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Virology

Displaying epitope B and epitope 7 of porcine reproductive and respiratory syndrome virus on virus like particles of porcine circovirus type 2 provides partial protection to pigs

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ABSTRACT. The Cap of porcine circovirus type 2 (PCV2) can be assembled into virus like particles (VLPs) *in vitro* that have multiple loops located on the particle surface. This would make it a good vehicle for displaying exogenous proteins or epitopes. We derived two epitopes, epitope B (EpB, S^{37}HIQLIYNL^{45}) and epitope 7 (Ep7, Q^{196}WGRL^{200}) from Gp5 of the highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV). We replaced the core region of Loop CD (L^{75}PPGGGSN^{82}) and the carboxyl terminus (K^{222}DPPL^{226}) of PCV2 Cap, respectively, to construct a bi-epitope chimeric PCV2 Cap. Its immunogenicity and protective effects were evaluated as one PRRSV subunit vaccine. The chimeric PCV2 Cap was soluble, efficiently expressed in an *Escherichia coli* expression system, and could be self-assembled into chimeric virus like particles (cVLPs) with a diameter of 12–15 nm. Western blotting confirmed that the cVLPs could be specifically recognized by anti-PCV2, anti-EpB and anti-Ep7 antibodies. The cVLPs vaccine could alleviate the clinical symptoms and reduce the viral loads after HP-PRRSV challenge in 100–120 days old pigs. These data suggest that the cVLPs vaccine could provide pigs with partial protection against homologous PRRSV strains, and it provides a new design for additional PRRSV subunit vaccines.

KEY WORDS: chimeric porcine circovirus type 2 Cap, epitope, highly pathogenic porcine reproductive and respiratory syndrome virus, surface display, virus like particle

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Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-stranded enveloped RNA virus in the family *Arteriviridae* and order *Nidovirales* [30]. Since its discovery in 1987, there have been outbreaks in all pig producing countries, and it is now one of the most important pathogens seriously harming the pig industry.

Based on differences in nucleic acid sequence and regional distribution, PRRSV is divided into two genotypes, European-like (PRRSV-1) and North American-like (PRRSV-2) [1]. These two genotypes share approximately 60% nucleotide sequence identity and exhibit serotype differences [15]. As a positive-stranded RNA virus, PRRSV has extensive variation and strain diversity. Based on open reading frame (ORF5) and ORF7 nucleotide sequence alignment analysis, PRRSV-1 can be divided into three subtypes: Pan-European Subtype 1, Eastern European Subtype 2 and Eastern European Subtype 3 [40]; PRRSV-2 can be divided into at least nine different subtypes or lineages [38, 41]. In 2006, a pig disease characterized by high fever, high morbidity and high mortality appeared in China, which was later confirmed to be caused by the highly pathogenic PRRSV (HP-PRRSV) strain. This strain is mainly characterized by the deletion of 30 amino acid (aa) in the Nsp2 region [2, 42]. However, its pathogenicity is not related to this deletion, but it is related to the variation of Nsp9 and Nsp10 [19, 26, 59]. PRRSV in China has the characteristics of complex and diverse strains, and HP-PRRSV and NADC30-like are the dominant epidemic strains [41, 52, 58].

Vaccines are important for the prevention and control of PRRSV. The current PRRSV vaccine types mainly include inactivated vaccines, modified live virus (MLV) vaccines, DNA vaccines, subunit vaccines and virus-vectored vaccines. Several MLV vaccines have been commercialized for use against both PRRSV-1 and PRRSV-2 and licensed in various countries depending on circulating

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viral genotypes. These include the MLV vaccine strains of HuN4-F112 [57] and TJM-F92 [56] against HP-PRRSV. In China, they are effective against HP-PRRSV and can provide complete protection to pigs. However, it should not be ignored that the safety of MLV vaccines and the partial protection or lack of protection against heterologous strains hinder their wider application [10, 37]. Some commercial inactivated vaccines have been introduced such as Suvaxyn® PRRS (Zoetis, Parsippany, NJ, USA) against PRRSV-1 and PRRomi® (Bayer, Berlin, Germany) against PRRSV-2. Inactivated PRRSV vaccine is relatively safe, and there is little risk of recombination with wild strains [10, 49], but it cannot induce adequate humoral and cellular immunity, and provides only limited protection against homologous and heterologous strains [7, 10, 21, 62]. As such, it is not widely used in clinical practice. DNA vaccines [39], subunit vaccines [36], and virus-vectored vaccines [60] represent new directions in vaccine development. They have some advantages over MLV vaccines and inactivated vaccines, but they all have a common problem: limited protection efficacy.

In this study, two different regions on the surface of the PCV2 Cap, the core region of Loop CD and the carboxyl terminus, were replaced with epitope B (SHIQLIYNL) and epitope 7 (QWGRL) derived from the HP-PRRSV NVDC-JXA1 strain, respectively. We then evaluated its protection effects against HP-PRRSV.

MATERIALS AND METHODS

Ethics statement

All of the animal protocols used were approved by the Institutional Animal Care and Use Committee of Yangtze University (approval no. 2019-01-002). All the animals were anesthetized using xylazine hydrochloride, resulting in minimal suffering.

