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Original article

Evaluation of the protective efficacy of virus-like particles based on PCV 2b and 2d subtypes against mixed challenge in mice

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Abstract

Porcine circovirus type 2 (PCV2) is an economically important swine pathogen and, although small, it has the highest evolution rate among DNA viruses. Commercial PCV2 commercial vaccines are inactivated PCV2 isolates or a subunit vaccine based on the Cap protein of PCV2. Currently, PCV2 VLPs of individual subtype vaccines are available. Although the main prevalent genotype worldwide is PCV2b, the emerging subtype PCV2d subtype is also increasingly associated with PCV disease. The aim of the study was to evaluate the protective efficacy of VLP based on the PCV2b and 2d subtypes against the mixed challenge of two hypotype PCV2 in mice. Thirty-six female SPV Kunming mice were immunized twice with PCV2b and 2d VLPs, then challenged with PCV2b and PCV2d, to assess the immunogenicity and effectiveness of the VLPs. Vaccination of the mice with PCV2b and 2d VLPs elicited a robust antibody response specific for the PCV2. The virus load detected in the 2b and 2d spleen vaccine group was the lowest compared to other groups. Furthermore, there was no pathological damage in the HE stained sections of the 2b and 2d spleen vaccine, and no virus was detected in the immunohistochemical sections. Our data suggest that the mixed PCV2b and 2d VLP vaccine could protect mice from challenge with the mixed infection of PCV2b and PCV2d.

Key words: type 2, virus-like particles, vaccine, mouse

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Introduction

Porcine circovirus type 2 (PCV2) is the pathogenic agent of postweaning multisystemic wasting syndrome (PMWS), proliferating and necrotizing pneumonia (PNP), and porcine dermatitis nephropathy syndrome (PDNS), also collectively regarded as porcine circovirus associated diseases (PCVADs) (Guo et al. 2010, Ge et al. 2012), recognized as a main cause of severe economic losses in porcine husbandry worldwide (Segales et al. 2005, Fraile et al. 2012).

The genome structure of PCV2, a member of the genus *Circovirus* of the family *Circoviridae*, is very minimalistic. This small, non-enveloped DNA virus has a single-stranded circular genome of 1766-1768 nucleotides containing two major open reading frames (ORFs): ORF1 and ORF2, which encode the replicase proteins (Rep and Rep') associated with the replication of the circoviral genome and the capsid protein (Cap), which is the main immunogenic protein of PCV2 (Nawagitgul et al. 2000). Despite its minimalistic design, PCV2 has the highest genetic variability among single-stranded DNA viruses and is characterized by a rate of nucleotide substitution in the order of 1.2×10^{-3} per site per year (Firth et al. 2009). In 2008, the EU consortium on porcine circovirus diseases proposed a standardized nomenclature for the PCV2 definition based on pairwise sequence comparison analysis applied to ORF2 nucleotide sequences (Segales et al. 2009). To date, PCV2 can be divided into five different genotypes, PCV2a, PCV2b, PCV2c, PCV2d and PCV2e (Wang et al. 2009, Guo et al. 2010, Guo et al. 2012, Xiao et al. 2015). Among all PCV2 genotypes, PCV2a was the predominant strain prior to 2000 and then there appeared to be a global genetic shift from PCV2a to PCV2b with the latter being the predominant genotype seen in the past decades (Wang et al. 2009, Guo et al. 2010). Recently, some studies have suggested that there was an ongoing genotype shift that occurred from PCV2b to PCV2d (Xiao et al. 2015, Yang et al. 2018), which was first identified in 2002 in China first and gradually emerged in essentially all pig herds in North America, South America, Europe, and Asia (Segales 2015, Xiao et al. 2016, Kwon et al. 2017). In 2010, a variant strain of the PCV2 mutant designated mPCV2b, now grouped in PCV2d, with an elongation of its ORF2 by one amino acid, lysine (K), was identified in several cases of PCVAD in China and other countries and recent studies showed that the prevalence rate of mPCV2b appeared to have increased in China and a similar trend was evident in the US (Guo et al. 2010, Xiao et al. 2012, Xiao et al. 2015, Jiang et al. 2017).

Virus-like particles (VLPs) are self-assembling, non-replicating particles lacking the viral genome. They can be produced from one or several viral structural proteins depending on the complexity of the virus and are often formed spontaneously when the structural proteins are expressed in different types of eukaryotic cells. VLPs are attractive candidates to elicit nAb responses as their resembling structure and are much safer than the wild-type virus from which they are derived (Jourdan et al. 2006, Stewart et al. 2010). VLPs have recently come into special focus with the FDA approval of a VLP-based vaccine against human papilloma viruses (HPV) (Mohsen et al. 2017). In addition to the HPV VLP vaccines, the VLP approach has been used to recently develop a chikungunya virus vaccine that was effective in non-human primates (Akahata et al. 2010). The term virus-like refers to the morphological similarity between VLPs and the corresponding virions, and VLPs can be purified after expression in yeast cells, insect cells using baculoviruses, Escherichia coli or mammalian cells (Salunke et al. 1986, Forstova et al. 1993, Palkova et al. 2000, Szecsi et al. 2006). The VLP-based vaccine is a rapidly growing field and includes a variety of different applications, mostly for protection against virus infections, but also in the field of cancer immune therapy, gene therapy, and treatment of different disorders such as rheumatoid arthritis and high blood pressure (Ramqvist et al. 2007). A VLP-based veterinary vaccine will be a new development in the future.

The aim of this study was to construct a PCV2b and 2d VLPs vaccine, with a focus on its efficacy and immunogenicity in mice models.

