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The C3d-fused Porcine circovirus type 2d virus-like particle induced early and enhanced immune response and protected pigs against challenge

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ABSTRACT

Porcine circovirus type 2 (PCV2) is an economically significant pathogen affecting the global swine industry. Vaccination is considered the most effective and best way to prevent PCV2-associated disease. The PCV2d genotype has become predominant by replacing the previous PCV2b genotype. The potential increase in the virulence of PCV2d has drawn attention, spurring the development of PCV2d vaccines. Virus-like particle (VLP) is an ideal vaccine candidate for its safety and potent immunogenicity. C3d is a molecular adjuvant that can be used to promote the protective efficacy of the PCV2 vaccine. In this study, we expressed PCV2d Cap protein fused with C3d epitope using *E. coli* expression system. The purified recombinant Cap protein assembled into VLP, which was designated as PCV2d-C3d-VLP. Through assessments in mice and piglets, we demonstrated that the PCV2d-C3d-VLP elicited robust humoral responses, notably accelerating antibody production one week earlier compared to a commercial PCV2d subunit vaccine. Furthermore, vaccination substantially reduced PCV2d viral load in piglets. These results present an innovative strategy for developing a more efficacious and cost-effective PCV2d VLP vaccine.

1. Introduction

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, circular, single-stranded DNA virus classified within the *Circoviridae* family (Tischer et al., 1982). PCV2 is the primary causative pathogen of PCV-associated disease (PCVAD), which is an economically significant disease in the global swine industry. In European Union countries, PCV2 has contributed to economic losses more than 600 million per year (Chae, 2012, Liu et al., 2020, Guo et al., 2022). PCV2d-infected pigs are characterized by immunosuppression and hampering production efficiency (Maity et al., 2023). Phylogenetic tree analysis showed that PCV2 can be divided into nine distinct genotypes, PCV2a to PCV2i (Franzo and Segales, 2020). Initially, PCV2a was the predominant strain until 2003, when a shift from PCV2a to PCV2b occurred. Since 2014, PCV2d has become the predominant strain worldwide (Ma et al., 2021, Gong et al., 2023, Maity et al., 2023). In addition, PCV2d is more virulent and can lead to more severe clinical symptoms (Guo et al., 2012, Opriessnig et al., 2014). Despite cross-immunity between different PCV2 genotypes, vaccines based on PCV2a or PCV2b induce incomplete cross-protection against PCV2d (Yu et al., 2023). The recent rise of PCVAD cases in PCV2 vaccine-immunized pigs was partially attributed to incomplete cross-genotype protection (Xiao et al., 2016, Kang et al., 2020). Thus, developing a PCV2d-based vaccine is of great importance in PCV2d prevention.

PCV2 genome contains two major open reading frames (ORFs), ORF1 and ORF2. ORF1 encodes replicase-associated proteins (Rep and Rep') and ORF2 encodes the viral capsid (Cap) protein. Cap protein is the sole structural protein forming the icosahedral virion and contains PCV2-specific neutralizing epitopes (Mo et al., 2019). Additionally, Cap protein can self-assemble into virus-like particle (VLP), which efficiently elicit both humoral and cell-mediated immune responses in pigs (Hemmati et al., 2022). Consequently, Cap protein is the prime target for

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developing novel recombinant vaccines. These vaccines could be used to protect pigs against PCV2 infections.

C3d is the final degradation product of complement component 3 and could be covalently attached to antigens. CD21 is a co-receptor on the surface of B cells. The C3d binding to CD21 improves the proliferation of B cells. When a C3d-labeled pathogen simultaneously interacts with BCR and CD21, the pathway crosstalk can lower the antigen threshold necessary for stimulating B-cell activation. Consequently, this interaction enhances adaptive immune responses to the labeled pathogens (Lee et al., 2022). Previous studies have shown that C3d, as a molecular adjuvant, can improve both humoral and cellular immune responses (Zhang et al., 2011, Zhao et al., 2017, Hou et al., 2019). Therefore, C3d can be used as an immune adjuvant to improve the immune efficacy of vaccines.