Cell, virus and plasmid

The HP-PRRSV strain NVDC-JXA1 (F4) was provided by the State Key Laboratory of Animal Genetic Engineering Vaccine, and propagated on the African green monkey kidney cell line Marc-145 cells (kindly provided by Dr. Du Y., Qingdao Yebio Biological Engineering Co., Ltd., Qingdao, China) maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and 5% CO_2. The NVDC-JXA1 was titrated as described previously [54]. The plasmid pET28a-PCV2 Cap (subtype 2b) was constructed in our previous study [51], and the PCV2 Cap could be self-assembled into virus-like particles in the *E. coli* expression system. Its purification was conducted as previously described [51].

Construction of chimeric PCV2 Cap

The overlapping-PCR method was used to construct the chimeric PCV2 Cap. Briefly, the N-Cap and C-Cap were cloned using the plasmid pET28a-PCV2 Cap as the template with the primers of PCV2 Cap F/ PCV2 plus EpB R and PCV2 plus EpB F/ PCV2 plus C-Ep7 R, respectively. And then the full length of chimeric PCV2 Cap was obtained with the primers of PCV2 Cap F/ PCV2 plus C-Ep7 R by adding equimolar amounts of N-Cap and C-Cap. After double digestion with *Nco* I and *Hind* III, they were ligated by T4 DNA ligase (Vazyme Biotech, Nanjing, China) and transformated into *E. coli* BL21 (DE3) competent cells (TransGen, Beijing, China) according to manufacturer instructions. The recombinant plasmid (named as pET28a-chimeric PCV2 Cap) was confirmed by DNA sequencing. The sequences of primers used are listed in Table 1.

Protein expression and purification

The positive transformant was grown in Luria-Bertani medium containing 50 µg/ml kanamycin at 37°C until the OD_{600} reached 0.8. Then α-lactose was added to a final concentration of 20 mM to incubate for an additional 12 hr at 32°C. The cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and re-suspended in PBS. Cells were lysed by sonication followed by centrifugation at 15,000 × *g* for 10 min at 4°C, and the supernatant was loaded into a GE Sepharose 6 Fast Flow column (2.6 cm in diameter and 90 cm in length) according to the AKTA Purfier100 manufacturer's manual. The protein was collected at the elution volume from 150 ml to 170 ml. Purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantified using a BCA Protein Assay Kit (CWBIO, Beijing, China).

Transmission electron microscopy

Purified samples with a concentration of 0.2 mg/ml were viewed under transmission electron microscopy (TEM, JEM-1200EX) as described previously [24].

Table 1. Primer sets for amplification of full length chimeric porcine circovirus type 2 (PCV2) Cap

The name of primers	Sequence (5′ to 3′)
PCV2 Cap F	TATACCATGGGCAGCAGCCATCATC
PCV2 plus EpB R	TAAGTTATAAATCAACTGAATATGAGAAAAATCATTAATATTAAATCT [a)]
PCV2 plus EpB F	TCTCATATTCAGTTGATTTATAACTTACCCCTCACTGTGCCCTTTGAA [b)]
PCV2 plus C-Ep7 R	CGCAAGCTTTTAGAGCCTACCCCATTGAAGATTAAATTCTCTGAATTG [c)]

[a)]The underscore represents the reverse complementary sequences that encode the EpB. [b)]The underscore represents the sequences that encode the EpB. [c)]The underscore represents the reverse complementary sequences that encode the Ep7.

Western blotting

Purified chimeric PCV2 Cap was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (PALL, Westborough, MA, USA) using a semi-dry transfer system (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skimmed milk at 37°C for 1 hr, and incubated at 37°C for 3 hr with a 1:200 dilution of porcine anti-PCV2 positive serum and a 1:200 dilution of mouse anti-EpB and mouse anti-Ep7 polyclonal antibody (obtained by immunizing mice with EpB or Ep7 coupled with keyhole limpet hemocyanin). After washing three times with PBST, HRP-conjugated goat anti-porcine IgG (KPL, Milford, MA, USA) or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) secondary antibody (1:20,000) was added and incubated at 37°C for 1 hr. Finally, signals were developed with Super ECL Detection Reagent (Yeasen, Shanghai, China). Purified PCV2 Cap was used for the control.

Vaccination and challenge study in pigs

The animal study was conducted in Qingdao Yebio Biological Engineering Co., Ltd. The 100–120 days old fattening pigs were divided into 3 groups, cVLPs group, TJM-F92 (a commercial MLV vaccine against PRRSV) group and challenge control group, and each group had five pigs. Before the study, all these pigs were antibody-negative for PRRSV with commercial PRRSV enzyme-linked immunosorbent assay (ELISA) HerdChek PRRS X3 Ab test (IDEXX, Westbrook, MA, USA). Pigs in the cVLPs group and TJM-F92 group were intramuscularly injected with 100 µg of purified chimeric PCV2 Cap (cVLPs) mixed with an equal amount of ISA201 adjuvant (SEPPIC, Paris, France) and 1 ml of the commercial MLV vaccine (TJM-F92), respectively. Pigs received boost immunizations (equivalent doses via the same route) 3 weeks after the primary vaccination. Pigs in the challenge control group were injected intramuscularly with 1 ml PBS as control group. At 35 days post-vaccination (dpv), all the pigs were challenged intranasally with 3 ml (2×10^5 $TCID_{50}$/ml) of NVDC-JXA1 strain. At 47 dpv (or called 12 days post-infection), the pigs were euthanized and necropsied.

Clinical observations

The pigs were monitored daily for their physical condition, and the clinical respiratory disease severity was scored on a scale from 0 (normal) to 6 (severe dyspnea and abdominal breathing) as previously described [16]. Rectal temperature was also recorded daily at the same time.

