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Protective humoral immunity in guinea pigs induced by PCV2 virus-like particles displaying the B cell linear epitope (^{228}QQITDA^{233}) of PPV1

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ABSTRACT

Although PCV2 infections generally cause mild disease in pigs, concurrent co-infections with other pathogens can damage the immune system and cause more severe diseases, collectively termed porcine circovirus associated diseases (PCVAD). Involvement of porcine parvovirus (PPV, a common cause of reproductive failure in naïve dams) in PCVAD caused by PCV2, has been reported. As this co-infection can be difficult to eliminate, there is a critical need to develop an effective vaccine to protect against PPV or synergistic effects of PCV2 and PPV under field conditions. In this study, we designed chimeric PCV2 virus-like particles (cVLPs) displaying a B-cell epitope derived from PPV1 structural protein around the surface of the 2-fold axes of PCV2 VLPs, based on 3D-structure analysis of the PCV2 capsid. The cVLPs were successfully prepared, verified by transmission electron microscopy and chromatography, with robust antibody titers against PCV2 and PPV1 produced in mice and guinea pigs. In addition, in guinea pigs challenged with 10^6 $TCID_{50}$ PCV2, cVLPs conferred more effective immune protection (based on viral load) than a commercial PCV2 vaccine. Finally, antibody responses and immune protection against PPV were also evaluated. In guinea pigs vaccinated with cVLPs, although PPV antibodies detected by a hemagglutination inhibition (HI) assay appeared later after vaccination in the PCV2 cVLPs group than in the commercial PPV vaccine group, there were fewer PPV genomic DNA copies in the PCV2 cVLPs group than in a PBS group. In conclusion, guinea pigs vaccinated with cVLPs developed effective protective immunity against PCV2 challenge, with some protective immunity against PPV. This study provided valuable research data to pursue molecular design of chimeric epitopes PCV2 VLPs.

1. Introduction

Porcine circovirus type 2 (PCV2) is the main etiologic agent for a multi-factorial clinical disease (porcine circovirus associated disease, PCVAD), causing major losses in swine production (Segalés et al., 2013). In addition, co-infection of PCV2 and other pathogens (viral or bacterial) increase clinical severity of PCVAD, perhaps through synergistic effects, which may modulate PCV2 replication or clearance by alteration of cytokine production and profiles (Darwich and Mateu, 2012; Ellis, 2014; Opriessnig and Halbur, 2012).

Porcine parvovirus (PPV) causes SMEDI (S, stillbirth; M, mummification; ED, embryonic death; I, infertility) when embryos or fetuses in seronegative dams are infected; in addition, it is also considered a co-factor for PCVAD (Ellis et al., 1999; Kim et al., 2003). Genomes of PCV2 and classical PPV or PPV2, PPV3 and PPV4 were concurrently present in pigs (Cadar et al., 2013a,b; Saekhow et al., 2016). Moreover, prevalence of classical PPV and PPV2 DNAs in PCVAD cases were higher than that of non-PCVAD cases (Saekhow et al., 2016). Since circovirus and parvovirus are both single-stranded DNA viruses, requiring actively proliferating target cells for productive viral replication, virus-induced lymphoproliferation or immunosuppression could enhance susceptibility to other viral

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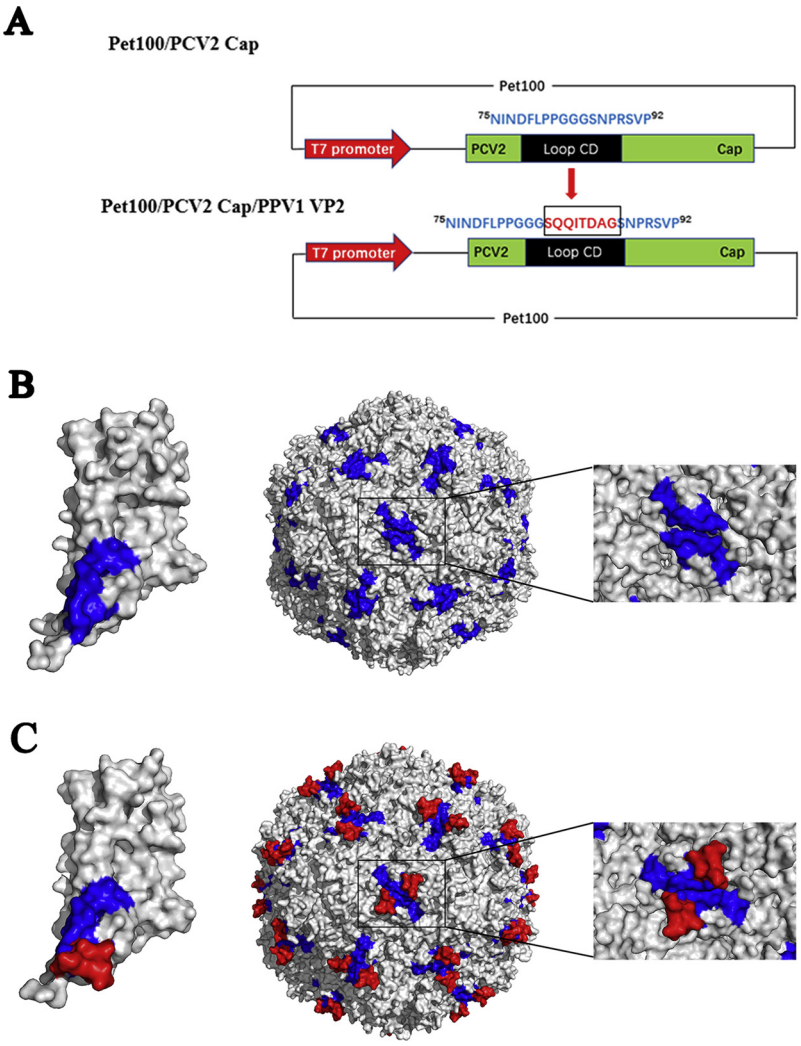