In this study, we expressed PCV2d Cap protein fused with a C3d-specific epitope (13 amino acids) in *E. coli*. After purification, the PCV2d Cap proteins were assembled into VLP. Subsequently, we assessed its immune efficacy in mice and pigs. In addition, we evaluated the protective efficacy of PCV2d-VLP in pigs against PCV2d challenge. The results showed that the novel C3d-fused PCV2d-VLP vaccine induced significant immune protection capacity compared to a commercial PCV2d subunit vaccine.

2. Materials and methods

2.1. Animal welfare statement

All animal experiments were conducted following the recommendation in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS) and the Animal Ethics Committee of Heilongjiang Province, China (Permission number: 221206–01-GR for mice and 230626–02-GR for pigs).

2.2. Cells and viruses

PK-15 cells were cultured in Dulbecco's modified eagle medium (DMEM, Gibco, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco, USA) and maintained at 37 ℃ with 5 % CO_2. PCV2d isolate FJ/2022 (Genbank accession no. PP554892) was kept in our lab.

2.3. Expression and purification of PCV2d Cap protein fused with C3d epitope

For plasmid construction, the ORF2 of PCV2d with the C3d epitope (corresponding to the amino acid sequence, GKQLYNVEATSYA) sequence was codon-optimized and synthesized by Genscript Corporation, which was cloned into the pET28a vector. The resulting recombinant plasmid was designated as pET28a-PCV2d-C3d-Cap.

The pET28a-PCV2d-C3d-Cap plasmid was transformed into *E. coli* BL21 (DE3). The monoclonal colonies were selected on kanamycin-containing plates (50 μg/mL), and incubated in LB medium with kanamycin (50 μg/mL) for 12 h. 4 mL of the bacterial solution was placed into 200 mL of LB medium containing kanamycin (50 μg/mL) and incubated for 2.5 h. Expression of PCV2d-C3d-Cap was induced with isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 20 hours at 25 ℃. After centrifugation at 5,000 g for 10 min, the bacterial pellets were resuspended in lysis buffer (0.02 M-phosphate buffer, 0.2 mM-EDTA, 1 %-Tween80, pH 7.5). Bacteria were crushed with an ultrasonic cell crusher (Cole Parmer, Vernon Hills, IL, USA) and centrifuged at 12,000 g for 10 min. The cell lysate and the supernatant were collected. The expression of soluble PCV2d-C3d-Cap in the supernatant was evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie Brilliant Blue.

The saturated ammonium sulfate solution was added drop by drop to the supernatants and mixed thoroughly to achieve a final concentration of 50 %. The protein precipitate was collected after centrifugation at 12,000 g for 30 min. The protein precipitate was resuspended in resuspension buffer (0.02 M-phosphate buffer, pH 7.5), mixed thoroughly, and centrifuged at 12,000 g for 30 min to collect the supernatant, filtered through a 0.45 μm filter. The clarified supernatant was loaded onto a Rose Plus Q XP column (Nanomicrotech, China) within an automated FPLC system (AKTA avant, GE-Healthcare Life Sciences, USA). The column was initially washed with buffer A (0.5 M NaCl, 0.02 M phosphate buffer, pH 7.5), and PCV2d-C3d-Cap proteins were subsequently eluted with buffer B (1 M NaCl, 50 mM phosphate buffer, pH 7.5). The purity of the PCV2d-C3d-Cap proteins collected from the second peak was assessed using SDS-PAGE. Subsequently, the proteins were further purified using a Sepharose 6FF 16/96 column (Bestchrom, China) equilibrated with equilibration buffer (0.02 M phosphate buffer, pH 7.5). The assembled PCV2d-C3d-VLP was observed by transmission electron microscopy (TEM).