Indirect ELISA

Sera were tested by indirect ELISA at 35 dpv and 47 dpv. Briefly, the peptide epitope B (SHIQLIYNL) and epitope 7 (QWGRL) were synthesized by Shanghai Sangon Biological Engineering and Technology Co., Ltd., and coated on high binding capacity microplate (Corning Inc., Corning, NY, USA) at 10 µg/ml diluted with 50 mM carbonate buffer (pH 9.6) at 4°C overnight. Following washing with PBST buffer (PBS containing 0.5% Tween-20, pH 7.4), wells were blocked using 2% bovine serum albumin at 4°C overnight. One hundred microliters of sera samples were added to each well at a 1:100 dilution in PBS containing 1.2 mg/ml *E. coli* lysate and was subsequently incubated at 37°C for 1 hr. After washing five times at an interval of 1 min by PBST buffer, a 100 µl volume of HRP-labeled goat anti-porcine IgG (KPL, Milford, MA, USA) secondary antibody (Sigma, Burlington, MA, USA) was added at 1:20,000 dilutions, following which the plates were further incubated at 37°C for 1 hr. The wells were washed as before described and then incubated with a 100 µl volume of TMB substrate solution (Tiangen, Beijing, China) at room temperature for 10 min in the dark. The reaction was stopped by adding a 100 µl volume of stop solution (2 M H_2SO_4). The absorbance at 450 nm was recorded using an ELISA plate reader (Bio-Rad).

Determination of neutralizing antibody titer

The neutralizing antibody titers against PRRSV were conducted as previously described [18]. Briefly, the serum samples were complement-inactivated at 56°C for 30 min before testing, and then two-fold serially diluted sera (50 µl) were mixed with an equal volume of 100 $TCID_{50}$ of the NVDC-JXA1 strain in 96-well culture plates and incubated for 1 hr at 37°C and 5% CO_2. Following incubation, the mixtures were dispensed into Marc-145 cells in 96-well plates and incubated for 1 hr at 37°C. After washing with PBS, the cells were maintained in DMEM with 2% fetal bovine serum. The plates were examined daily for up to six days for the appearance of a PRRSV-specific cytopathic effect (CPE). The neutralization titers were expressed as the reciprocal of the highest serum dilution that neutralized 100 $TCID_{50}$ of PRRSV in 50% of the wells.

Quantification of viral loads

At 12 days post-infection (dpi), the pigs were euthanized and necropsied. Absolute quantitative RT-PCR (qRT-PCR) was performed to determine the viral loads of serum, lung, bronchial lymph node, spleen and kidney. The total RNA was extracted using an Ultra-Pure Total RNA Extracting Kit (Simgen, Zhejiang, China) according to manufacturer instructions. The forward primer (5′-GAAGCCCCATTTCCCTCT-3′) and reverse primer (5′-CTTATCCTCCCTGAATCTGACA-3′) were used to amplify a 144-bp fragment (ORF7) with a HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme, Nanjing, China) under the following conditions: 50°C for 15 min, 95°C for 2 min, 40 cycles of 94°C for 15 sec and 60°C for 30 sec. The sensitivity and specificity of the assay were determined with a dilution series of the positive plasmid (containing the ORF7, cloned into pTOPO vector). The Cq values determined from the plasmid dilution series were used to create a standard curve to determine the genomic copy number. The other serum, lung, bronchial lymph node, spleen and kidney from PRRSV-negative pigs (negative control) were preserved in our laboratory and used to analyze viral loads differences. Each sample was run in triplicate.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using the GraphPad Prism 5 software. Values of $P<0.05$ were considered statistically significant.

RESULTS

Soluble expression of chimeric PCV2 Cap

The overlapping-PCR method was used to replace the Loop CD core region (aa 75–82, LPPGGGSN) of the PCV2 Cap with the epitope B (SHIQLIYNL) of HP-PRRSV strain NADC-JXA1. The C-terminal (aa 222–226, KDPPL) of PCV2 Cap was also replaced with the epitope 7 (QWGRL). The construction strategy of two epitopes chimeric PCV2 Cap is shown in Fig. 1A. The chimeric PCV2 Cap was expressed by *E*. coli BL21 (DE3)/pET28a system, and the results of SDS-PAGE (Fig. 1B) showed that the protein was highly expressed and mainly existed in soluble form. During the purification process, we found that the elution volume of the chimeric PCV2 Cap (150–170 ml) was small in a size exclusion chromatography column (GE Sepharose 6 Fast Flow) with about 500 m*l* column volume (Fig. 1C), indicating that the apparent molecular weight of the chimeric PCV2 Cap was relatively large and high aggregates might be formed.

Assembled into nanoparticles

TEM observation results showed that the PCV2 Cap displayed with epitope B and epitope 7 formed nano-scale particles with a particle size of about 12–15 nm (Fig. 2A), indicating that it could self-assemble into virus-like particles during the expression process. However, its particle size was significantly smaller than that of PCV2 Cap with 17–19 nm (Fig. 2B). Although the particle morphology of the chimeric PCV2 Cap was dominated by spherical particles, elliptic or irregular spherical particles were also observed.

Chimeric epitopes could be recognized by specific antibodies

Western blotting was used to identify the chimeric PCV2 Cap. Both cVLPs and PCV2 VLPs can be recognized by swine anti-PCV2 positive sera (Fig. 3A), while only cVLPs can be recognized by mouse anti-EpB and anti-Ep7 polyclonal antibodies (Fig. 3B and 3C). This indicates that epitope B and epitope 7 were successfully displayed in the PCV2 cap and had immunological functions.

