A commercial PCV2a-based vaccine is effective in protection from experimental challenge of PCV2 mutant with two amino acids elongation in capsid protein

Long-Jun Guo^{1,†}, Yu-Jie Fu^{1,2,†}, Li-Ping Huang¹, Yi-Ping Wang¹, Yan-Wu Wei¹, Hong-Li Wu¹ and Chang-Ming Liu^{1,*}

1 Swine Infectious Diseases, State Key Laboratory of Veterinary Biotechnology,
Harbin Veterinary Research Institute of the Chinese Academy of Agricultural
Sciences, Harbin 150001, China, 427 Maduan Street, Nangang region, Harbin
150001, P.R. China.

2 Shenyang Institute of Technology, NO.1, East Binhe Road, Development Zone,
Fushun, Liaoning, 113122, P.R. China.

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: lcm@hvri.ac.cn;
Tel.: (+86) 18946066094; Fax: (+86 451) 82733132.

Abstract

Current commercial PCV2 vaccines are almost based on PCV2a and have been shown to be effective in reducing PCV2a and PCV2b viremia and PCV2-associated lesions and diseases. The recent emergence of novel mutant PCV2 (mPCV2) strains and linkage of mPCV2 with cases of porcine circovirus associated disease (PCVAD) in pig herds have raised concerns over emergence of vaccine-escape mutants and reduced efficacy of PCV2a-based vaccines. The aim of this study was to determine the ability of a commercial PCV2a-based vaccine developed by our laboratory to protect conventional pigs against experimental challenge with mPCV2 at 9 weeks of age. Twenty 4-week-old pigs free of PCV2 infection were randomly divided into four treatment groups with 5 pigs each. Two groups were unvaccinated as positive and negative controls. Another two groups were vaccinated with the commercial PCV2a-based vaccine (PCV2-LG strain, China) at 4 weeks of age and identical booster immunization was conducted 3 weeks post primary immunization. At 9 weeks of age, all pigs except the negative control were challenged with a mutant PCV2b/YJ(mPCV2b/YJ) with two amino acids elongation in capsid protein. The experiment was terminated 28 days after challenge. Under the conditions of this study, vaccinated pigs were protected against PCV2viremia and lesions whereas unvaccinated pigs were not. Moreover, mPCV2b/YJ infection was demonstrated in positive control and almost all had macroscopic or microscopic lesions consistent with PCVAD while negative control did not develop PCVAD. This study indicate that mPCV2b/YJ infection alone can trigger PCVAD development and that the

commercial vaccine (PCV2-LG) is still effective in protecting conventional pigs against the emerging mPCV2b/YJ strain in China.

Keywords: porcine circovirus type 2 (PCV2); mutant PCV2; vaccine; PCV2a; PCV2b

1. Introduction

Porcine circovirus type 2 (PCV2) has been recognized as the primary causative agent of porcine circovirus associated disease (PCVAD), and has become a serious threat to the swine industry worldwide [1]. Although clinical signs are highly variable, characteristic PCVAD symptoms are wasting, respiratory dysfunction, enlargement of superficial inguinal lymph nodes, diarrhea, and a generalized depletion of lymphocytes. [2]. PCV2 is the single-stranded and circular DNA genome with a rather small size (1767-1768 nts) and the non-enveloped spherical virus particles, which belongs to the genus *Circovirus* of the family *Circoviridae*. [3-5]. Rep and cap, associated with replication and virus capsid, respectively, are the only two major proteins encoded by PCV2 [6, 7]. Based on pair-wise sequence comparisons (PASC) of the open reading frame 2 (ORF2) PCV2 can be divided into three genotypes (PCV2a, PCV2b, and PCV2c). Among them PCV2a and PCV2b are the most prevalent genotypes in the pig raising farms worldwide [8, 9]. In the past less than one decade, more genetic variations and recombination associated with PCV2 have been reported worldwide [10-20]. It is important that a 1766 nt PCV2 strain (YJ) with ORF2 of 708 nt has been first isolated from the clinically infected pig with PCVAD in china. The deletion at position 1039 of the YJ strain contributed to the elongation of

two amino acids (N and E) at the C terminus of the ORF2-encoded Cap protein, which proved that PCV2 undergone variations frequently [16, 20]. To date, all of the currently commercial PCV2 vaccines have been demonstrated to be effective at reducing clinical signs and improving production parameters in farms with PCV2 infection [21, 22], however, whether the commercial PCV2 vaccine can completely prevent mPCV2b/YJ infection remains unknown.