2.4. Western blot

Proteins from SDS-PAGE gels were transferred to PVDF membranes (Millipore, MA, USA) and blocked with 5 % skimmed milk in PBS. The PVDF membranes were washed three times with PBS containing 0.1 % Tween-20 (PBS-T) for 15 min and incubated with PCV2-specific MAb 2G8 (Sun et al., 2022) (1: 500) for 1 h at 37 ℃. The PVDF membranes were washed three times with PBST and incubated with fluorescently labeled goat anti-mice secondary antibody (Biodragon, Beijing, China; 1: 10,000) at 37 ℃ for 50 min. Finally, the PVDF membranes were washed three more times under light-avoidance conditions, and the PVDF membranes were scanned using a near-infrared fluorescence scanning imaging system (Odyssey CLX, USA) according to the manufacturer's instructions.

2.5. Detection of PCV2d-C3d-VLP using TEM

Ten microliters of purified protein suspension were dripped onto a copper mesh and incubated at 25 ℃ for 10 min. After the copper mesh was completely dried, it was stained with 10 microliters of 2.5 % tungsten phosphate negative staining solution and was carefully placed under TEM (HITACHI, Tokyo, Japan) to observe the PCV2d-C3d-VLP.

2.6. Preparation of PCV2d-C3d-VLP vaccine and mice immunization

The purified PCV2d-C3d-VLP protein solution was filtered through a 0.22 μm filter and mixed with MONTANIDE ISA 15 A VG adjuvant (Seppic, Paris, France) at a ratio of 85:15, and emulsified after stirring at 25 ℃ for 15 min to prepare PCV2d-C3d-VLP vaccine. The final protein concentration was 50 μg/mL and stored at 4 ℃. Besides, a Subunit Vaccine of Porcine Circovirus Type2 (Recombinant Baculovirus Strain OKM), designated as VLP-C, from Zhengye Biotechnology Holding Limited was used as a control group to compare the immunogenicity with PCV2d-C3d-VLP.

Fifteen 6-week-old female BALB/c mice (Changsheng, Benxi, China) were randomly divided into three groups (n = 5). Mice in the PCV2d-C3d-VLP group, VLP-C group, and negative control group were vaccinated with 200 μL of the PCV2d-C3d-VLP vaccine, VLP-C, and PBS, respectively. The second immunization was performed three weeks after the first immunization at the same dose. Sera samples were collected at 14, 21, 28, and 35 days post-immunization (dpi) and analyzed for Cap protein-specific antibodies using indirect enzyme-linked immunosorbent assay (I-ELISA). Peripheral blood mononuclear cells (PBMCs) were collected at 24 dpi. All mice were sacrificed at 35 dpi.

2.7. Determination of T lymphocyte subpopulations in peripheral blood of mice

From each mouse, 100 μL of mouse blood was collected into an anticoagulation blood collection tube (Becton Dickinson, USA) and mixed gently to prevent coagulation. Then, 50 μL of blood was transferred to a microcentrifuge tube (Corning, Shanghai, China) and incubated with 1 μL each of CD3, CD4, and CD8 antibodies (Biosciences, Brisbane, CA, USA) for 30 min at 25 ℃ in dark conditions. Subsequently, 1.8 mL of erythrocyte lysate (Beyotime, China) was added and incubated for 40 min at 4 ℃ in dark conditions, then centrifuged at 4 ℃ and 2000 rpm for 5 min, and the supernatant was discarded. The cells were resuspended by adding 1.8 mL of PBS, centrifuged at 2000 rpm for 5 min at 4 ℃, and the supernatant was discarded. Cells were resuspended with 200 μL PBS, filtered through a strainer, and analyzed using a flow cytometer (Becton Dickinson, USA) according to a previously published method (Shen et al., 2007).

2.8. Piglet immunization and PCV2d challenge

Twenty 4-week-old SPF piglets were randomly divided into four groups and immunized (Table 1). Piglets were vaccinated at the same dose three weeks after the first immunization. Groups 1–3 were challenged with PCV2d (10^5 $TCID_{50}$/mL) nasally (2 mL) and intramuscularly (2 mL) two weeks after the second immunization, and group 4 served as the negative control.