Fig. 1. PPV1 epitope infusion design and 3D structures of Loops CD of PCV2 Cap. (A) Sketch Map of PCV2 infusion Cap protein containing PPV1 B cell liner epitope B5-E1 (^{228}QQITDA^{233}). Residues in red represent foreign peptides inserted into Loop CD of PCV2 Cap. PCV2 Cap surface and PCV2 capsid. Loop CD is blue (B) whereas epitope B5-E1 is red (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

replications and infections (Darwich and Mateu, 2012; Ellis et al., 2004; Saekhow et al., 2016, 2014).

Recently identified parvoviruses (PPV3 and PPV4) have apparently already spread worldwide (Saekhow et al., 2016). Co-existence of multiple PPV strains on swine farms and co-infection of pigs with various PPV strains complicate vaccine design strategies. The minimal B cell linear epitope ^{228}QQITDA^{233} (designated B5-E1) was defined as a core epitope using a PEPSCAN analysis, based on PPV1 VP2 protein specific monoclonal antibodies (8H6) (Sun et al., 2015). The epitope of VP2 protein is highly conserved (except a single amino acid substitution at residue 233) among PPV1 strains and is very flexible, with random coiling exposed on the PPV1 virion surface (Kamstrup et al., 1998; Sun et al., 2015). Due to presence of a core epitope B5-E1 on the virion surface, an ELISA based on the VP2 epitope (covered B5-E1) has been developed and may be effective for evaluating anti-PPV1 antibodies in porcine sera (Sun et al., 2015). Therefore, it was concluded that epitope B5-E1 was PPV specific and elicited an effective immune reaction.

We determined that the location between 85G and 86S of PCV2 Cap Loop CD was an ideal insertion site for a foreign epitope (Hu et al., 2016). Insertion of the PRRSV GP5 epitope B into this site of the Loop CD did not affect assembly of PCV2 VLPs, but could form PCV2 chimeric virus-like particles (cVLPs) that elicited immunoreactions specific to PCV2 Cap and GP5 epitope B (Hu et al., 2016). Based on this location on PCV2 VLPs surface, PCV2 cVLPs containing epitopes of various classical and newly identified PPV may be effective vaccine strategies for preventing PPV or synergistic effects of PCV2 and PPV. In this study, a PCV2 cVLPs containing the ^{228}QQITDA^{233} (Sun et al., 2015) was developed and its immunogenicity and protective effects evaluated in guinea pigs and compared to inactivated vaccines.

2. Materials and methods

2.1. Cell lines and virus

PK-15 cells (ATCC CCL-33) were cultured in Dulbecco's modified eagle medium (DMEM, Thermo Fisher Scientific, Beijing, China) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in 5% CO_2. The PCV2 isolate (GenBank accession number KJ867555) was isolated from a diseased pig with clinical signs of post-weaning multisystemic wasting syndrome (PMWS) on a farm in Hunan province, China. The PPV TJ strain (GenBank accession number KX233726, isolated from the liver of an aborted fetus in Tianjin, China) was kindly provided by Dr. Shangjin Cui. Murine polyclonal antibodies against PCV2 were stored in our laboratory (Dong et al., 2016). Epitope B5-E1 was synthesized commercially (GenScript, Piscataway, NJ, USA).