The objective of this study was to investigate the ability of a commercialPCV2a-based vaccine (PCV2-LG strain, China) developed in our laboratory to protect experimental challenge of mPCV2b/YJ.

2. Materials and methods

2.1. Ethics statement

The animal experiment was approved by Harbin Veterinary Research Institute and performed in accordance with animal ethics guidelines and approved protocols. The animal ethics committee approval number is Heilongjiang-SYXK-2006-032.

2.2. Cells and viruses

The porcine kidney cell line (PK15), free of PCV1 contamination, was obtained by successive subcloning from the American Type Culture Collection (ATCC CCL-33). It was maintained at 37°C with 5% CO₂ in minimum essential medium (MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin. Two clinical isolates of PCV2, which were termed PCV2a/CL and mPCV2b/YJ with

GenBank accession numbers HM038033 and HM038032, respectively, were used for challenge experiments in this study. Experimental challenge of animals was performed with the 10th passage of the both viruses in culture, with the virus infectious dose adjusted to 10^{4.5} TCID₅₀/mL. The detailed information about PCV2a/CL and mPCV2b/YJ was summarized in Table 1.

2.3. Animals and experimental design

Twenty healthy, weaned, 28-day-old, commercial Large White–Dutch Landrace crossbred piglets were obtained from a pig farm that was negative for PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV) infections using commercial enzyme-linked immunosorbent assay (ELISA) kits for PRRSV antibody detection (IDEXX Labs Inc., Westbrook, ME, USA) and PCV2 antibody detection (INGEZIM CIRCOVIRUS IgG/IgM kit; Ingenasa, Madrid, Spain) and using RT-PCR or PCR for viral nucleic acid detection. Using serological methods and/or RT-PCR/PCR, the animals were shown to be negative for classical swine fever virus, porcine parvovirus, pseudorabies virus, swine influenza virus and *Mycoplasma hyopneumoniae* infections prior to this study. The piglets were transported to the national level 2 animal facilities at the Harbin Veterinary Research Institute. The pigs were randomly assigned to one of four groups with 5 pigs in each and were raised separately in different isolation rooms with individual ventilation. The animals received food and water *ad libitum*. At 4 weeks of age, pigs in two groups were vaccinated with the commercial PCV2a-LG vaccine according to the manufacturer's instructions. An identical booster immunization was conducted 3

weeks post primary immunization as recommended by the vaccine manufacturer. Negative and positive control pigs were not vaccinated. Serum samples were collected to detect antibodies against PCV2 from 0 d before primary vaccination through 28 d post challenge. At 9 weeks of age, two weeks after the second immunization, all pigs from two vaccinated groups were challenged with mPCV2b/YJ strain ($10^{4.5}$ TCID₅₀/mL) and PCV2a/CL strain ($10^{4.5}$ TCID₅₀/mL), respectively, 1.0 mL intraperitoneally and 1.0 mL intranasally. One unvaccinated group was challenged with mPCV2b/YJ as positive control as mentioned above and another unvaccinated group was designed as negative control with no treatments. After challenge, serum samples were collected for viral nucleic acid and antibody analysis, at 0, 7, 14, 21 and 28 days post-challenge (DPC) and all piglets were examined clinically during the experimental period. On the day of challenge and at the end of the experiment, all the piglets were weighed, and the average daily weight gain (ADWG) (kg/day), expressed as (body weight at 28 DPC – body weight at 0 DPC)/28, was determined. At 28 DPC, all pigs were euthanized and postmortem examinations were performed.