2.9. Detection of PCV2-specific antibodies and high-avidity antibodies

To measure PCV2-Cap-specific IgG in the sera of immunized mice and pigs, we performed using an in-house PCV2d ELISA and a commercial PCV2 ELISA Kit (PCV2-rCap Antibody Test Kit, JBT, South Korea). Briefly, for PCV2d ELISA, ELISA plates were coated with PCV2d-Cap proteins and incubated overnight at 4 ℃. Each well was added 100 μL 5 % skimmed milk and incubated for 1 h at 25 ℃. After washing, the plates were incubated with serum samples (1:100) for 30 min at 25 ℃. Subsequently, 100 μL/well HRP-labeled goat anti-mouse IgG (1:10,000, Biodragon, China) and HRP-labeled goat anti-pig IgG (1:10,000, Biodragon, China) were added to the corresponding wells based on the serum species. After washing, 100 μL/well of TMB solution was added and incubated for 15 min at 25 ℃. The HRP-TMB reaction was stopped with 1 M HCl, and OD_{450} was measured using a spectrophotometer (PE, USA). For the commercial PCV2 ELISA, sera samples were tested using a commercial PCV2-Cap-ELISA Kit (PCV2-rCap Antibody Test Kit, JBT, South Korea) following the manufacturer's instructions. The samples were considered positive if the calculated S/P ratio was $\geq$0.4.

To assess the avidity of antibodies produced by immunized piglets, we performed avidity ELISA as described in a previous study (Chang et al., 2021). After incubation with the same serum, two plates were processed in parallel: one was washed three times with PBST-8 M urea by 5 min incubation, and the other with PBST. The avidity index was calculated as the ratio of S/P value (PBST-8 M urea) to S/P value (PBST) with the same dilution.

Table 1
The vaccination and challenge status of experimental piglets.

Groups	Number of pigs	Formulation	Vaccine/ PBS Dose	Challenge Dose ($TCID_{50}$)
PCV2d-C3d-VLP	5	PCV2d-C3d-VLP	1 mL	4 mL,1×10^5/mL
VLP-C	5	A commercial PCV2d subunit vaccine	1 mL	4 mL, 1×10^5/mL
Challenge control	5	PBS	1 mL	4 mL,1×10^5/mL
Negative control	5	PBS	1 mL	-

2.10. Virus neutralization (VN) assay

The sera collected at 14, 28, and 35 dpi were assessed for neutralizing titers against PCV2 according to a previous method with minor modifications (Kim et al., 2020). Briefly, the sera were inactivated by heating at 56 ℃ for 30 min, twofold serially diluted in DMEM, and mixed with an equal volume of PCV2d (200 $TCID_{50}$). The mixture was inoculated into PK-15 cells that were 70–80 % confluent. After 72 h, the cells were fixed with 4 % paraformaldehyde and penetrated with 0.2 % triton-100. An immunofluorescence assay (IFA) using pig serum against PCV2-Cap was performed as described previously. The VN titers were determined to have the highest serum dilution, which resulted in greater than 70 % neutralization of the well under a fluorescence microscope.

2.11. Quantification of PCV2 DNA

All pigs were sacrificed at 28 days post challenge (dpc). Viral DNA from the inguinal lymph nodes was extracted using a Viral DNA/RNA Extraction Kit (Tiangen, China). Quantitative polymerase chain reaction (qPCR) was conducted to detect viral DNA using Premix Ex Taq (Probe qPCR, TaKaRa, Dalian, China). The primer pair for qPCR amplification was described previously (Gagnon et al., 2010).

2.12. Immunohistochemistry

The inguinal lymph nodes were harvested and fixed in 10 % neutral buffered formalin. The presence of PCV2 antigen in the lymph nodes was determined using immunohistochemistry (IHC). Briefly, tissue sections were incubated at 37 ℃ for 1 hour with a 1:100 dilution of PCV2 polyclonal antibodies (Thermo Fisher Scientific, Waltham, MA, USA), followed by a 1:100 dilution of HRP-labeled goat anti-rabbit antibody at 37 ℃ for 1 hour. The freshly prepared Diaminoamphetamine was applied to the sections at 25 ℃ to visualize color development. Positive cells were visualized using a light microscope (Nikon, Tokyo, Japan).