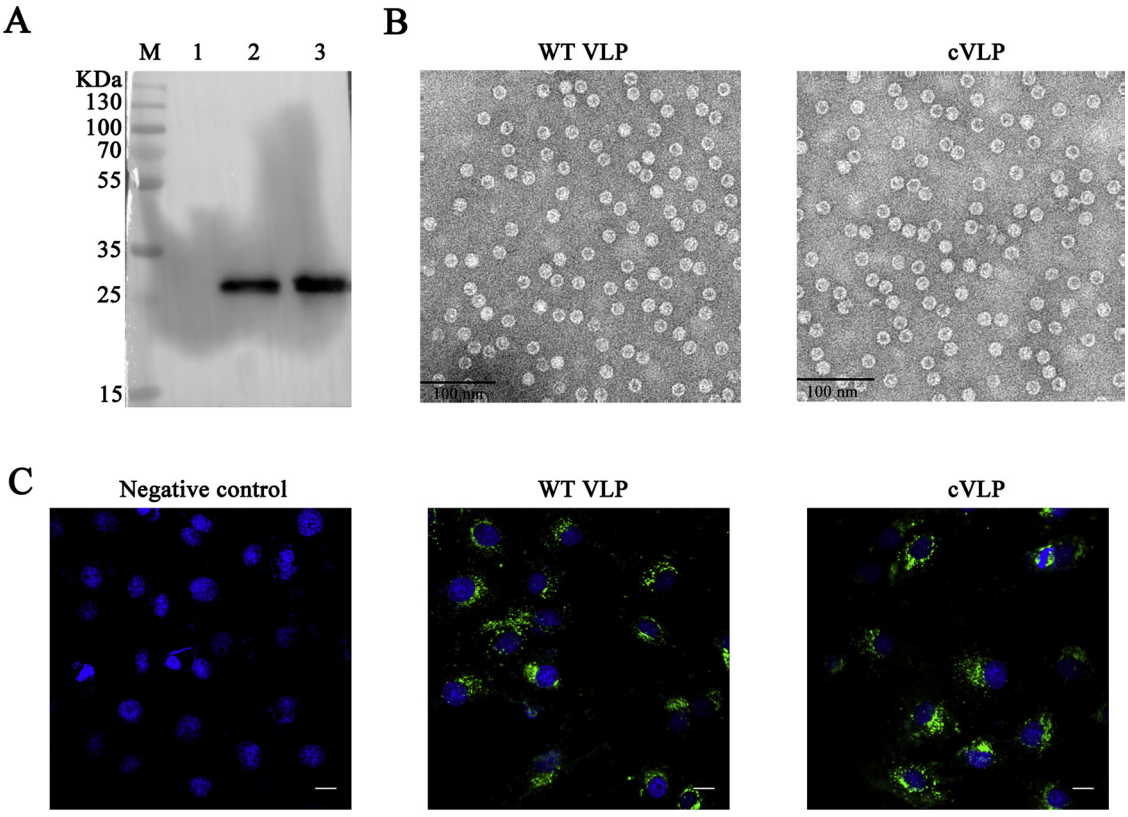

Fig. 2. Identification of PCV2 VLPs assembled *in vitro* (A) Western blots of supernatants from bacterial lysates containing expressed Cap-WT and Cap-PPV epitope. Lane M, protein marker; Lane 1, normal bacterial lysates; Lane 2 Cap-WT; and Lane 3 Cap-PPV epitope. (B) PCV2 VLPs and cVLPs formations assessed with TEM. (C) Entry of PCV2 VLPs and cVLPs into PK-15 cells confirmed by confocal microscopy. PBS as negative control. Green fluorescence represents PCV2 Cap in PK15 cells. Nuclei (blue) of PK-15 cells were stained by DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Construction of recombinant PCV2 cap gene

First, homology modeling of recombinant Cap 3D structure was performed using a known PCV2 Cap crystal structure (PDB accession number: 3R0R) template structure, as described (Hu et al., 2016; Wang et al., 2018) to predict potential effects of PPV1 B cell liner epitope B5-E1 (^{228}QQITDA^{233}) inserted between ^{85}G and ^{86}S of Loop CD on the Cap and Capsid structure, using Modeller (http://salilab.org/modeller/). Then, structure of the icosahedral capsid was generated based on a monomeric PCV2 Cap structure by applying a VMD 1.9 matrix transformation using Tcl script (Humphrey et al., 1996). Three-dimensional structures of the recombinant Cap and capsid and distribution of the epitope were viewed using Pymol molecular graphics software (Version 1.7.4.4, http://www.pymol.org/).

Based on structure predictions, we inserted the following DNA segment encoding epitope B5-E1 between 85 G and 86S of the optimized PCV2 *cap* gene (GenBank accession number: JF504708) by overlap polymerase chain reaction (PCR) with various primers (Table S1), as described (Hu et al., 2016; Wang et al., 2018). Following PCR amplification, resultant DNA fragments were cloned into pET100 vectors (Invitrogen, Carlsbad, CA, USA) and recombinant plasmids (Fig. 1A), confirmed using a DNA sequencer, were transformed into competent *E. coli* BL21 (DE3) cells (TransGen, Beijing, China) to express Cap and recombinant Cap (rCap) proteins, respectively, as reported, (Hu et al., 2016; Wang et al., 2018).

2.3. Self-assembly of PCV2 VLPs

PCV2 VLPs and cVLPs (containing epitope B5-E1) assembled *in vitro* by Cap and recombinant Cap were produced and purified using immunoaffinity columns, as described (Hu et al., 2016). VLPs were characterized by western blots and transmission electron microscopy (TEM), whereas cell entry of VLPs were determined with IFAs, as described (Wang et al., 2018).

For western blots, whole-cell lysates containing protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA, USA), then transferred electrophoretically onto a polyvinylidene difluoride membrane (PVDF; Life Technologies, Carlsbad, CA, USA) in a Bio-Rad transfer apparatus by applying 80 V for 1 h. The membrane was blocked for 1 h with 3% bovine serum albumin (BSA; Roche, Basel, Switzerland) in phosphate-buffered saline (1 × PBS, pH 7.4), and subjected to primary antibody (rabbit anti-PCV2 Cap, 1:500) and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000, Promega, Madison, WI, USA), with bound antibodies visualized using High-sig ECL western blotting substrate (Tanon, Shanghai, China).