Induction of a specific antibody response and neutralizing antibody

Based on indirect ELISA, the specific anti-EpB and anti-Ep7 antibodies were measured at 35 dpv and 47 dpv (12 dpi). As shown in Fig. 4A and 4B, specific anti-EpB and anti-Ep7 antibodies could be detected in the cVLPs group and the TJM-F92 group, and the antibody level at 47 dpv was higher than that at 35 dpv in these two groups. The TJM-F92 group had a higher level of anti-EpB and anti-Ep7 antibodies than the cVLPs group at both 35 dpv and 47 dpv.

The titers of PRRSV-specific neutralizing antibodies present in the sera of inoculated pigs at 47 dpv were evaluated in Marc-145 cells. Neutralizing antibodies could be detected in the cVLPs group (geometric mean value: 3.014 ± 0.282) and the TJM-F92 group (4.687 ± 0.315), while no obvious neutralizing antibodies were detected in the challenge control group (1.372 ± 0.322) (Fig. 4C). In addition, the average neutralization titer of the TJM-F92 group was higher than that of the cVLPs group ($P<0.05$). Overall, the neutralizing antibody titer in the cVLPs group was relatively low.

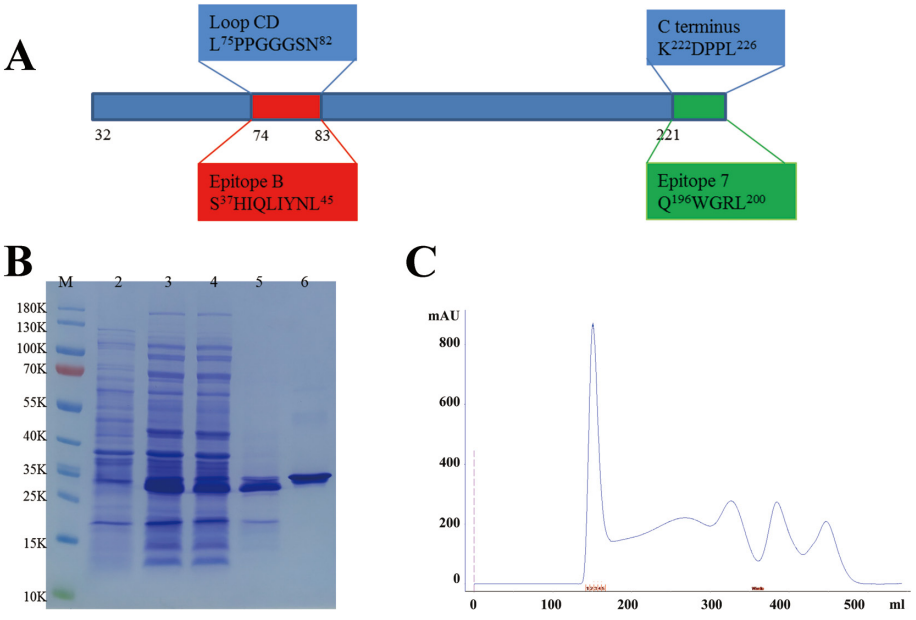

Fig. 1. The construction, expression and purification of chimeric porcine circovirus type 2 (PCV2) Cap. **(A)** The construction strategy of two epitopes chimeric PCV2 Cap. **(B)** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Lane 2: soluble extracts of BL21 (DE3)/pET28a (negative control); lane 3: total extracts of BL21 (DE3)/pET28a-chimeric PCV2 Cap; lane 4: soluble extracts of BL21 (DE3)/pET28a-chimeric PCV2 Cap; lane 5: purified chimeric PCV2 Cap; lane 6: purified PCV2 Cap virus like particles (VLPs). **(C)** The elution curve of soluble extracts of BL21 (DE3)/pET28a-chimeric PCV2 Cap in a GE Sepharose 6 Fast Flow column. The elution volume of the chimeric PCV2 Cap was in 150–170 ml.

Regardless of the challenge control group, there was a significant positive correlation (Fig. 4D, r=0.621, $P<0.05$) between anti-EpB antibody level and the neutralizing antibody titer, however, there was no obvious correlation between anti-Ep7 antibody level and the neutralizing antibody titer (Fig. 4E, r=0.446, $P=0.197$) and between anti-EpB and anti-Ep7 antibody level (Fig. 4F, r=0.126, $P=0.728$).

Relief of clinical symptoms after the challenge

Prior to the PRRSV challenge, no clinical signs were observed. After the challenge, including the control group, none of the pigs died. At 4 dpi, 1/5 of the pigs in the TJM-F92 vaccine group had an elevated rectal temperature and mild respiratory symptoms, and this pig lasted for two days (5 dpi and 6 dpi) with rectal temperature of more than 40.5°C; after 7 dpi, the rectal temperature of this pig returned to normal. Other pigs in TJM-F92 vaccine group had normal body temperature, smooth breathing, normal feeding and normal mental state. All the pigs in the challenge group showed signs of fever, dyspnea, and loss of appetite. From 5 dpi to 9 dpi, the mean of rectal temperature exceeded 41°C for five consecutive days, and from 5 dpi to 10 dpi, the mean of respiratory scores exceeded 3 for six consecutive days. Overall, the pigs in the challenge group had more severe symptoms. The cVLPs group showed similar, but less severe symptoms than the challenge control group after 2 dpi. The mean of rectal temperature exceeded 41°C for only one day (5 dpi), and the mean of respiratory scores exceeded 3 for only three days (from 6 dpi to 8 dpi). One-way ANOVA revealed that the rectal temperature at 6 dpi, 8 dpi, 9 dpi and 11 dpi and the respiratory scores from 5 dpi to 11 dpi in the cVLPs group were significantly lower ($P<0.05$) compared to the challenge control group (Fig. 5). Therefore, these clinical symptoms indicated that the cVLPs vaccine could alleviate the symptoms after PRRSV challenge and they could provide partial protection to the pigs.