2.13. Statistical analysis

The results were expressed as the mean ± standard deviation (SD). Data were analyzed by t-test using GraphPad Prism version 9.00 (GraphPad, USA). A P value of <0.05 was considered significant.

3. Results

3.1. Purified PCV2d-C3d-Cap assembled into VLP

Recombinant plasmid pET28a-PCV2d-C3d-Cap was constructed and transformed into *E. coli* BL21 (DE3) competent cells. After induction with 0.1 mM IPTG for 20 h, the bacteria were harvested and sonicated. PCV2d-C3d-Cap protein was predominantly soluble when expressed in *E. coli* (Fig. 1A). Subsequently, the proteins were purified by ion exchange chromatography (IEC) (Fig. 1B). SDS-PAGE (Fig. 1C) analysis showed high purity of the PCV2d Cap protein. Western blot analysis showed that PCV2-specific MAb 2G8 recognized PCV2d-C3d-Cap protein, indicating the PCV2d-C3d-Cap protein has good immunoreactivity (Fig. 1D). In addition, TEM revealed PCV2d-VLP is about 20 nm in diameter (Fig. 1E). Furthermore, PCV2d-C3d-Cap protein was purified by size exclusion chromatography (SEC). SDS-PAGE and TEM results showed that the purified PCV2d-C3d-Cap assembled into VLP and the VLP were homogeneous and intact with no visible impurities (Fig. 2A-B).