2.4. Vaccination

The pigs were vaccinated intramuscularly in the right neck and intranasally at 4 weeks of age with 1 ml of commercial PCV2-LG strain inactivated vaccine (lot number: 080011071, Weike, Harbin, China), respectively. An identical booster immunization was conducted 3 weeks post primary immunization.

2.5. Clinical examination

Piglets in vaccination-challenge groups as well as the negative and positive control groups were examined clinically and a physical condition score was assessed. The clinical signs evaluated included the presence of respiratory and digestive clinical signs (wasting, coughing, dyspnea and diarrhea), behavior (depression) and pathological condition. The clinical parameters were scored using a numeric value ranging between 0 and 2 (none, 0; mild, 1; severe, 2) [23]. The clinical examination of physical condition was recorded daily post challenge and the final score was given after overall evaluation during this period by a blinded researcher. On the basis of the recorded parameters, a clinical score (CS) was calculated using the formula: CS = [(numeric value of wasting + numeric value of coughing + rough hair-coat + numeric value of dyspnea + numeric value of diarrhea + numeric value of behavior)].

2.6. Serology

Serum samples obtained throughout the study at a week interval until 28 DPC were used to determine the changes with time in PCV2-specific antibody *in vivo* post PCV2 vaccination and/or mPCV2b/YJ challenge, using an IPMA, as previously described [23-25].

2.7. Detection of PCV2 viremia in serum samples by PCR

Serum samples were collected from all animals at 0, 7, 14, 21 and 28 DPC, respectively, and PCV2 DNA extract and viremia detection were performed as the method described previously in our laboratory [26].

2.8. Real-time qPCR to detect PCV2 nucleic acid in different organs/tissues

The experimental piglets were euthanized at the end of the trial at 28 DPC, and

various organs/tissues were collected for detection of PCV2 nucleic acid. Viral DNA was extracted from the samples using a DNA extraction kit (Tiangen) according to the manufacturer's instructions. All DNA extracts were tested for the presence of PCV2 DNA by a quantitative real-time PCR assay targeting a conserved region as described previously [23].

2.9. Pathological studies

The macropathological lesions (i.e. the pathological condition) and the micropathological lesions (i.e. histological lesions) were considered together in the pathological studies. During postmortem examination, the pathological condition was observed, including ascites or edema, icterus, pale or congested liver, atrophy of lymph nodes, and enlargement or hemorrhage of peripheral lymph nodes. Each pathological condition was scored from 0 (none) to 2 (severe). At postmortem examination, inguinal lymph nodes were obtained and were fixed by immersion in 10% buffered formalin. The fixed samples were dehydrated and embedded in paraffin wax in a single block per animal. Sections 4 µm thick were cut from each block. Each section was processed for routine histopathology and stained with hematoxylin and eosin. The pathological studies had two objectives. The first was to establish the diagnosis of PCVAD at the moment of occurrence of clinical signs compatible with the disease, as reported previously [27]. In such a scenario, a pig was diagnosed with PCVAD when all three criteria of the accepted international individual case definition for the disease (presence of clinical signs, mainly wasting, plus moderate to severe lymphoid lesions) were present [28]. Second, lymphocyte depletion of inguinal lymph

nodes was assessed between different treatment groups [23]. The clinical examinations of pathological condition and histological lesions were performed at 28 DPC by a blinded researcher.

3. Results

3.1. Clinical examination

No apparent gross lesions were observed in the control group, however, clinical signs compatible with PCVAD, characterized by wasting, coughing, dyspnea, diarrhea, rough hair-coat and depression, were observed in the mPCV2b/YJ challenge group without vaccination mainly from 10 to 28 days post-challenge (DPC). The mean clinical score (CS) was significantly higher ($P < 0.05$) in the positive mPCV2b/YJ challenged group compared with those of the PCV2-LG vaccination groups throughout the study period, which confirmed only mPCV2b/YJ infection can induces the clinical diseases compatible with PCVAD. Based on the CS in the piglets, however, no significant difference was demonstrated between the PCV2a/CL and mPCV2b/YJ challenged groups post the commercial PCV2-LG vaccination ($P > 0.05$). Based on the CS value, it indicate that the commercial PCV2-LG vaccine can completely protect the piglets from the infection of the mPCV2b/YJ. The detailed CS values are shown in Table 2. However, significantly more severe clinical manifestations were demonstrated in the mPCV2b/YJ challenged group compared with those induced in the remaining vaccination-challenge groups and the control. For example, there were more severe pathological lesions in the inguinal lymph nodes (Figure 1).