Viral loads decreased after the challenge

The viral loads in the lungs, hilar lymph nodes, spleen, kidneys and serum were determined at 12 dpi, and the lung, hilar lymph nodes, spleen, kidney, and serum samples from PRRSV-negative pigs were also used as negative controls to determine the sensitivity. A high viral load was detected in the lung, hilar lymph nodes, spleen, kidney and serum in the challenge control group (Fig. 6). Among solid organs, hilar lymph nodes had the highest viral load, followed by lungs, spleen and kidneys. Among these organs or tissues, hilar lymph nodes had the highest viral loads, followed by lungs, spleen, kidneys and serum. The data dispersion was relatively small (SD was between 0.33 and 0.63) in the challenge control group.

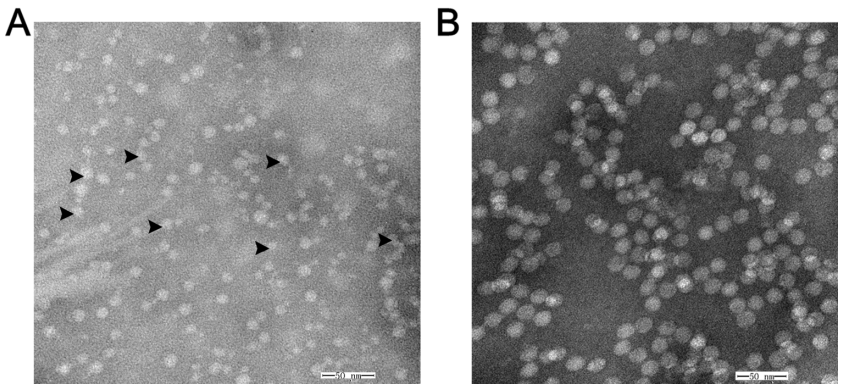

Fig. 2. Transmission electron microscopy (TEM) images of chimeric porcine circovirus type 2 (PCV2) Cap **(A)** and PCV2 Cap virus like particles (VLPs) **(B)**. The black arrows indicated the elliptic or irregular particles. Scale bars=50 nm.

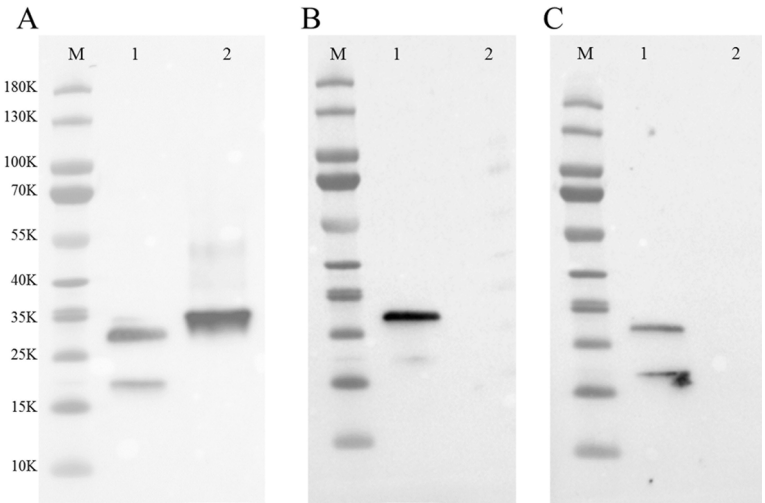

Fig. 3. Western blotting. The primary antibody was swine anti-porcine circovirus type 2 (PCV2) antibody **(A)**, mouse anti-keyhole limpet hemocyanin (KLH)-EpB antibody **(B)** and mouse anti-KLH-Ep7 antibody **(C)**, respectively. Lane 1: chimeric PCV2 Cap, lane 2: PCV2 Cap.