For TEM, samples containing VLPs were adsorbed on to carbon-coated glow-discharged copper grids for 10 min and negatively stained for 10 min with 1% phosphotungstic acid. Thereafter, VLPs were imaged using a CM100 instrument (Philips Electron Optics, Zurich, Switzerland).

For the indirect immune fluorescence assay (IFA), PK15 cells were incubated with PCV2 VLPs (2 μg) in each well (24-well plate) for 1 h. To remove unbound VLPs, PK15 cells were then washed 3 times with PBS. At 12 h post-inoculation of PCV2 VLPs, PK15 cells were fixed in 4% (wt/vol) paraformaldehyde in PBS, and permeabilized with 0.1% Triton X-100 for 5 min. After washing cells 3 times with PBS, expression of PCV3 Cap was detected using primary antibodies of rabbit anti-PCV2 serum (1:1000) and fluorescein isothiocyanate (FITC) conjugated donkey anti-rabbit IgG (1:2,000, Life Technologies). Finally, specific

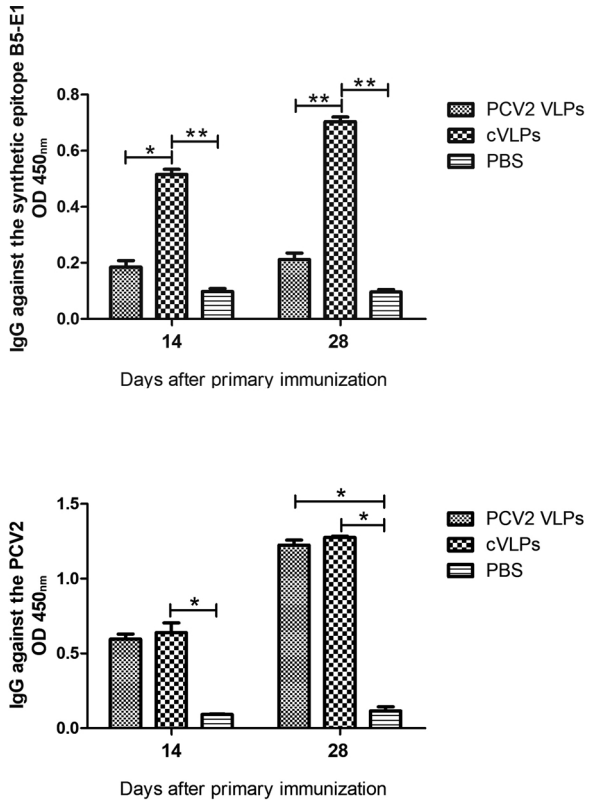

Fig. 3. Polyclonal antibody titres against the synthetic epitope B5-E1or PCV2 using PCV2 VLPs and cVLPs-indirect ELISA. Guinea pig polyclonal antibodies against synthetic epitope B5-E1 at 14 and 28 d post immunization were detected by indirect ELISA (PCV2 VLPs and cVLPs as antigen); mouse polyclonal antibodies against PCV2 at 14 and 28 d post immunization were detected by indirect ELISA (PCV2 VLPs and cVLPs as antigen); Optical densities were read at 450 nm. Bars represent arithmetic means ± SD of antibody titers (*P < 0.05).

fluorescence in infected cells were observed using confocal microscopy (LSM 710 NLO & DuoScan System, Carl Zeiss, Germany).

2.4. Vaccination of mice and guinea pigs with VLPs

Fifteen female Kunming mice (3–4 wk old), were purchased from Hunan Slaccas Jingda Laboratory Animal Company (Hunan, China) with license number SCXK (Xiang) 2013-0004. All procedures and protocols in animal studies were performed in accordance with guidelines of the Animal Care and Use Committee of Hunan Agricultural University and animal use was approved by the Institutional Ethics Committee (IEC) of Hunan Agricultural University. Mice were randomly allocated into 3 groups (n = 5/group). Mice were immunized with subcutaneous injections of: 1) PCV2 VLPs; 2) cVLPs [containing 50 μg protein, diluted in 50 μl PBS and emulsified with 50 μl Complete Freund's adjuvant (Sigma-Aldrich, St Louis, MO, USA)]; or 3) PBS (negative control). Mice were boostered 2 wk later with the same dose suspended in 50 μl Incomplete Freund's adjuvant (Sigma-Aldrich, St Louis, MO, USA). Blood samples were collected 0, 14, 28 and 42 d post-primary immunization (dpi). Mice immunized with VLPs and Freund's adjuvant were treated with iodine on a daily basis, with no lesions observed at the site of injection.