3.2. Average daily weight gain (ADWG)

As shown in the Figure 2, a significantly lower ADWG was observed in the mPCV2b/YJ challenged group without vaccination than that in the other two vaccination-challenge groups and the control ($P < 0.05$), which could represent persuasive proof of the effective protection of the widely used commercial vaccine (PCV2a-LG) in China against the mPCV2b/YJ mutant. Meanwhile, no significant difference was found between the PCV2a/CL and mPCV2b/YJ-challenged groups post vaccination on the basis of the ADWG ($P > 0.05$).

3.3. Viremia

For the detection of viremia, viral DNA was extracted from serum samples collected at 0, 7, 14, 21, and 28DPC and investigated using routine PCR. No viremia was detected in the piglets of the control group, in contrast, all piglets in the mPCV2b/YJ challenge group without vaccination showed viremia, which persisted for about 7 days from around 21 to 28 DPC (Table 3).

3.4. Detection of PCV2-specific antibody

The antibody titers to PCV2 were detected by IPMA, and the data are displayed in Figure 3. The antibodies began to appeared from 21 dpi in the PCV2 vaccinated groups and the titers gradually increased with time following booster immunization. After challenge, the PCV2 vaccinated groups exhibited enhanced antibody titers and achieved a peak titer, with a mean antibody titer of 1200-fold and 1280-fold, respectively. As expected, the antibodies were absent in the challenge group without vaccination prior to challenge, whereas the antibodies were detectable with a mean

antibody titer of 50-fold at 28 DPC.

3.5. Virus distribution and quantification by real-time quantitative PCR (qPCR) in different organs/tissues

PCV2 was localized mainly in the inguinal, submandibular and mesenteric lymph nodes, tonsils and spleen. The viral loads in all the organs/tissues investigated from the mPCV2b/YJ challenged group without vaccination were significantly higher than those in the PCV2 vaccinated groups post challenge at 28 DPC($P < 0.05$). In addition, there was no significant difference in the viral loads of the organs/tissues mentioned above between the PCV2a/CL and mPCV2b/YJ challenge groups post vaccination($P > 0.05$). The results of qPCR performed on the organ/tissue samples are summarized in Figure 4.

3.6. Pathological lesions

The pathological condition was observed, including ascites or edema, icterus, pale or congested liver, atrophy of lymph nodes, and enlargement or hemorrhage of peripheral lymph nodes, which were consistent with a diagnosis of PCVAD. The score for the pathological condition was significantly higher in the mPCV2b/YJ challenged group without vaccination than that in the other two vaccination-challenge groups ($P < 0.05$). In contrast, no lesions were observed in both the vaccination-challenge groups and the control group, which indicated that the commercial PCV2a vaccine can provide protection from the mPCV2b/YJ infection (Table 2). In addition, more severe depletion of lymphocytes in the inguinal lymph nodes was present in the mPCV2b/YJ challenge group without vaccination (Figure5).

4. Discussion

In our previous studies, we isolated several emerging PCV2 mutant stain [16]. Under the experimental conditions, the mutant PCV2 strains demonstrate high virulence compared to that of the conventional PCV2a and PCV2b genotypes [23]. In addition, emerging PCV2 mutants have been also identified in U.S. [21, 22] raising concerns about vaccine efficacy. The large-scale vaccination in the pig producing industry might contribute to the PCV2 variations or mutations, which might be the important mechanism to escape immune elimination initiated by host immune response. Recent evidence from field trials in Japan indicates that efficacy of PCV2 vaccination may depend on the PCV2 genotype [29]. Moreover, Opriessnig et al. has demonstrated a similar study that PCV1-2b vaccination significantly reduced the prevalence and amount of PCV2 viremia compared to the PCV1-2a vaccination [30]. However, the latest studies from the same research group indicated that the current commercial vaccines are efficient against the emerging variant PCV2 strain in U.S. [21, 22]. The objective of this study was to determine the efficacy of the leading commercial PCV2a-based vaccine (PCV2a-LG) in China against the infection of the emerging PCV2 mutant with two amino acids elongation (N and E) in capsid protein.