Materials and Methods

Ethics approval and consent to participate

The study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Protocols for the animal studies were approved by the Committee on the Ethics of Animal Experiments of the College of Veterinary Medicine, Hunan Agricultural University.

Cell lines and viruses

Porcine kidney cell line (PK-15, ATCC: CCL-33), purchased from the American Type Culture Collection, was free of PCV1 and PCV2. PK-15 cells were cultured at 37 ° C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, *life*, USA) supplemented with 8% newborn calf serum (NBCS, *life*, USA). The PCV2b used in the study was isolated from the disease material of our laboratory and named YiY-3-27 (GenBank No. KU317482); it was stored at -80 ° C and propagated

Table1. Experimental design. Thirty six female SPV Kunming mice were randomly divided into six groups (6 per group), and were immunized twice on days 0 and 14. The dosage and time of the primary inoculation (PI), secondary inoculation (SI) and challenge for each group can be seen in the table. "S" represents PBS sham-vaccinated or PBS sham-Challenged. "C" stands for challenge with PCV2b+2d.

Group	Vaccine	Vaccine dose/day				Challenge	Challenge dose/day	
		PI	SI	PI	SI			
NC	PBS	-	-	-	-	-	-	-
S+C	PBS	-	-	-	-	PCV2b+2d	10^4	21
2b+C	PCV2b VLP	50 µg	100 µg	0	14	PCV2b+2d	10^4	21
2d+C	PCV2b VLP	50 µg	100 µg	0	14	PCV2b+2d	10^4	21
2b2d+C	PCV2b+2d VLP	50 µg	100 µg	0	14	PCV2b+2d	10^4	21
2b2d+S	PCV2b+2d VLP	50 µg	100 µg	0	14	PBS	-	-

in PK-15 cells prior to use. The viral stock had a titer of $1\times10^{-5.5}$ 50% tissue culture infective dose ($TCID_{50}$) /mL in the supernatant of the PK-15 cell culture. The PCV2d was also isolated in the laboratory and named ChenZ-2-1 (GenBank No. MH718995). The virus titer was $TCID_{50}$ of $1\times10^{-5.6}$/mL.

PCV2 virus-like particle preparation

PCV2b and 2d virus-like particles were constructed as previously described (Zhang et al. 2016). The constructed virus-like particles were observed and identified using an electron microscope.

Mouse Immunization Schedule

The experimental design of mouse challenge protection is shown in Table 1. All experimental protocols involving mice, approved by the Hunan province Laboratory Animal Monitoring Committee, were negative for PCV2 antibodies analyzed by enzyme-linked immunosorbent assay (ELISA). Thirty-six female SPV Kunming mice purchased from the Shrek Jingda Hunan Changsha Company were randomly assigned to six groups of six mice and were immunized twice on days 0 and 14 with (1) Negative control. (NC, PBS sham--vaccinated / PBS sham-Challenged); (2) S+C group (PBS sham-vaccinated / PCV2b+2d challenge); (3) 2b+C (PCV2b VLPs vaccine / PCV2b+2d challenge); (4) 2d+C (PCV2d VLPs vaccine / PCV2b+2d challenge); (5) 2b2d+C (PCV2b+2d VLPs vaccine / PCV2b+2d challenge); (6) 2b2d+S (PCV2b+2d VLPs vaccine / PBS sham-Challenged). The protein concentration was determined using a NanoDrop 2000 spectrophotometer. The experimental groups were vaccinated intramuscularly after mixing the protein with Freund's incomplete adjuvant (Sigma, USA). Serum samples were collected from the animals on day 21, 35, and 49 after primary immunization (DPI) for antibody titration. On day 21 after immunization, the mice in the experimental group were infected with wild-type PCV2b and PCV2d, whose doses were mixed 1:1 ($10^{-4}TCID_{50}$ in 200 µL PBS per mouse). The negative control group (PBS sham-vaccinated, PBS sham--challenged) was neither vaccinated nor challenged with virus. On 49 DPI, the mice were sacrificed using carbon dioxide asphyxiation euthanasia, the spleens were sampled and processed for histological and immunohistochemical analysis, and used for the testing of virus titration. PCV2 infection was confirmed using a quantitative real-time PCR (qPCR) based on the PCV2-Cap gene using the primers: PCV2 Cap-97 GGGTTATGGTATGGCGGGAG and PCV2 Cap-98 CCCTCACTGTGCCCTTTGAA.

Ethics statement

The study was carried out following the Guide for the Care and Use of Animals in Research of the People's Republic of China.

Antibody titer measurement

Antibody titers in sera from immunized mice were measured using ELISA as described previously (Yuan et al. 2017). The PCV2d Cap protein was used to coat the ELISA plates. The second antibody was HRP-conjugated goat anti-mouse IgG.

Transmission electron microscopy (TEM)

After dialysis, the assembled sample was adsorbed on a 200-mesh carbon-coated copper grid. It was allowed to act for 8-10 min at room temperature. The excess liquid was removed using filter paper and negatively stained with 3% crane acid for 8-10 min. The prepared samples were observed under TEM to observe the morphological structure of PCV2b and 2d Cap VLPs.