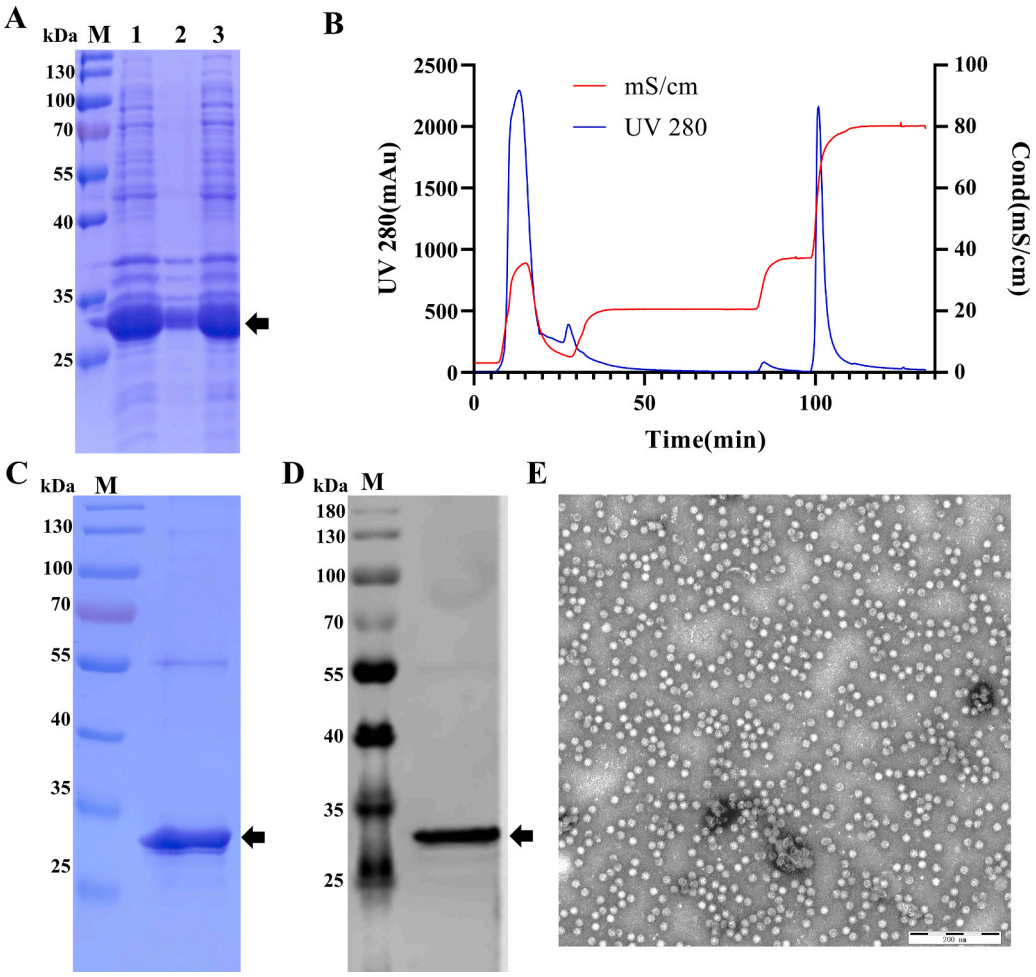

Fig. 1. Analysis and IEC purification of PCV2d-C3d-Cap and PCV2d-C3d-VLP. (A) SDS-PAGE analysis of PCV2d-C3d-Cap expression in *E. coli*. (B) Ion exchange chromatography (IEC) profile of self-assembled PCV2d-C3d-VLP. (C) Coomassie staining of the PCV2d-C3d-Cap purified by IEC. (D) Western blot analysis of PCV2d-C3d-Cap purified by IEC. (E) The TEM image of PCV2d-C3d-VLP. M: Marker; Lane 1: Total cell lysate after fragmentation; Lane 2: the supernatant of the centrifuged cell lysate; Lane 3: the pellets of the centrifuged cell lysate.

3.2. PCV2d-C3d-VLP induced both humoral and cellular immune responses in mice

To evaluate the immunogenicity of the PCV2d-C3d-VLP vaccine, we measured antibodies induced by PCV2d-C3d-VLP and VLP-C in mice. All the mice were immunized using the same vaccination regimen (Fig. 3A). Serum IgG antibodies specific for the Cap were detected by ELISA (Fig. 3B). The IgG antibody titers of the PCV2d-C3d-VLP and VLP-C groups increased rapidly from 14 dpi, reaching the highest levels at 35 dpi. Furthermore, PCV2d-C3d-VLP IgG antibody titers were significantly ($p < 0.001$) higher than that of VLP-C at 21, 28, and 35 dpi.

In addition, we determined the ratio of CD3+CD4+T cells and CD3+CD8+T cells (CD4/CD8 ratio) in mice PBMCs at 24 dpi. Both the PCV2d-C3d-VLP group and the VLP-C group showed significantly ($p < 0.01$) higher CD4/CD8 ratios compared to the negative control group (Fig. 3C).

3.3. PCV2d-C3d-VLP induced a potent humoral immune response in pigs

We evaluated the humoral immune response of the PCV2d-C3d-VLP vaccine in pigs. Piglets were immunized with the PCV2d-C3d-VLP or VLP-C (Fig. 4A). To assess PCV2d-specific IgG titers, we developed an ELISA using PCV2d-Cap proteins. Seroconversion was observed in the sera of the PCV2d-C3d-VLP group from 14 dpi, and the VLP-C group from 21 dpi. The PCV2-Cap-specific IgG in the sera of the PCV2d-C3d-VLP group were significantly higher than those in the VLP-C vaccinated group at 7 dpi, 14 dpi, 21 dpi ($P < 0.001$) and 28 dpi ($P < 0.01$) (Fig. 4B).

To confirm this result, the induction of humoral immune response in each vaccinated group was monitored by measuring serum IgG antibodies using a commercial ELISA kit. We observed seroconversion at 21 dpi in the PCV2d-C3d-VLP group, and at 28 dpi in the VLP-C group. The PCV2d-C3d-VLP group also showed significant ($P < 0.05$) higher IgG titers than those in the VLP-C group at 14, 21, 28, 35, 42, 56, and 63 dpi (Fig. 4C). This result is similar to the PCV2d-ELISA result.