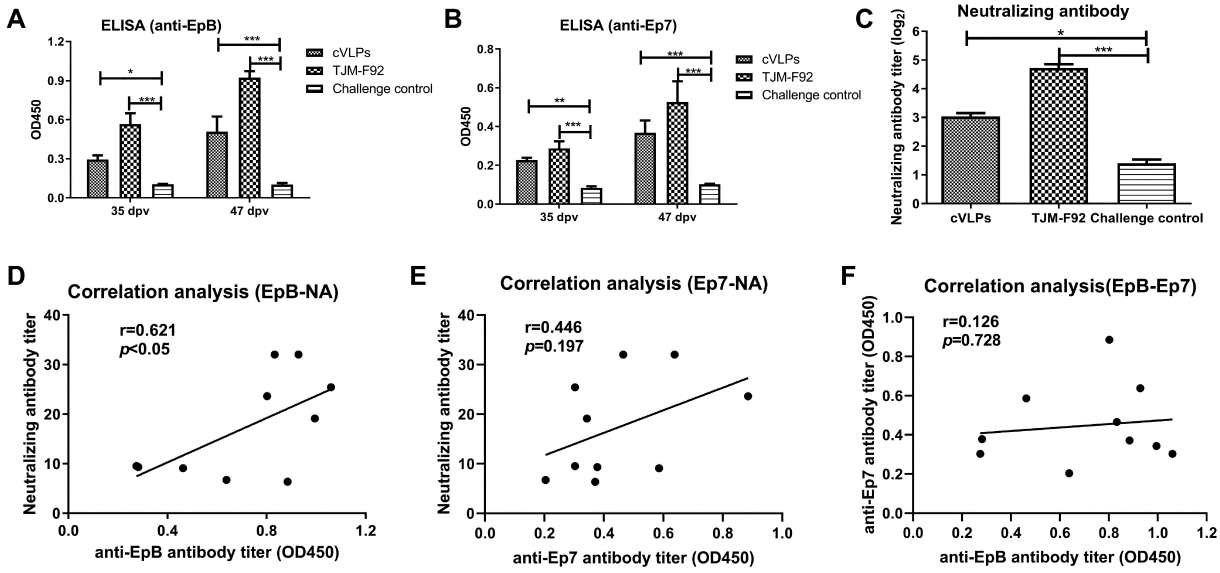

Fig. 4. The specific antibody response and neutralizing antibody. The specific anti-EpB antibody **(A)** and anti-Ep7 antibody **(B)** responses were detected by indirect enzyme linked immunosorbent assay (ELISA) at 35 dpv and 47 dpv. **(C)** The porcine reproductive and respiratory syndrome virus (PRRSV)-specific neutralization antibody at 47 dpv. The correlation analysis between anti-EpB antibody and neutralization antibody tier **(D)**, between anti-Ep7 antibody and the neutralizing antibody tier **(E)**, and between anti-EpB and anti-Ep7 antibody level **(F)** in the chimeric virus like particles (cVLPs) group and the TJM-F92 group at 47 dpv.

As expected, the viral loads in TJM-F92 vaccine group were significantly lower than those of pigs in the challenge control group ($P<0.01$). In the lung and spleen of the TJM-F92 group, the logarithmic mean of viral loads in 3/5 pigs was not different from that of the negative controls, while 2/5 pigs had a high viral load of 1 $\log_{10}$ and 1/5 pig had a high viral load of 2 $\log_{10}$ compared with the negative controls. Also, similar results were found in the serum, hilar lymph nodes and kidney. These data indicate that the TJM-F92 vaccine was effective against HP-PRRSV NVDC-JXA1 strain to pigs around 120 days old, but some pigs still had low viral copies at 12 dpi.

Compared with the challenge control group, the viral load in the cVLPs group decreased to different degrees. The viral loads dropped the most in the lungs (2.75 $\log_{10}$), followed by the serum (1.39 $\log_{10}$), the spleen (0.91 $\log_{10}$), hilar lymph nodes (0.32 $\log_{10}$), and kidneys (0.24 $\log_{10}$). In the lungs, 3/5 pigs had lower viral loads than a pig which had the highest viral loads in the TJM-F92 group, and 1/5 pig had no difference compared with the negative controls. Expected for the hilar lymph nodes, the data of viral loads in other tissues or organs were highly dispersed (SD between 0.64 and 1.69). These results indicated that the cVLPs vaccine could reduce viral loads and provide partial protection to the 100–120 days old pigs.

DISCUSSION

It is a feasible method to use virus like particles as transfer vehicles to display foreign proteins or antigen epitopes to produce chimeric VLPs for bivalent or multivalent vaccines. A variety of VLPs can be used as vehicles, such as Hepatitis B virus core protein VLPs [5, 23], PCV2 VLPs [13, 18, 47], foot-and-mouth disease virus VLPs [27, 28], and avian influenza virus VLPs [33]. By displaying foreign proteins or antigen epitopes on the surface of particles and assembling them into nanoscale particles, they will be more easily recognized and presented by the immune system, and more effective in activating humoral and cellular immunity.

PCV2 is one of the smallest animal viruses, and Cap is the only structural protein. Cap can be efficiently expressed in *E. coli* expression system [53], yeast expression system [8] and insect expression system [29]. It can be assembled into virus like particles with T=1, making it an ideal vehicle for the display of foreign sequences. Crystal structure analysis [20, 31] showed that PCV2 VLPs were composed of 60 Cap subunits. Each Cap monomer is composed of eight anti-parallel β-sheets, and a total of seven loops were formed between adjacent folded sheets, including Loop BC (aa 58–66), Loop CD (aa 75–93), Loop DE (aa 108–117), Loop EF (aa 124–147), Loop FG (aa 152–156), Loop GH (aa 162–194) and Loop HI (aa 204–208) [20, 48]. With the exception of Loop FG, the core regions of other loops were exposed on the surface of PCV2 VLPs, which played important roles in antigen recognition and presentation, neutralizing antibody production, capsid assembly and stabilization [20]. Based on amino acid sequence alignment of PCV1, PCV2 and PCV3, eight key continuous residues in Loop CD (called core region of Loop CD in this paper) were identified, and these residues were enriched in glycine which is the smallest amino acid and is often present in random loops [18]. This core region of Loop CD had high tolerances and high capacity for the insertion or replacement of foreign peptides, and it might not affect VLPs assembly and cell entry [47]. Besides the core region of Loop CD, the carboxyl terminus of the Cap played critical roles in the immune-recognition, pathogenesis and proliferation of PCV2 [55], and the carboxyl