Thirty-five female guinea pigs (400–500 g) (Hunan Slaccas Jingda Laboratory Animal Company), were randomly allocated into 4 groups and individually immunized with: 1) 0.5 ml (50 ug) cVLPs mixed with Montanide ISA 15A (Seppic, Paris, France; 2) 0.5 ml (150 ug) peptides (PPV1 VP2 B cell liner epitope B5-E1) mixed with Montanide ISA 15A; 3) 0.5 ml standard inactivated PPV (Haili, China) and 0.5 ml PCV2

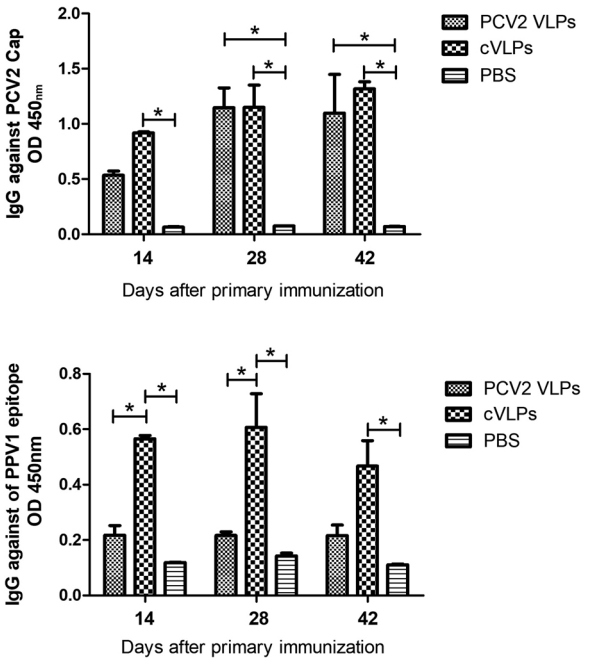

Fig. 4. Humoral immune responses to VLPs in mice (A) PCV2 Cap-specific IgG antibody in mice at 14, 28 and 42 d post immunization were detected by indirect ELISA (PCV2 VLPs as antigen). (B) PPV1 epitope-specific IgG antibodies in mice at 14, 28 and 42 d post immunization were detected by indirect ELISA (PPV epitope peptide as antigen). Optical densities were read at 450 nm. Bars represent arithmetic means ± SD of antibody titers (*P < 0.05).

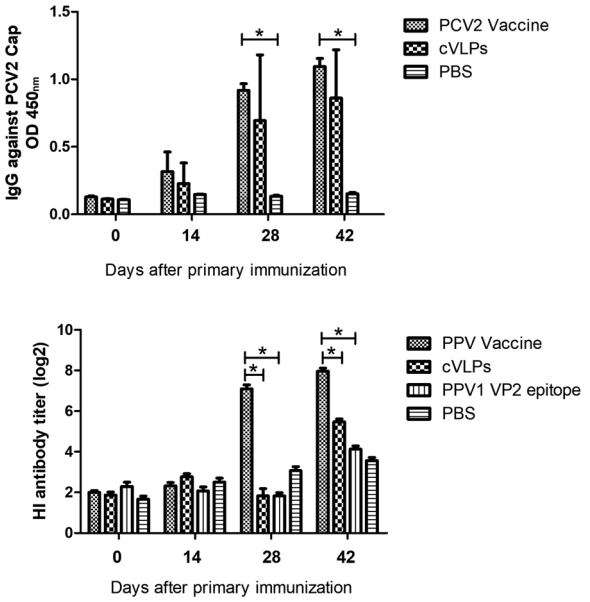

Fig. 5. Humoral immune responses to VLPs in guinea pigs (A) PCV2 Cap-specific IgG antibody in mice at 0, 14, 28 and 42 d post immunization were detected with a commercial PCV2 Cap ELISA kit. (B) PPV antibody in guinea pigs at 14, 28 and 42 d post immunization was detected with an HI assay. Optical densities were read at 450 nm. Bars represent arithmetic means ± SD of antibody titers (*P < 0.05).

vaccine (Qilu Animal Health Products Co., LTD., China) as positive control; or 4) 0.5 ml PBS (negative control). All guinea pigs were boostered with the same dose, 28 d after primary vaccination. Blood samples were collected at 0, 14, 28 and 42 dpi.

At 42 dpi, cVLPs and PBS groups received an inoculum of 100 μl 10^6 $TCID_{50}$/ml PPV and PCV2, respectively. The PCV2 vaccine group was