Under the study conditions, the Chinese wildlly used commercial PCV2 vaccine (PCV2a-LG) significantly prevented detectable PCV2 viremia, weight gain loss and pathological lesions. This is in accordance with the study mentioned above where a commercial vaccine based on PCV2a is effective in protecting pigs against challenge with PCV2 mutant prevailing in U.S. during 2012 and 2013 [21, 22]. Taken all

previous studies together, the efficiency of the current PCV2 vaccination may depend on the PCV2 genotypes or the individual strains with different degrees of variations.

The significance of PCV2 mutations in terms of PCV-associated pathogenesis is unclear. The virulence properties of PCV2 appear to be located in the capsid protein. For example, it has been reported that the attenuation that occurred following serial passage of an isolate of PCV2 was the result of mutations in the capsid protein [31]. However, the same group reported that a chimera incorporating ORF1 from PCV1 and ORF2 from PCV2 was non-pathogenic in pigs [32]. It is not a single ORF1 gene or ORF2 gene that contributes to changes in the virulence and pathogenicity of PCV2, but the involvement of the whole virus genome [33]. Therefore, mutation of the capsid protein may contribute to enhanced virulence, but may not be the only factor. It still remains unknown that the efficiency of the Chinese commercial PCV2a based vaccine (PCV2a-LG) against the emerging PCV2 (mPCV2b/YJ) with two amino acids elongation in the capsid protein. In this study, we answered the question that the Chinese commercial PCV2a based vaccine (PCV2a-LG) can provide effective protection against the challenge with mPCV2b/YJ. The findings of this study may facilitate future use of the current commercial PCV2a-LG vaccine for controlling the PCV2 infection in China, including the different PCV2 genotypes as well as the emerging PCV2 mutant.

5. Conclusions

In this study, vaccination with a commercial PCV2a-based vaccine (PCV2a-LG) in China was effective in protecting against mPCV2b/YJ with two amino acids

elongation in the ORF2 encoded capsid protein.

Acknowledgments

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Figure legends

Figure 1. Clinical lesions of inguinal lymph nodes in the different treatment groups at 28 DPC. A: mPCV2b/YJ challenge group without vaccination; B: Control without vaccination and challenge; C: PCV2a/CL challenge group post vaccination; D: mPCV2b/YJ challenge group post vaccination.

Figure 2. Average daily weight gain (ADWG) in different treatment groups. The ADWG (kg/day), expressed as (body weight at 28 DPC – body weight at 0 DPC)/28, was determined. ADWG $\pm$ SD was calculated for each treatment group throughout the experiment, with (*) indicating significant differences between the specified pairs. No significant difference was seen between the PCV2a/CL and mPCV2b/YJ challenged groups post vaccination ($P > 0.05$).

Figure 3. Changes of PCV2-specific antibodies in different treatment groups. At 4 weeks of age, pigs in two groups were vaccinated with the commercial PCV2a-LG vaccine according to the manufacturer's instructions. An identical booster immunization was conducted 3 weeks post primary immunization as recommended by the vaccine manufacturer. Negative and positive control pigs were not vaccinated. At 9 weeks of age, two weeks after the second immunization, all pigs except the negative control were challenged with mPCV2b/YJ strain ($10^{4.5}$ TCID₅₀/mL) and PCV2a/CL strain ($10^{4.5}$ TCID₅₀/mL), respectively, 1.0 mL intraperitoneally and 1.0 mL intranasally. Serum samples were collected to detect antibodies against PCV2 at indicated time point from 0 d before primary vaccination through 28 d post challenge. Detection of PCV2-specific antibodies in sera at different times using the IPMA

method as previously described [23].