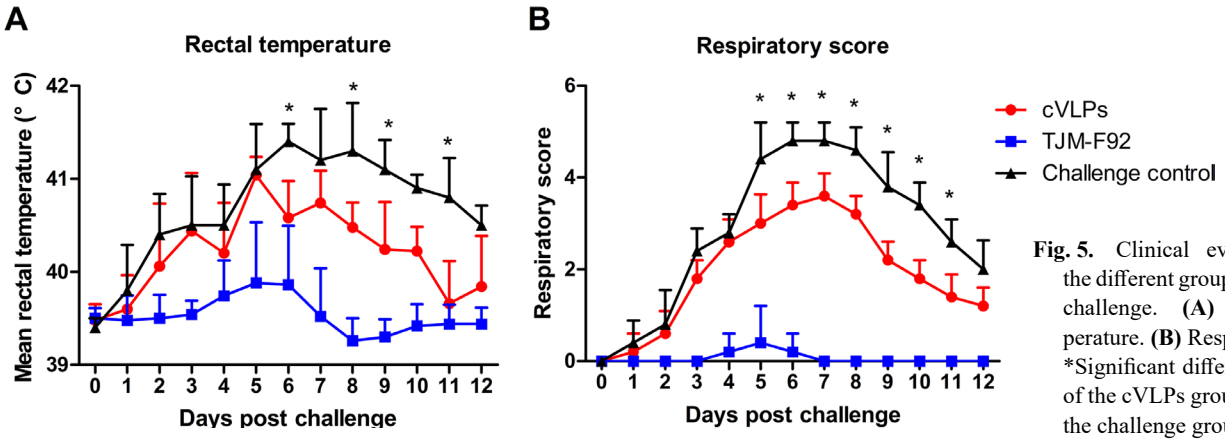

Fig. 5. Clinical evaluation from the different groups post-PRRSV challenge. **(A)** Rectal temperature. **(B)** Respiratory scores. *Significant difference (P<0.05) of the cVLPs group compared to the challenge group.

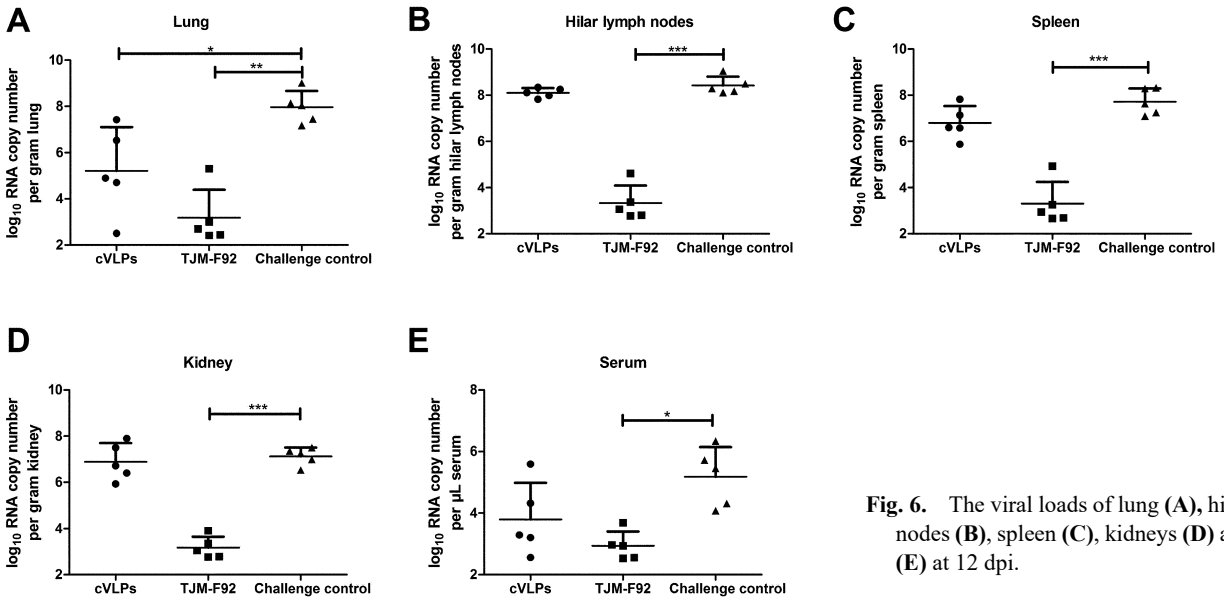

Fig. 6. The viral loads of lung **(A),** hilar lymph nodes **(B)**, spleen **(C)**, kidneys **(D)** and serum **(E)** at 12 dpi.

terminus which was exposed on the particle surface was composed of two critical linear and conformational epitopes [20, 25]. Foreign peptides fused at the carboxyl terminus could assemble into chimeric virus like particles [25]. Therefore, the core region of Loop CD and the carboxyl terminus of PCV2b Cap were chosen to be replaced with epitope B and epitope 7 of HP-PRRSV, respectively. The bi-epitopes chimeric PCV2 Cap could be soluble and be expressed efficiently in *E. coli* expression system, and could self-assembly virus like particles in the expression process rather than in some particular assembly buffer *in vitro*. Although there were some differences in particle size and morphology compared with the PCV2 VLPs, the characteristics of nano-size and water solubility indicated that hydrophobic residues were embedded in the inside of the particles and polymers were assembled through hydrophobic interaction and hydrogen bonding force. Therefore, the final polymers might have secondary and tertiary protein structures similar to PCV2 Cap. However, there were some differences in details, such as the formation process of the protein assembly, the number of monomers, the degree of polymer looseness, the distribution of surface charge and hydrophobic groups and the quaternary protein structure. This might be improved by means of computer structure simulation, selection of other insertion sites, optimization of insertion sites, flexible connection of exogenous epitopes and optimization of the linker.