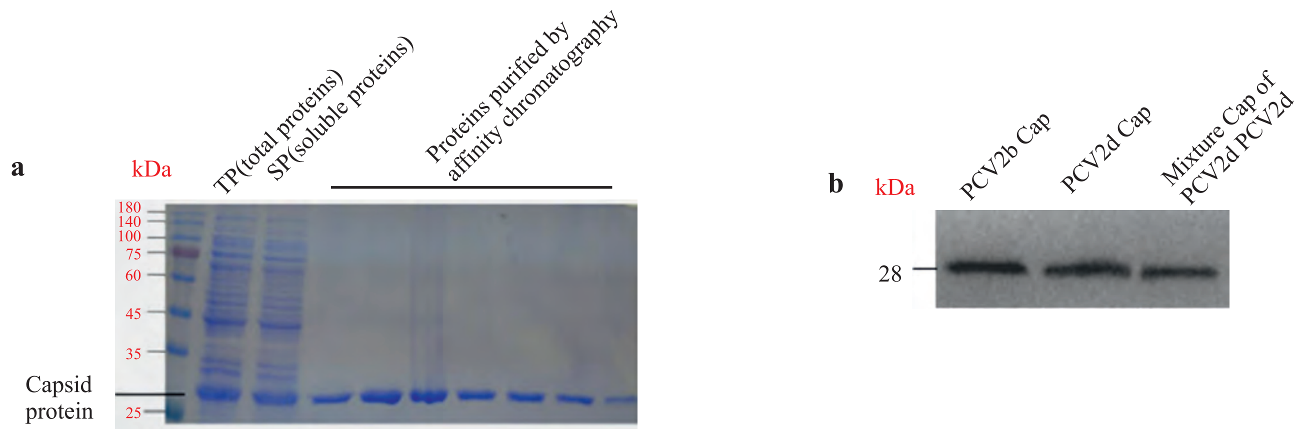

Fig. 1. Purification and verification of soluble PCV2 Cap. The expression of the Cap protein with molecular 406 weights of about 28 kDa was analyzed by SDS-PAGE in Fig.1a, and by Western-blotting using two anti-PCV2 specific antibodies in Fig.1b.

Statistical analysis

All data were analyzed by one-way ANOVA using SPSS statistical analysis software (version 13.0) to calculate statistical significance ($p<0.05$, significant difference; $P<0.01$, extremely significant difference). Statistical analysis was performed using Prism v6.0 (GraphPad Software, La Jolla, CA, United States), and expressed as mean values with standard deviations.

Results

Engineering of PCV2b and 2d of Cap VLPs

In order to engineer an effective PCV2b and 2d of Cap VLPs, we used E. coli BL21 (DE3) cells to express and assemble. The expression of the Cap protein in E. coli lysates were analyzed by SDS-PAGE and Western-blotting using the anti-PCV2 specific antibodies as primary antibodies. SDS-PAGE analysis showed that most of the Cap protein with molecular weights of about 28 kDa existed in the supernatant from the lysate of E. coli, while little in the precipitation, indicating that the Cap was soluble protein (Fig. 1a). The results of Western-blotting revealed that the specific 28 kDa band corresponding to that of the Cap could be recognized by both the anti-PCV2 specific antibodies (Fig. 1b). Then the Cap protein was purified by nickel affinity chromatography. The supernatant from the lysate of the E. coli was loaded onto the nickel affinity column and multiple elution fractions were obtained. The Cap protein with high purity of approximately 28 kDa in the elution fractions was visible on the SDS-PAGE gel.

Transmission electron microscopy analysis of VLPs

To examine whether the PCV2b and 2d Cap protein can be assembled into VLPs, TEM analysis was performed. The assembled PCV2b and 2d Cap protein could be observed under TEM using PCV2b and 2d complete virion as control. The PCV2b and 2d Cap proteins could form homogeneous and tight VLPs (Fig. 2). In comparison, the complete virion PCV2b and 2d was looser than the Cap VLPs, the diameter of which formed by both the PCV2b and the 2d Cap protein was 17-20 nm.

Immune response in mice

The immune response to vaccination with PCV2b and 2d VLPs was assessed by indirect ELISA. The mice received two vaccinations, on days 0 and 14, respectively. The Cap-specific immune response gradually increased over time in animals immunized with PCV2b and 2d VLP (Fig. 3a). The IgG titers in animals vaccinated with PCV2b and 2d VLPs were significantly higher than the other groups ($p<0.01$; $n=5$). The qPCR dates showed that PCV2 DNA copies of the 2b2d + C group in spleens during 14 days and 28 days were lower than the other groups (Fig. 3b). The average weight curves for mice inoculated with a virus titer of 1×10^{-4} $TCID_{50}$ /mL challenge are shown in Fig. 3c. All VLP groups inoculated into mice resulted in no adverse reactions. The PCV2b and 2d challenge data indicate that PCV2b and 2d VLPs had the least weight loss after immunization. The clinical presentation statistics of all groups are in Table 2. The microscopic pathology of HE staining of the experimental mouse spleen section is shown in Fig. 4. The types and positive rates of histopathological damage to the spleen are shown in Table 3 and Table 4. The result of immunohistochemical detection of PCV2 antigen in the spleen is shown in Fig. 5. The results show that no PCV2 virus was detected in the spleen sections of all mice in the 2b2d+S group. The spleen sections of 6 mice in the challenge control group (S+C) all showed PCV2 positive. Taken together, these results show that the PCV2b and 2d VLPs immunized the mice and provided complete protection