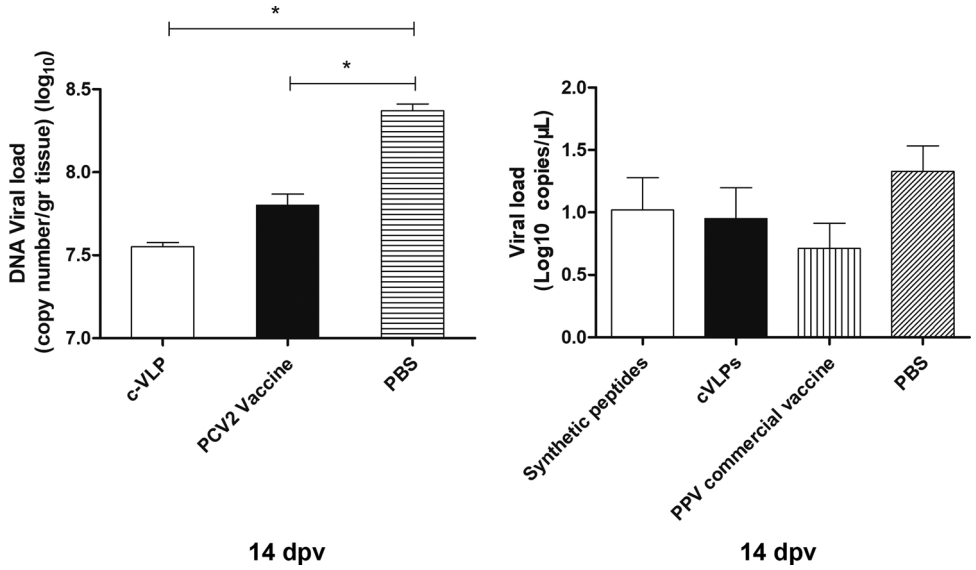

Fig. 6. Virus copies in guinea pigs at 14 dpi. (A) PCV2 virus copies in cVLPs, PBS and vaccine groups. (B) PPV virus copies in cVLPs, PBS, synthetic epitope peptides and vaccine groups. Bars represent arithmetic means ± SD of antibody titers (*P < 0.05).

infected with 100 μl 10^6 $TCID_{50}$/ml PCV2, whereas PPV vaccine and peptide groups were infected with 100 μl 10^6 $TCID_{50}$/ml PPV.

2.5. ELISA

Sera of mice in PCV2 cVLPs and VLPs groups were assessed using our established PCV2 VLPs indirect enzyme-linked immunosorbent assay (ELISA), as described (Zhang et al., 2016). Secondary antibody was HRP-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For detection of antibodies specific to PCV2 in guinea pigs vaccinated with PCV2 cVLPs or PCV2 vaccine, commercial PCV2 antibody test ELISA (Biochek, Reeuwijk, The Netherlands) was used. Secondary antibody was goat anti-guinea pig IgG conjugated to alkaline phosphatase (Southern Biotech, Birmingham, AL, USA) with 1:2000 dilution.

2.6. Hemagglutination inhibition (HI) assay

The HI antibody titers of serum from mice in PPV vaccine, PCV2 cVLPs and peptide groups were measured using standard methods (Joo et al., 1976). Briefly, 2-fold serial dilution (range, 1:2 to 1:2048) of 25 μL serum after inactivation at 56 °C for 30 min in a 96-well, V-shaped bottom microtiter plate (containing 50 μL CMF-PBS). Then 25 μL PPV antigen (4 HA units) was added into all wells, except for the last row (controls). The serum-antigen mixture was incubated at room temperature for 20 min and then 25 ml of 1% rooster erythrocytes suspension was added into each well, followed by incubation at room temperature for 30 min. End points were defined as the highest serum dilution causing complete inhibition. The geometric mean HI titer of serum antibody was reported as reciprocal log2 values of the highest dilution.

2.7. Virus copies

DNA was extracted from mixed tissue samples (0.21 g liver, 0.21 g spleen, 0.45 g lung, 0.25 g kidney and 0.15 g mesenteric lymph node) of guinea pigs (cVLPs, PCV2 vaccine and PBS groups) in 2 ml PBS, using a commercial kit (TIANGEN Biotech Co, Ltd., Beijing) at 14 d post-inoculation. A quantitative real-time PCV2 assay was used to determine PCV2 genomic copy numbers in DNA samples, as reported (Olvera et al., 2004).

DNA was extracted from 0.29 g spleen samples of guinea pigs (cVLPs, PPV vaccine, epitope and PBS groups) in 2 ml PBS, using a commercial kit (TIANGEN Biotech) at 14 d post-inoculation. Copy numbers of PPV genome were measured by quantitative real-time PCR assay with specific primers for the NS1 gene (Cao et al., 2017).

2.8. Statistical analyses

All data were subjected to one-way ANOVA to detect differences among groups (VLPs, cVLPs, epitope, vaccine or PBS) using SAS Version 9.2 and GraphPad Prism Version 5 (GraphPad Software, La Jolla, CA, USA). When there was a difference ($P < 0.05$), Dunnett's test was used to locate differences.

3. Results

3.1. G and ^{86}S of Loop CD of PCV2 VLPs tolerated insertions of PPV epitope B5-E1

In a previous study, insertion of the PRRSV GP5 epitope B between ^{85}G and ^{86}S of Loop CD of the PCV2 Cap did not alter folding of the recombinant Cap (rCap) and assembly of PCV2 VLPs (Hu et al., 2016). In this study, the ideal insertion site for a foreign epitope was used to efficiently present PPV1 B cell liner epitope B5-E1 (^{228}QQITDA^{233}) on the exterior surface of PCV2 VLPs. Structural modeling of the PCV2 rCap and VLPs was done to predict the location of the epitope B5-E1 in its Loop CD. The inserted epitope B5-E1 protruded from the rCap surface and formed a relatively independent unit from the rCap backbone (Fig. 1C); 60 copies of the insertion were distributed on the surface of PCV2 cVLPs, without altering the original PCV2 VLPs structure (Fig. 1B).