PRRSV Gp5 is a 25 kDa glycosylated protein that is the main antigen for inducing neutralizing antibodies. The Gp5 protein is composed of four functional domains, including the signal peptide (31 aa) at the N-terminus, the ectodomain (35 aa), the triple transmembrane domain (60 aa) and the intracellular domain (70 aa) at the C-terminus [3]. Monoclonal antibody screening demonstrated that a non-neutralizing epitope A (27–30 aa) and a neutralizing epitope B (37–45 aa) exist in the ectodomain of the GP5 protein. Epitope A, as an immune-dominant epitope, can block the exposure of epitope B and prevent the production of neutralizing antibodies [14, 22, 34, 35]. Near the neutralizing antibody epitope B, the N-linked glycosylation N30, N34, N44 and N51 might shield the recognition of epitope B with the immune system and influence the production of neutralizing antibodies [4, 11, 46]. Therefore, removal of glycosylation, deletion of epitope A and efficient display of epitope B were important considerations for PRRSV subunit vaccine design. Except for epitope A and epitope B, another three minimal epitopes (epitope 3: R^{152}LYRWR^{156}, epitope 5: E^{169}GHLIDLKRV^{178} and epitope 7: Q^{196}WGRL^{200}) were identified in the North American type isolates, and they

might be involved in the antiviral process [50, 61]. In this study, epitope B and epitope 7 were chosen to display on PCV2 VLPs. Considering the position effect, and the length and properties of epitopes, these two epitopes replaced the core region of Loop CD and the carboxyl terminus, respectively. The animal experiments showed that it could induce the production of neutralizing antibodies and specific antibodies against epitope B and epitope 7. It also showed a significant positive correlation between the anti-EpB antibody level and the neutralizing antibody titer. However, it induced a lower neutralizing antibody titer than the commercial MLV vaccine. These data indicated that the display of EpB on the chimeric PCV2 VLPs was less effective than that of the virion of the TJM-F92 strain. Cellular immunity [32] and other neutralizing antibody epitopes [43–45] might also play important roles in the TJM-F92 vaccine.

It also has been found that some sera samples with high levels of anti-EpB antibody detected by ELISA showed low titers of neutralizing antibody. There may be the following four factors. First, in addition to the neutralizing EpB of Gp5, several other neutralizing epitopes had been identified [12, 45] that could induce the production of neutralizing antibodies. In our work, only one of the neutralizing epitopes (EpB from GP5) has been displayed. Second, it is possible that only EpB in the correct conformation can induce high-affinity neutralizing antibodies, and the spatial structure of EpB displayed on PCV2 Cap may be different from the natural structure. Third, after the challenge, the neutralizing antibody neutralized the virus, which would also cause a decrease of the neutralizing antibody to a certain extent. Finally, PAM cells, as the target cells of PRRSV infection, had a greater advantage than Marc145 in detecting neutralizing antibody.

Pigs of all ages, especially piglets, were highly susceptible to the HP-PRRSV and showed obvious clinical symptoms and high mortality. The 100–120 days old fattening pigs were chosen as experimental animals, and they displayed the typical clinical symptoms after virus challenge, including viremia, high viral loads, persistent fever, difficulty breathing, and decreased drinking and eating. But there was no death within 12 days after the challenge, which might be related to the age of the pigs and the challenge dose. The pigs in the cVLPs group had severe clinical symptoms after the challenge, but compared with the challenge control group, the rectal temperature and breathing were improved, and the viral load in tissues or serum was reduced. The viral loads in the lungs decreased most significantly, and there was also a small decrease in other tissues, but there was no statistical difference. HP-PRRSV has a wide range of tissue tropism, and the lung is also one of the target organs for infection. However, the tonsils or lymph nodes had higher viral loads for a period of time after the infection [17]. This difference may make the virus in the lungs relatively quick to be cleared by the immune system. In addition, the lung, as one of the important barriers against foreign pathogens, is rich in macrophages. The cVLPs vaccine may activate cellular immunity and induce macrophages to produce some cytokines, and accelerate the clearance of viruses in the lung.

However, there was one major limitation in this study that could be addressed in future research. In this study, there are only 5 pigs in each group, so that some conclusions cannot be accurately and reliably obtained when the deviation of the measured data is relatively large.

In a conclusion, the cVLPs vaccine provided a degree of protection to pigs and had some positive effects in alleviating clinical symptoms. Although the protective effect was limited, this might be improved through additional chimeric epitopes, optimization of surface display, use of IL15/18 immune enhancers [9] and more efficient adjuvants [6]. Boosting immunization may also be another important option for improving vaccine effectiveness. In this work, the animal had received two inoculations with the cVLPs vaccine. Considering the low immunogenicity of the subunit vaccines, three even four inoculations are acceptable. Although multiple inoculations may be difficult in clinical practice, it is also meaningful from the perspective of the efficacy and safety of the vaccine.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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