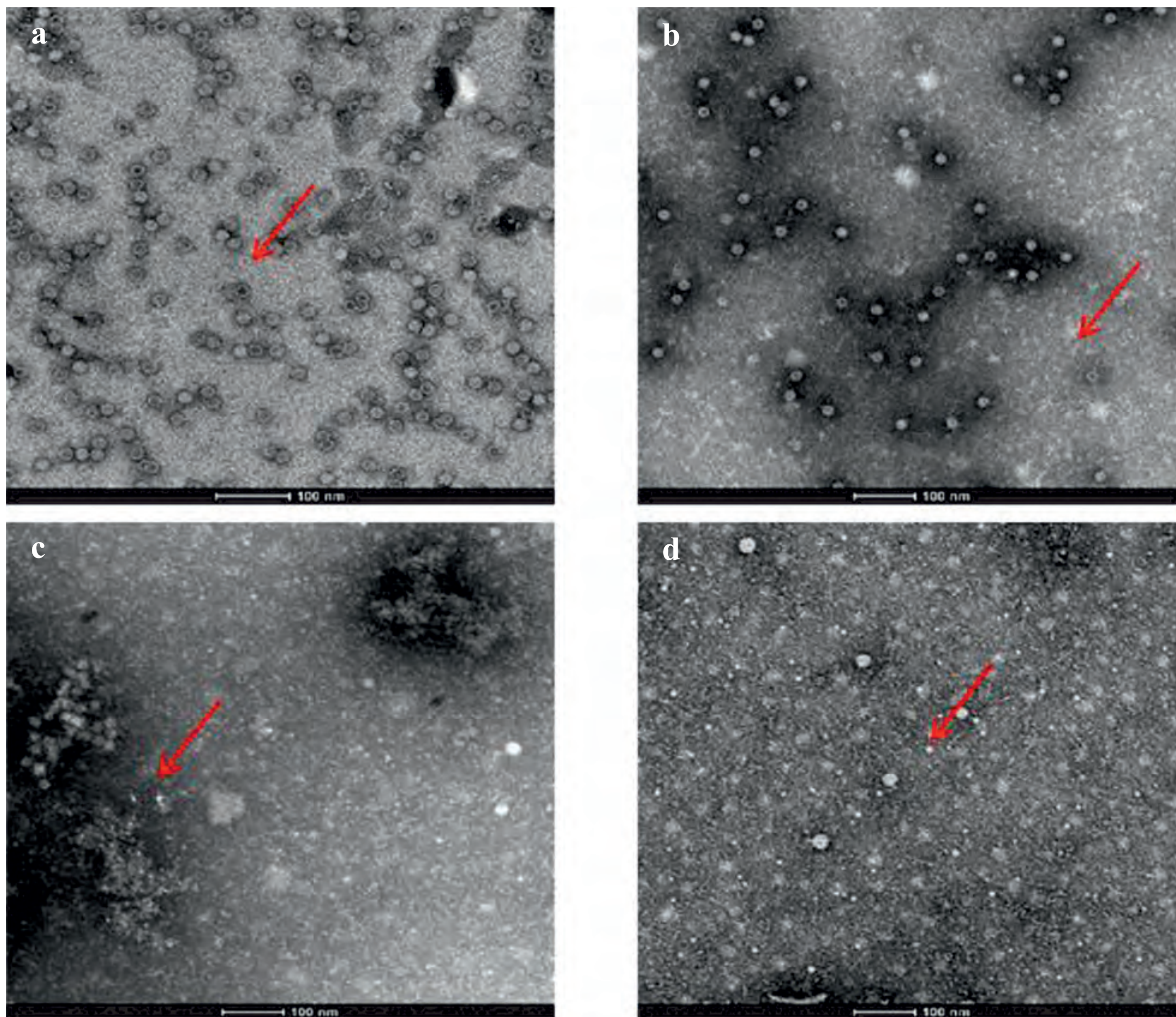

Fig. 2. Transmission electron microscopy analysis of VLPs. The structure of PCV2 VLPs and natural virions were demonstrated by transmission electron microscopy. Red arrows indicate VLP. (a) PCV2b VLPs, (b) PCV2d 412 VLPs, (c) natural virions of PCV2b, (d) natural virions of PCV2d.

against infection with the PCV2b and 2d mixed infection.

Safety of VLPs in mice

The safety of the PCV2b and2d VLPs vaccine was assessed in six-week-old mice for up to 7 weeks post-immunization. There were no indications of abnormal body temperature or appetite in any of the vaccinated animals. These results demonstrate that PCV2b and 2d VLPs were well tolerated in mice.

Sequence and structure differences in the Cap protein of PCV 2b and 2d

The amino acid sequences in the Cap proteins of the two viruses were compared. Sequence alignment indicated that the amino acid sequence identity between the two strains was 94% (Fig. 6). The prediction structure of the Cap protein is shown in Fig. 7 using three-dimensional structural modeling. The main differences in the three-dimensional structure diagram are marked with different colors. It can be noted that PCV2b and 2d of the Cap protein are different in key areas, which have been marked in Fig. 7.

Discussion

We engineered PCV 2b and 2d VLPs vaccine that expressed the Cap protein. Two kinds of virus-like particles added together that induced a strong immune response to the Cap antigen of PCV2 in mice models and provided full protection against infection of two subtype viruses. Our results suggest that two types of virus-like particles mixed together is a promising candidate for bivalent vaccine against PCV2b and 2d mixed infection.