In addition, we assessed the avidity of PCV2-specific antibodies by avidity ELISA. Since 8 M urea can wash away low-avidity antibodies, the avidity index was calculated as the ratio of ELISA readouts with 8 M urea wash to those with PBST wash. The avidity index of the PCV2d-C3d-VLP vaccinated group was significantly ($P < 0.001$) higher than that of the VLP-C vaccinated group at 21 dpi, 28 dpi and 35 dpi (Fig. 5).

The serum samples were analyzed using an IFA to evaluate VN titers against the PCV2d in PK-15 cells. As shown in Fig. 6, PCV2-neutralizing antibody titers were first detected in pigs immunized with PCV2d-C3d-VLP at 21 dpi and VLP-C at 28 dpi. The peak neutralizing antibody titers were $2^{7.4}$ (PCV2d-C3d-VLP) and $2^{6.4}$ (VLP-C) at 35 dpi. The neutralizing antibody titers induced by PCV2d-C3d-VLP were significantly higher than those of the VLP-C group.

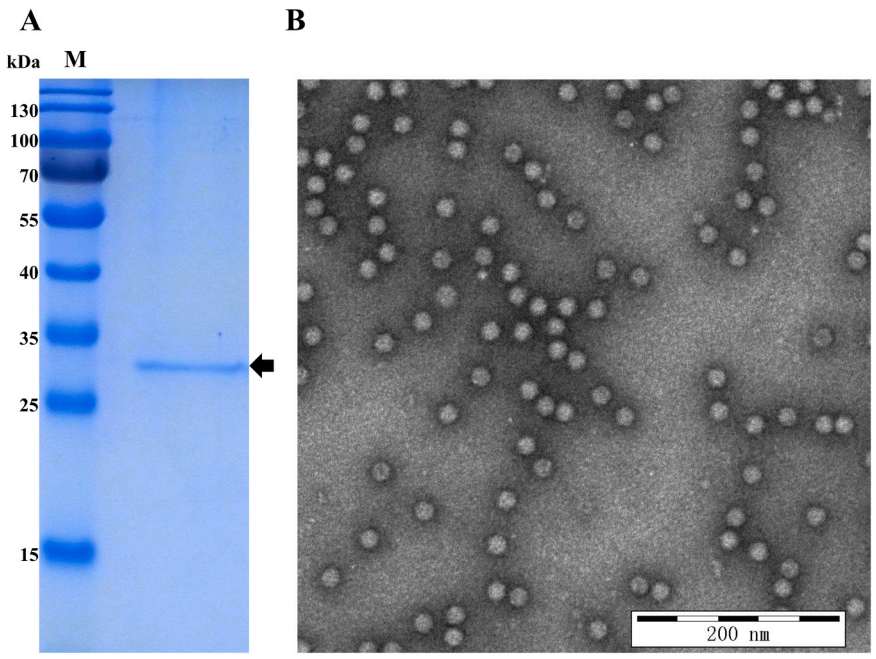

Fig. 2. Analysis and further purification of PCV2d-C3d-Cap and PCV2d-C3d-VLP. (A) Coomassie staining of the PCV2d-C3d-Cap after further purification. (B) The TEM image of PCV2d-C3d-VLP.

