccine-induced neutralising antibody titres in swine fever. Vet Microbiol 1988;16:123–8.

[20] van Oirschot JT. Vaccinology of classical swine fever: from lab to field. Vet Microbiol 2003;96:367–84. https://doi.org/10.1016/j.vetmic.2003.09.008.

[21] Li H, Yang H. Infection of porcine reproductive and respiratory syndrome virus suppresses the antibody response to classical swine fever virus vaccination. Vet Microbiol 2003;95:295–301. https://doi.org/10.1016/S0378-1135(03)00158-5.

[22] Suradhat S, Kesdangsakonwut S, Sada W, Buranapraditkun S, Wongsawang S, Thanawongnuwech R. Negative impact of porcine reproductive and respiratory syndrome virus infection on the efficacy of classical swine fever vaccine. Vaccine 2006;24:2634–42. https://doi.org/10.1016/j.vaccine.2005.12.010.

[23] Suradhat S, Damrongwatanapokin S, Thanawongnuwech R. Factors critical for successful vaccination against classical swine fever in endemic areas. Vet Microbiol 2007;119:1–9. https://doi.org/10.1016/j.vetmic.2006.10.003.

[24] Wang X, Mu G, Dang R, Yang Z. Up-regulation of IL-10 upon PRRSV vaccination impacts on the immune response against CSFV. Vet Microbiol 2016;197:68–71. https://doi.org/10.1016/j.vetmic.2016.11.007.

[25] Maes D. Subclinical porcine circovirus infection: what lies beneath. Vet J 2012;194:9. https://doi.org/10.1016/j.tvjl.2012.06.029.

[26] McNair I, Marshall M, McNeilly F, Botner A, Ladekjaer-Mikkelsen AS, Vincent I, et al. Interlaboratory testing of porcine sera for antibodies to porcine circovirus type 2. J Vet Diagn Invest 2004;16:164–6. https://doi.org/10.1177/104063870401600214.

[27] Patterson AR, Johnson JK, Ramamoorthy S, Hesse RA, Murtaugh MP, Puvanendiran S, et al. Interlaboratory comparison of porcine circovirus-2 indirect immunofluorescent antibody test and enzyme-linked immunosorbent assay results on experimentally infected pigs. J Vet Diagn Invest 2011;23:206–12.

[28] Grasland B, Loizel C, Blanchard P, Oger A, Nignol A-C, Bigarré L, et al. Reproduction of PMWS in immunostimulated SPF piglets transfected with infectious cloned genomic DNA of type 2 porcine circovirus. Vet Res 2005;36:685–97. https://doi.org/10.1051/vetres:2005024.

[29] Kennedy S, Moffett D, McNeilly F, Meehan B, Ellis J, Krakowka S, et al. Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone or in combination with porcine parvovirus. J Comp Pathol 2000;122:9–24. https://doi.org/10.1053/jcpa.1999.0337.

[30] Reed LJ, Muench H. A simple method of estimating fifty percent endpoint. Am J Epidemiol 1938;27:493–7. https://doi.org/10.1093/oxfordjournals.aje.a118408.

[31] Kristensen CS, Baadsgaard NP, Toft N. A meta-analysis comparing the effect of PCV2 vaccines on average daily weight gain and mortality rate in pigs from weaning to slaughter. Prev Vet Med 2011;98:250–8. https://doi.org/10.1016/j.prevetmed.2010.11.015.

[32] Seo HW, Han K, Park C, Chae C. Clinical, virological, immunological and pathological evaluation of four porcine circovirus type 2 vaccines. Vet J 2014;200:65–70. https://doi.org/10.1016/j.tvjl.2014.02.002.

[33] Park C, Seo HW, Han K, Chae C. Comparison of four commercial one-dose porcine circovirus type 2 (PCV2) vaccines administered to pigs challenged with PCV2 and porcine reproductive and respiratory syndrome virus at 17 weeks postvaccination to control porcine respiratory disease complex under Korean field conditions. Clin Vaccine Immunol 2014;21:399–406. https://doi.org/10.1128/CVI.00768-13.

[34] Feng H, Blanco G, Segalés J, Sibila M. Can Porcine circovirus type 2 (PCV2) infection be eradicated by mass vaccination? Vet Microbiol 2014;172:92–9. https://doi.org/10.1016/j.vetmic.2014.05.003.

[35] Rose N, Andraud M. The use of vaccines to control pathogen spread in pig populations. Porc Health Manag 2017;3:8. https://doi.org/10.1186/s40813-017-0053-6.

[36] Huang Y-L, Pang VF, Lin C-M, Tsai Y-C, Chia M-Y, Deng M-C, et al. Porcine circovirus type 2 (PCV2) infection decreases the efficacy of an attenuated classical swine fever virus (CSFV) vaccine. Vet Res 2011;42. https://doi.org/10.1186/1297-9716-42-115.

[37] Brunborg IM, Moldal T, Jonassen CM. Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. J Virol Methods 2004;122:171–8. https://doi.org/10.1016/j.jviromet.2004.08.014.

[38] Fort M, Olvera A, Sibila M, Segalés J, Mateu E. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. Vet Microbiol 2007;125:244–55. https://doi.org/10.1016/j.vetmic.2007.06.004.

[39] Grau-Roma L, Hjulsager CK, Sibila M, Kristensen CS, López-Soria S, Enoe C, et al. Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark. Vet Microbiol 2009;135:272–82. https://doi.org/10.1016/j.vetmic.2008.10.007.

[40] Brunborg IM, Fossum C, Lium B, Blomqvist G, Merlot E, Jørgensen A, et al. Dynamics of serum antibodies to and load of porcine circovirus type 2 (PCV2) in pigs in three finishing herds, affected or not by postweaning multisystemic wasting syndrome. Acta Vet Scand 2010;52:22. https://doi.org/10.1186/1751-0147-52-22.

[41] Cortey M, Napp S, Alba A, Pileri E, Grau-Roma L, Sibila M, et al. Theoretical and experimental approaches to estimate the usefulness of pooled serum samples for the diagnosis of postweaning multisystemic wasting syndrome. J Vet Diagn Invest 2011;23:233–40. https://doi.org/10.1177/104063871102300206.

[42] Segalés J. Porcine circovirus type 2 (PCV2) infections: clinical signs, pathology and laboratory diagnosis. Virus Res 2012;164:10–9. https://doi.org/10.1016/j.virusres.2011.10.007.