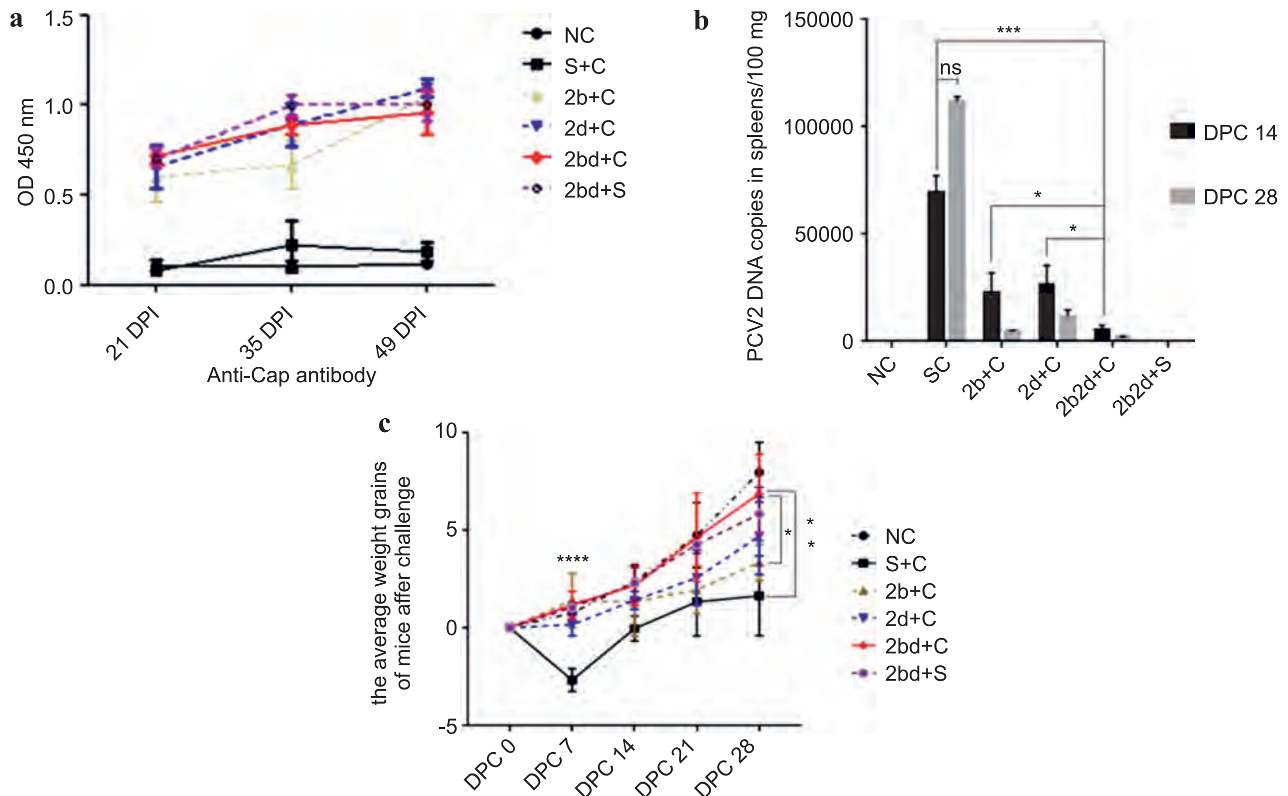

Fig. 3. Immune response to VLPs in mice. (a) Indirect ELISA assessesment of specific antibody levels. (b) The number of PCV2 copies in the spleen of mice was detected by qPCR using specific primes on days 14 and 28 post-primary challenge (DPC). (c) Mice gained weight on average on days 0, 7, 14, 21 and 28 post-primary challenge.

Table 2. Severity of disease in experimental mice. Degree of appetite loss indicates the rate of decrease in average daily food intake per mouse, compared to the control group (MOCK), –: relative food intake fell within 5 percent; +: relative food intake is down between 5 and 10 percent; ++: relative food intake is down between 10 and 20 percent. Loss of mental stability represents a sluggish and lethargic response from the mice: –: mental stability; +: mentally unstable. Thick fur disorderly indicates that the fur of the mice after challenge was rough and untidy: –: normal; +: slight disorderly.

Group	Mock	NC	2b+C	2d+C	2b2d+C	2b2d+S
Degree of appetite loss	-	++	+	+	-	-
Loss of mental stability	-	+	+	+	-	-
Thick fur disorderly	-	+	+	+	-	-

Table 3. Distribution of histopathological lesions in different tissues from experimental mice challenged with PCV2.

Group	LE	LD
NC	0/6	0/6
S+C	2/6	3/6
2b+C	2/6	3/5
2d+C	0/6	1/6
2b2d+C	0/6	0/6
2b2d+S	0/6	0/6

It has been shown that several epitopes are conserved among various PCV2 strains. However, it is unclear whether these different strains of PCV2 can induce a cross-immune response. The Cap protein is a crucial component of PCV2 and contains a few neutralizing epitopes, but it is genetically variable, which