Figure 4. Quantification and distribution of viral DNA loads in different tissues/organs. Detection and quantification of viral DNA loads in lymphoid tissues and spleen was performed using qPCR method as previously described [23]. Group mean logarithm viral genomic copies/gram of tissue ($\pm$ SD) was calculated as the corresponding value on the x axis for each treatment group. Pairs of treatments with (*) were significantly different ($P < 0.05$).

Figure 5. Microscopic lesions of inguinal lymph nodes observed in pigs from different treatment groups. Sections were processed as the method mentioned above in the materials section and stained with hematoxylin and eosin. There were no apparent lymphoid depletion in PCV2a/CL challenge group post vaccination (A), mPCV2b/YJ challenge group post vaccination (B), Control without vaccination and challenge (C). However, severe lymphoid depletion was observed in the mPCV2/YJ challenge group without vaccination (D). Magnification scale (200X).

Table 1: Characteristics of PCV2 strains used for challenge experiment in this study

Characteristics	PCV2 used for challenge	
	PCV2a/CL	mPCV2b/YJ
GenBank No	HM038033	HM038032
Genotype	PCV2a	PCV2b
Passage [△]	10	10
Year of isolation	2008	2008
Genome length (nt)	1768	1766
ORF2 (aa)	233	235
ORF1 (aa)	314	314
Number of amino acids*	0	2 (N and E)

Note: [△] represents the number of passages of the PCV2 strains used for the infection experiment in this study; * represents the number of additional amino acids encoded by ORF2 compared with classical PCV2 strains with 233 aa in the ORF2-encoded Cap protein.

Table 2: Scored values for clinical condition of piglets in different vaccination and challenge groups

	Vaccine ➡	PCV2a LG	PCV2a LG	None	None
	Challenge ➡	mPCV2b/YJ	PCV2a/CL	mPCV2b/YJ	None
Physical condition					
Wasting		0,0,0,0,0	0,0,0,0,0	2,1,1,2,2	0,0,0,0,0
Coughing		0,0,0,0,0	0,0,0,0,0	1,0,0,0,1	0,0,0,0,0
Rough hair-coat		0,0,0,0,0	0,0,0,0,0	0,1,0,1,1	0,0,0,0,0
Dyspnea		0,0,0,0,0	0,0,0,0,0	0,0,0,0,0	0,0,0,0,0
Diarrhea		0,0,0,0,0	0,0,0,0,0	1,0,0,1,0	0,0,0,0,0
Depression		0,0,0,0,0	0,0,0,0,0	0,0,0,1,1	0,0,0,0,0
Median score		0	0	4	0
Pathological condition					
Ascites or edema		0,0,0,0,0	0,0,0,0,0	0,0,0,0,0	0,0,0,0,0
Icterus		0,0,0,0,0	0,0,0,0,0	1,0,0,0,0	0,0,0,0,0
Liver pale or congested		0,0,0,0,0	0,0,0,0,0	1,1,0,0,0	0,0,0,0,0
Atrophy of lymph nodes		0,0,0,0,0	0,0,0,0,0	1,0,1,1,1	0,0,0,0,0
Enlargement and hemorrhage of peripheral lymph nodes		0,0,0,0,0	0,0,0,0,0	1,0,1,0,1	0,0,0,0,0
Median score		0	0	2	0

Table 3: Detection of PCV2 viral DNA using PCR in sera of pigs challenged experimentally with different PCV2 strains at different time points post challenge

Vaccination	Challenge	Days post challenge				
		0	7	14	21	28
Vaccine [#]	PCV2a/ CL	0/5 [*]	0/5	0/5	0/5	0/5
Vaccine	mPCV2b/YJ	0/5	0/5	0/5	0/5	0/5
None	mPCV2b /YJ	0/5	0/5	0/5	4/5	5/5
None	None	0/5	0/5	0/5	0/5	0/5

[#]: a PCV2a genotype based commercial PCV2a LG vaccine widely used in China

^{*}: Number of PCV2 positive/Number of pigs tested

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5