Western blot analysis detected 2 obvious bands corresponding to the Cap and rCap (~26 and ~27 kDa, respectively) recognized by rabbit anti-PCV2 Cap (Fig. 2A). Using TEM to confirm that the rCap had self-assembled cVLPs, small, spherical, virus-like particles (diameter ~20 nm) were observed (Fig. 2B). Furthermore, cVLPs entered PK15 cells as PCV2 VLPs (Fig. 2C).

3.2. The inserted PPV epitope B5-E1 was functionally displayed on the surface of PCV2 cVLPs

The PCV2 VLPs and cVLPs were recovered using gel filtration of dialysis product, as described (Zhang et al., 2016). Further analysis

with gel filtration-purified PCV2 VLPs and cVLPs-indirect ELISA confirmed that cVLPs were significantly bound to guinea pig polyclonal antibodies against the synthetic epitope B5-E1, compared to PCV2 VLPs and had the same reactivity with mouse polyclonal antibodies against PCV2 (Fig. 3). Therefore, epitope B5-E1 was inserted into cVLPs and functionally displayed on the surface of PCV2 cVLPs.

3.3. Induction of antibody against inserted epitope B5-E1 by immunization of mice with cVLPs

To evaluate humoral immune responses induced by cVLPs, PCV2 VLPs and epitope B5-E1 peptide-indirect ELISA were used to detect PCV2 and B5-E1-specific IgG antibodies, respectively, in mice. As expected, IgG antibodies were detected in cVLP and PCV2 VLPs groups at 14 dpv (Fig. 4). The IgG titers of PCV2 VLPs reached high levels at 28 dpv in PCV2 VLPs and cVLPs groups, whereas titers in the PCV2 cVLPs group further increased at 42 dpv (Fig. 4). In addition, VLPs and cVLPs groups had higher IgG titers compared to the PBS group ($P < 0.01$, Fig. 4), with no difference ($P > 0.01$, Fig. 4) in IgG titers between PCV2 VLPs and cVLPs. Anti-epitope B5-E1 antibodies were detected at 14 dpv in cVLPs group; cVLPs group had significantly higher IgG titers of epitope B5-E1 peptide than that in PCV2 VLPs and PBS groups ($P < 0.01$, Fig. 4) at 14, 28 and 42 dpv. No anti-epitope B5-E1 antibodies were detected in the PBS group. In mice, cVLPs induced antibodies that reacted with synthetic anti-epitope B5-E1 peptides and PCV2 VLPs.

3.4. cVLPs induced protective immunity in guinea pigs

To further evaluate anti-PCV2 humoral immune responses induced by cVLPs, PCV2 VLPs-indirect ELISA were used to detect cVLPs and PCV2 vaccine-specific IgG antibodies, respectively, in guinea pigs. In PCV2 ELISA analysis, antibody titers in cVLPs and PCV2 vaccine groups in guinea pigs steadily increased over 14, 28 and 42 dpv. Furthermore, titers in cVLPs group were overall slightly lower than that in PCV2 vaccine group at 14, 28 and 42 dpv. In addition, cVLPs and PCV2 vaccine groups had higher IgG titers compared to the PBS group ($P < 0.01$, Fig. 5) at 28 and 42 dpv. There was no difference (Fig. 5) in IgG titers between cVLPs and PCV2 vaccine groups, which suggested that guinea pig IgG antibodies induced by cVLPs had the same binding to PCV2 Cap with those induced by PCV2 vaccine.

To assess anti-PPV immune responses in guinea pigs induced by cVLPs, PPV antibodies in guinea pigs induced by cVLPs and the other 3 groups (PPV vaccine, PPV epitope B5-E1 and PBS as controls) were all tested with an HI assay. The HI value of antibodies from PPV vaccine immunized guinea pig groups increased with time and was higher than that of other groups. At 42 dpv, the HI value in cVLP and epitope B5-E1 group was highest among all time points (except for PPV vaccine group), whereas the HI value among cVLPs was higher than that of epitope B5-E1 and PBS groups.

Virus loads (virus copies) in tissues of PPV-infected guinea pigs were used as an important indicator of vaccine-induced, serum antiviral effects (Ji et al., 2017). Therefore, guinea pigs were immunized with cVLPs and commercial vaccine (PCV2 or PPV), challenged with PCV2 or PPV virus, respectively and ultimately, RT-PCR used to quantify virus copies (PCV2 or PPV) in tissue samples of all groups. PCV2 virus copies in cVLPs group were lower than that of PBS and PCV2 vaccine groups at 14 dpi (Fig. 6). Furthermore, PPV virus copies in cVLPs group were lower than that of PBS group, but higher than that in PPV vaccine group (Fig. 6), confirming that cVLPs vaccinated guinea pigs had effective protective immunity against challenge with PCV2 and some protective immunity against challenge with PPV.