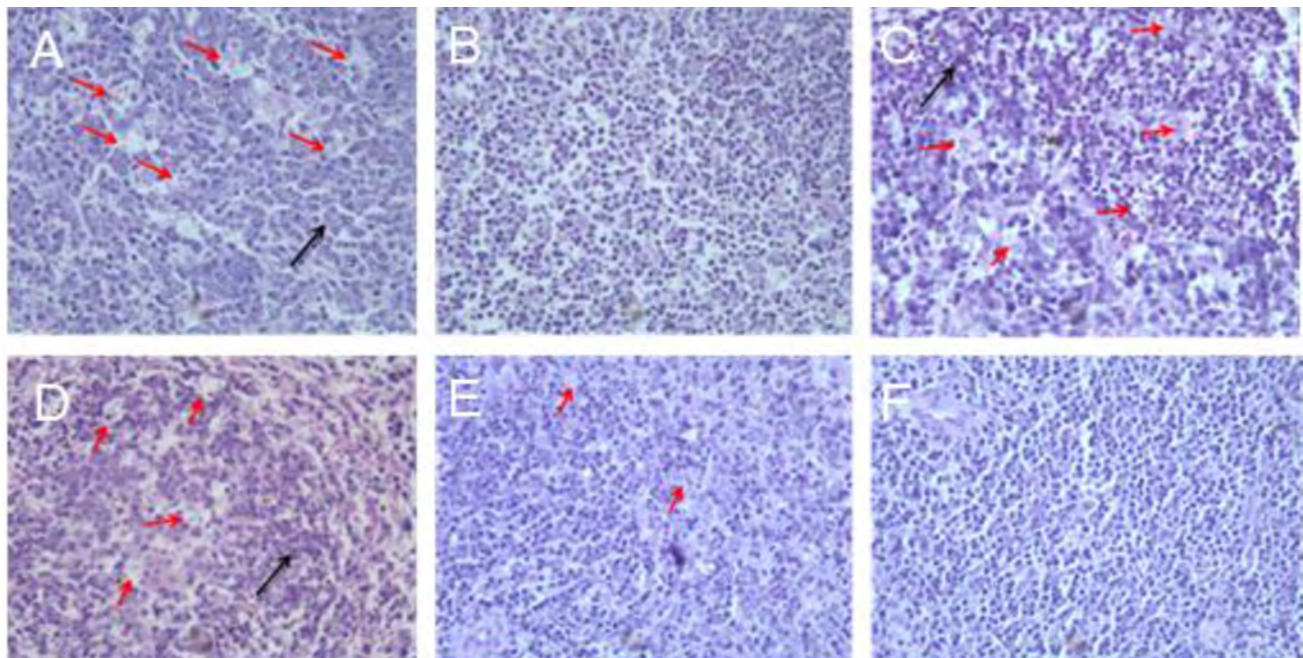

Fig. 4. Microscopic lesions in experimental mice spleens (×400). (A) Severe lymphoid depletion was observed in the spleen of three of six unvaccinated mice, a large number of apoptotic lymphocyte fragments, lymphocyte enlargement and intercellular apoptotic spaces can be observed. (B) Lymphocytes of the spleen are of moderate size and closely arranged, no remarkable microscopic lesions were found in the spleen of mice in the control group. (C) Similarly, severe lymphocyte apoptosis and lymphocyte enlargement was observed in three of the five mice in the 2b+C group. (D) One of the six mice in the 2d+C group was observed to have a large number of erythrocyte cells, which were arranged in a disorderly manner and were visible as cytolytic fragments. (E) All the mice spleen cells were well aligned and had regular nuclear morphology, with only a small number of apoptotic areas in the 2b2d+C group. (F) The spleen of the 2b2d+S group showed no obvious microscopic damage.

Table 4. Evolution of lesions in mice from different treatment groups. The macroscopic lesions score was obtained by summing I, II and III according to the following description and lesion scores from 0 (normal) to 10 (severe lesion). Shown are mean ± S.D. I. Congestive splenomegaly: 0 (none) to 4 (severe intumescentia). II. Nodular hemorrhage of the lung: 0 (no bleeding) to 3 (severe bleeding). III. Hepatic necrosis spot: 0 (no necrosis) to 3 (Several necrosis). The microscopic score was obtained by summing I to V according to the following description and lesion scores from 0 (normal) to 10 (severe lesion). Shown are mean ± S.D. I. Infiltrations of inflammatory cells: 0, none; 1 (mild); 2 (severe). II. Pulmonary interstitial hyperplasia: 0 (no lesion); 1 (mild); 2 (severe). III. Splenic corpuscle atrophy, lymphocyte body enlargement and lymphocyte apoptosis: 0 (none) to 4 (severe). IV. Pulmonary hemorrhage: 0, none; 1 (mild); 2 (severe).

Group	n	Macroscopic lesions score				Macroscopic lesions score			
NC	6	0.00±0.00				0.00±0.00			
S+C	6	6.81±0.33				5.83±0.45			
2b+C	5	4.60±0.35			***	5.10±0.22			***
2d+C	6	3.32±0.44	*	*		2.57±0.31	**	***	
2b2d+C	6	2.34±0.20				0.81±0.24			
2b2d+S	6	0.00±0.00				0.00±0.00			

added to the difficulty of generating a PCV vaccine. Cap protein has good virus-like particle assembly characteristics, and it produced VLPs have been used in clinical vaccine applications. This VLPs vaccine is currently recognized as the safest vaccine. Compared with attenuated vaccines, the VLP vaccine is expected to achieve the effect of eradicating viral diseases on pig farms. However, the high variability of the circovirus determines that the effect of a single type of VLPs will not be as good as that of two types of VLPs. These results can also provide insight into why commercial vaccines developed from single strains of the PCV2 subtype are no longer effective in China due to the emergence of mixed PCV 2b and 2d infection. Studies have shown that vaccination with either the conventional PCV2 or PCV1-2 vaccines can effectively reduce viremia and lymph node PCV2 load in experimentally infected pigs, but cannot prevent PCV2 infection and

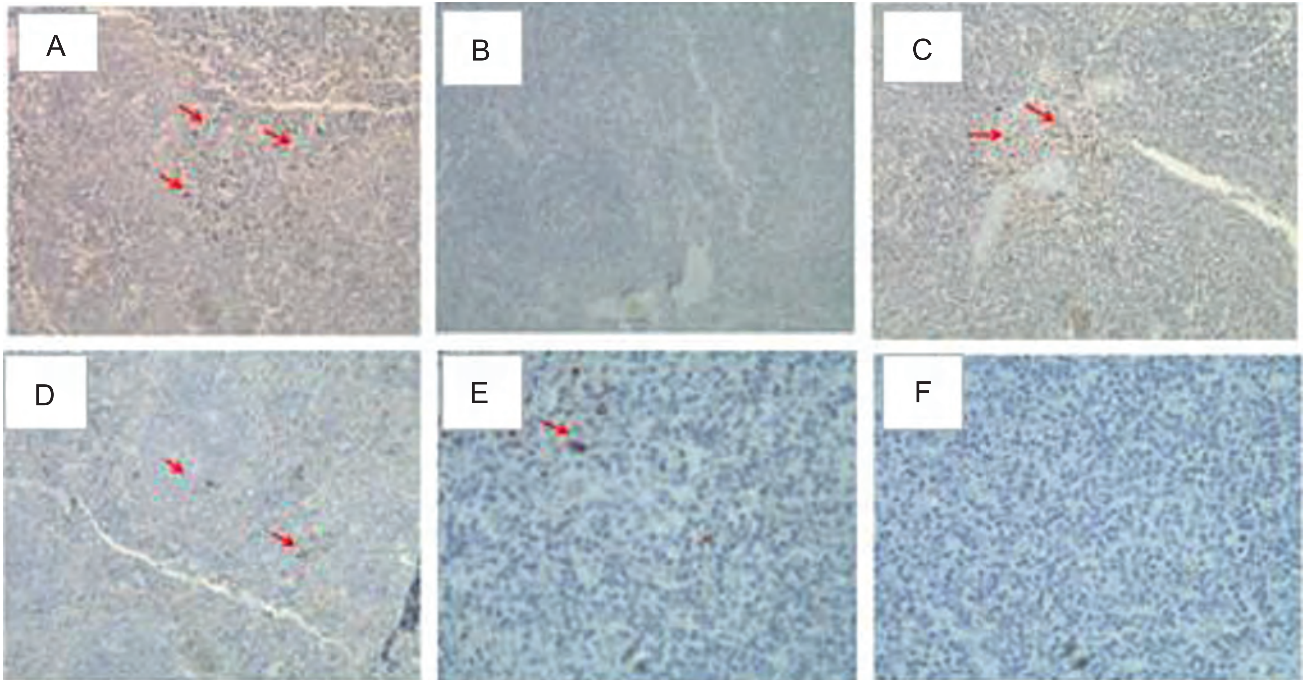

Fig.5. Immunohistochemical detection of PCV2 antigen in spleen (×400). (A) High amounts of PCV2 antigen in spleen lymphocytes of every non-vaccinated challenged mouse from S+C group. (B) Slight staining of the lungs of the NC group was obtained, and no detection of PCV2 antigen in spleens of six control mice. (C) PCV2 antigen signal was detected in the spleens of five mice from the 2b+C group. (D) Four of the six mice in 2d+C showed a clear brown positive signal in the spleen. (E) Notably, only one of the six mice in 2b2d+C showed a weak brown positive signal in the spleen. (F) The spleen of the 2bd+S group showed no obvious PCV2 antigen.

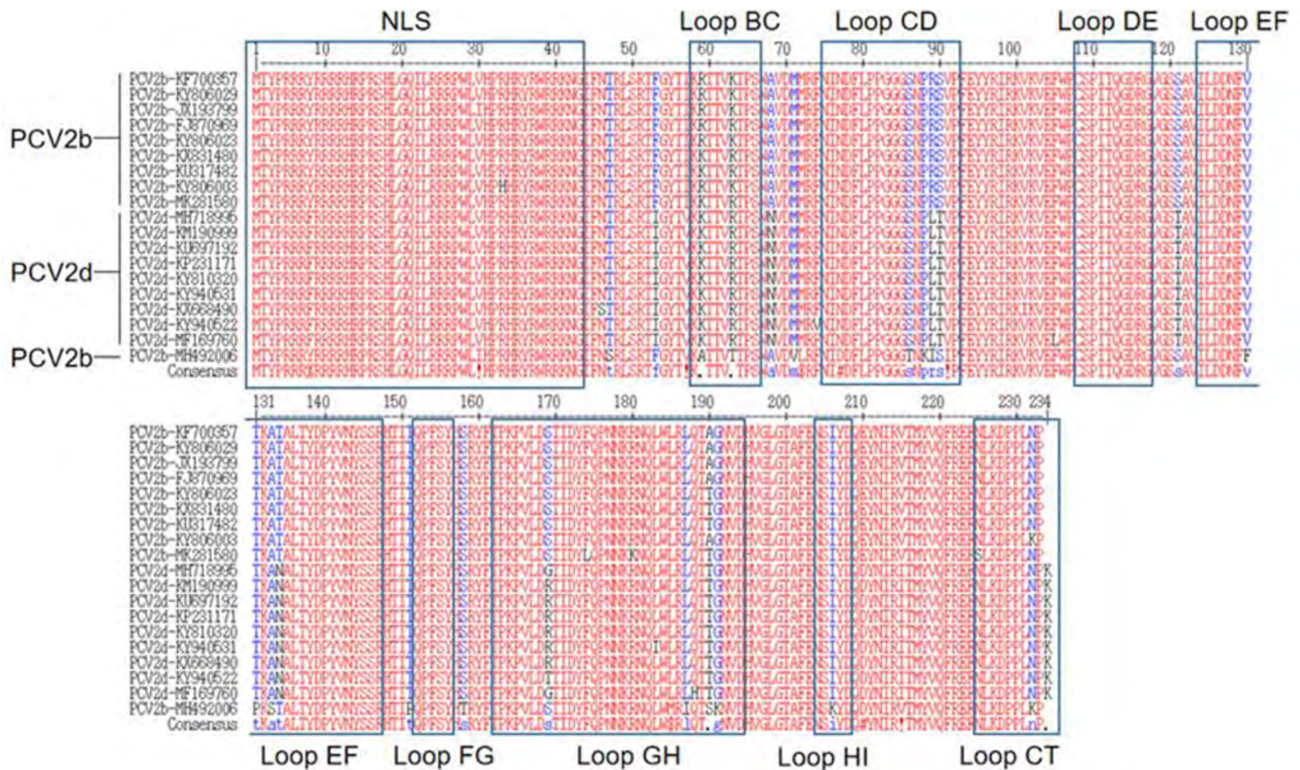

Fig. 6. Alignment of the aa sequences from the PCV2 strains PCV2b and PCV2d.