4. Discussion

Baculovirus and *E. coli*-expressed PCV2 VLPs with diameters of ~20 nm, developing a fully effective immune response, have been engineered as bivalent or multivalent vaccines. PCV2 has the highest evolution rate among DNA viruses (Karuppannan and Opriessnig, 2017) and currently has at least 5 distinct genotypes (designated a–e). Since 2005, the shift in genotype from PCV2a to PCV2b occurred in North America (Carman et al., 2006); fortunately, commercial PCV2a-based PCV2 vaccine is effective against PCV2b (Fort et al., 2008; Seo et al., 2014). Since 2012, the PCV2b genotype was replaced by the PCV2d genotype (as the most prevalent) in North America, China and South Korea (Xiao et al., 2016). The PCV2a vaccine was effective against experimental PCV2b and PCV2d challenges (Karuppannan and Opriessnig, 2017; Park et al., 2019). However, frequent reports of PCVAD cases have raised alarms. Current commercial PCV2 vaccines based on the PCV2a genotype or its capsid protein (Opriessnig et al., 2007) may minimize PCV2d replication, but do not eliminate viruses (Karuppannan and Opriessnig, 2017) and confer incomplete protection. In this study, VLPs and virus strains were PCV2b genotype whereas virus strains were PCV2b genotype, with VLPs conferring good protective effects. However, under field conditions, there are various genotypes or combinations of genotypes. Whether VLPs prepared in this study would have protective effects on other PCV2genotypes needs further study. Using VLPs of various genotypes combined to prepare a polygenic vaccine may achieve complete protection, as PCV2 VLPs can be updated and upgraded to match the clinical genotype.

PCV2 depletes lymphocytes and immunosuppresses pigs (Segales et al., 2004), making PCV2-infected pigs more susceptible to other pathogens, with greater probability to be co-infected with other pathogens, *e.g.* PPV (Ellis et al., 2004). These co-infections are more difficult to control than infections with PCV2 *per se*. Currently, vaccination is still the most effective means of defense. Therefore, it is attractive to explore a novel combined VLPs vaccine to prevent both PCV2 and PPV in swine. PPV VLPs have been used to display PCV2 epitopes. PPV cVLPs displaying immunoreactive epitopes (aa 165-200) from the PCV2 cap was constructed using an adenovirus vector system (Pan et al., 2008). This cVLPs elicited robust antibody responses against PCV2 in mice, making it a feasible candidate bivalent vaccine against both PCV2 and PPV. In this study, we demonstrated the feasibility of developing a novel PCV2 virus-like particles (85 G and 86S of PCV2 Loop CD) displaying the B cell linear epitope (^{228}QQITDA^{233}) of PPV1, providing a basis for future research and development of PCV2-PPV bivalent vaccines. We produced PCV2 rCap by inserting PPV1 B cell liner epitope B5-E1 (^{228}QQITDA^{233}) into the Loop CD of PCV2 Cap, which successfully self-assembled into cVLPs. In addition, the consistency of structure prediction results of cVLPs and ELISA results based on cVLPs inserted epitopes confirmed that epitope B5-E1 was functionally displayed on the surface of the cVLPs. These data confirmed that the site between 85 G and 86S of PCV2 Loop CD of PCV2 Cap accommodated insertion of PPV epitopes. Except for the site displaying effective neutralizing epitope GP5 B cell epitope (^{37}SHIQLIYNL^{45}) of PRRSV in a previous study (Hu et al., 2016), insertion of PPV epitopes displayed on the surface of PCV2 VLPs did not affect PCV2 assembly. This site on the surface of PCV2 capsid was an ideal candidate chimeric position, with potential to accommodate insertion of other pathogen epitopes. Maximum lengths of amino acid fragments accomodated by the site and effects of insertion of this site on stability of VLPs should be determined in future studies.

Antibodies from murine antisera obtained by immunization with cVLPs recognized peptides of PPV1 epitope and PCV2 Cap. There is increasing evidence that antisera from guinea pigs immunized with cVLPs efficiently neutralized PCV2s and protected these animals from experimental challenges. In addition, antisera in guinea pigs also neutralized a portion of PPV from experimental challenge, indicating that cVLPs retained immunogenicity of PCV2 and the selected PPV epitope, induced an effective and specific humoral immune response, and produced cross-neutralization for PCV2 and PPV1. Although cross-neutralization based on the current cVLPs may not seem very efficient,

these results confirmed that Loop CD could contribute to epitopes of vaccine-induced, cross-neutralizing antibodies. Potential explanations include: 1) efficacy of cVLPs vaccine may be affected by the epitope and cVLPs only contained 1 antigen epitope B5-E1 (^{228}QQITDA^{233}) of PPV, which may not have induced a strong immune response against PPV; and 2) folding and packaging of the PCV2-Cap protein may affect full exposure of epitope on the VLPs surface. Regardless, current results are an impetus to use PCV2 cVLPs with multiple potent explored PPV-epitopes as a vaccine antigen as a practical and effective strategy for induction of cross-neutralizing antibodies.

5. Conclusion

In conclusion, cVLPs vaccinated guinea pigs had protective immunity against challenge with PCV2, and some protective immunity against challenge with PPV. Ability to chimerize heterologous epitopes in PCV2 capsid protein was expanded, providing valuable research data for future efforts regarding molecular design of chimeric epitopes PCV2 VLPs.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.06.002.

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