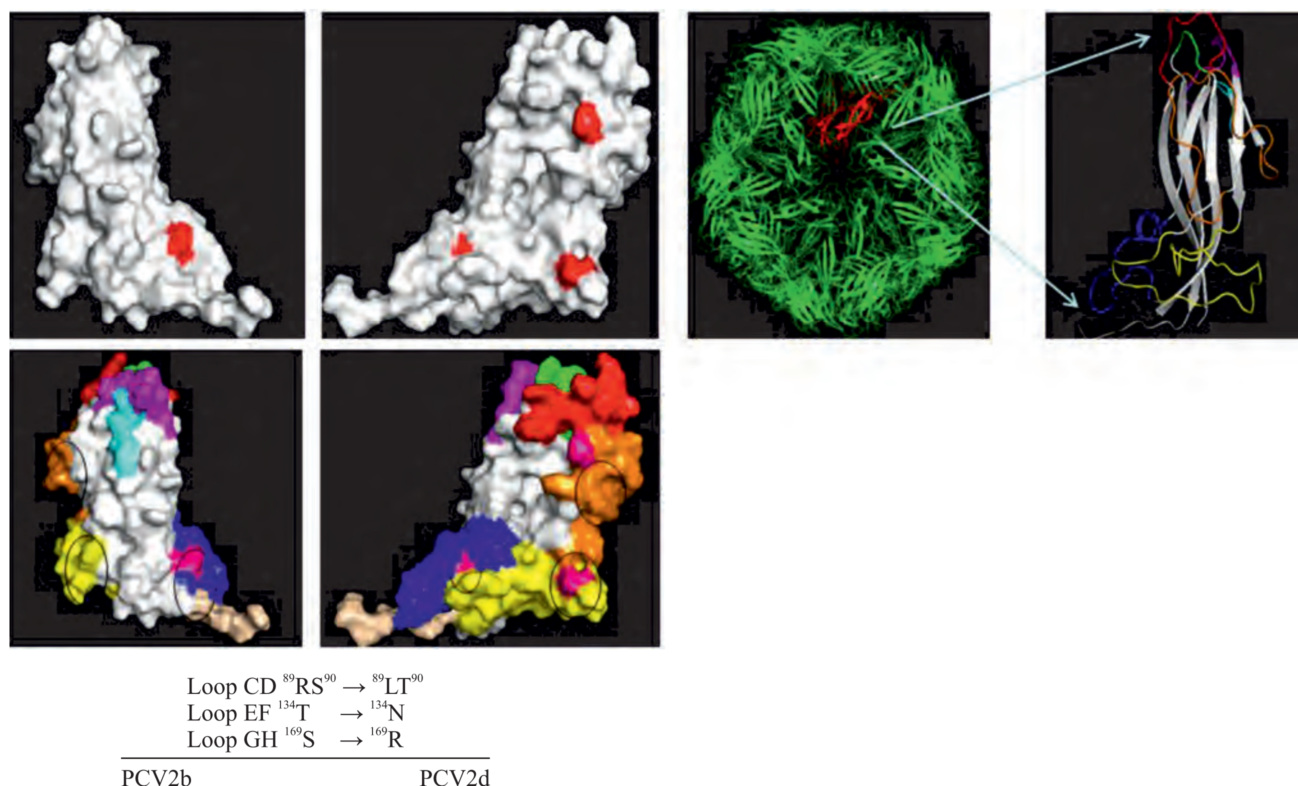

Fig. 7. The prediction structure of the CAP protein. Red: loop BC; blue: loop CD; purple: loop DE; orange: loop EF; cyan: loop FG; yellow: loop GH; green: loop HI; khaki: loop CT.

shedding (Kixmoller et al. 2008, Opriessnig et al. 2011, Hemann et al. 2012). Our results further indicate that vaccination with Cap of PCV 2b and 2d VLP resulted in effective defense against the same genotype virus challenge, but not the mixed challenge of PCV 2b and 2d.

Conclusions

Our data suggest that the PCV2b and 2d mixed VLP vaccine can completely protect mice from PCV2 viremia caused by PCV2b and 2d mixed infection, and significantly reduce the viral load of PCV2 in spleen tissues. Therefore, the PCV2b and 2d VLPs vaccine can offer cross-protection against both PCV2b and 2d subtypes, which indicates that the PCV2b and 2d VLPs vaccine is effective and safe in prevention of the current PCVADs caused by the two prevailing subgenotype PCV2 strains.

Abbreviations

PCV2: porcine circovirus type 2;
VLPs: Virus-like particles;
PMWS: post-weaning multisystemic wasting syndrome;
PNP: proliferating and necrotizing pneumonia;
PDNS: porcine dermatitis nephropathy syndrome;
PCVADs: diseases associated with porcine circovirus-associated diseases;
ORFs: open reading frames;
Rep: replicase;
Cap: capsid;
EU: European Union;
K: lysine;
FDA: Food and Drug Administration;
HPV: human papilloma virus;
PK-15: porcine kidney cell line;
DMEM: Dulbecco 's modified eagle medium;
NBCS: newborn calf serum;
TCID50: tissue culture infective dose 50;
ELISA: enzyme linked immunosorbent assay;
NC: negative control;
DPI: day XX after primary immunization;
qPCR: quantitative real-time PCR;
TEM: transmission electron microscopy;
PBS: phosphate buffered saline;
IMC: immunohistochemical;
TP: total proteins;
SP: soluble proteins;
IgG: immunoglobulin G;
HE: hematoxylin-eosin;
S: sham;
C: challenged;

PI: primary inoculation;
SI: secondary inoculation;
LE: lymphocyte enlargement;
LD: lymphoid depletion.

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