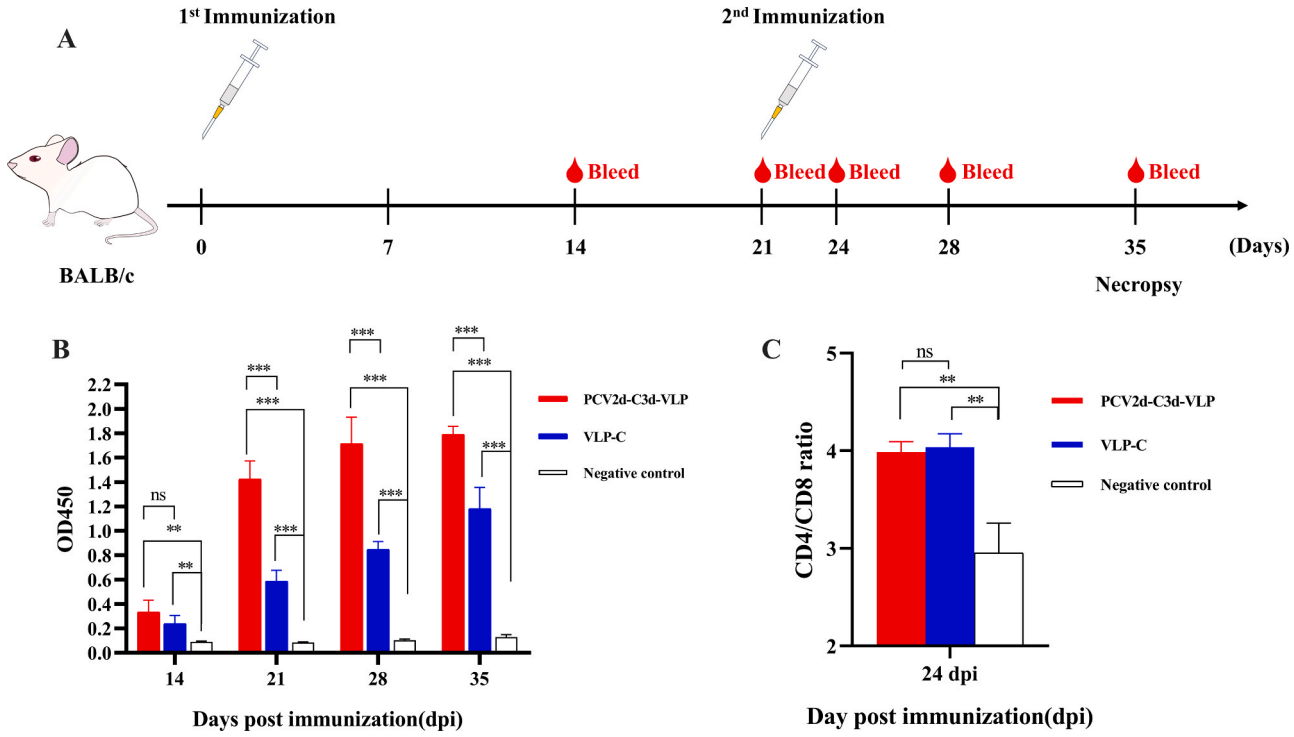

Fig. 3. Immunization strategies and immunogenicity in mice. (A) Mice immunization strategy. (B) ELISA for PCV2d-specific antibodies in mice after immunization. (C) Analysis of CD4/CD8 ratio of immunized pig PBMCs by flow cytometry. VLP-C refers to the commercial PCV2d subunit vaccine. **: $P < 0.01$, ***: $P < 0.001$, ns: not significant.

3.4. PCV2d-C3d-VLP decreased the PCV2 viral load in lymph nodes

To evaluate the efficacy of the vaccines, we detected the PCV2 viral load in pig inguinal lymph nodes at 28 dpc. PCV2 viral DNA copy numbers in inguinal lymph nodes of the PCV2d-C3d-VLP group (8.34×10^4 copies/g, $p < 0.001$) and the VLP-C group (3.30×10^5 copies/g, $p < 0.01$) were significantly lower than the challenge control group (7.27×10^7 copies/g) (Fig. 7A). This result indicated that the PCV2d-C3d-VLP effectively reduced PCV2d replication in pigs.

We also conducted immunohistochemistry (IHC) using PCV2 polyclonal antibodies to examine the PCV2 virions in the inguinal lymph nodes of pigs. The result showed a higher number of positively stained cells in the inguinal lymph nodes of the challenge control group (Fig. 7B). In contrast, no detectable staining for the PCV2 antigen was

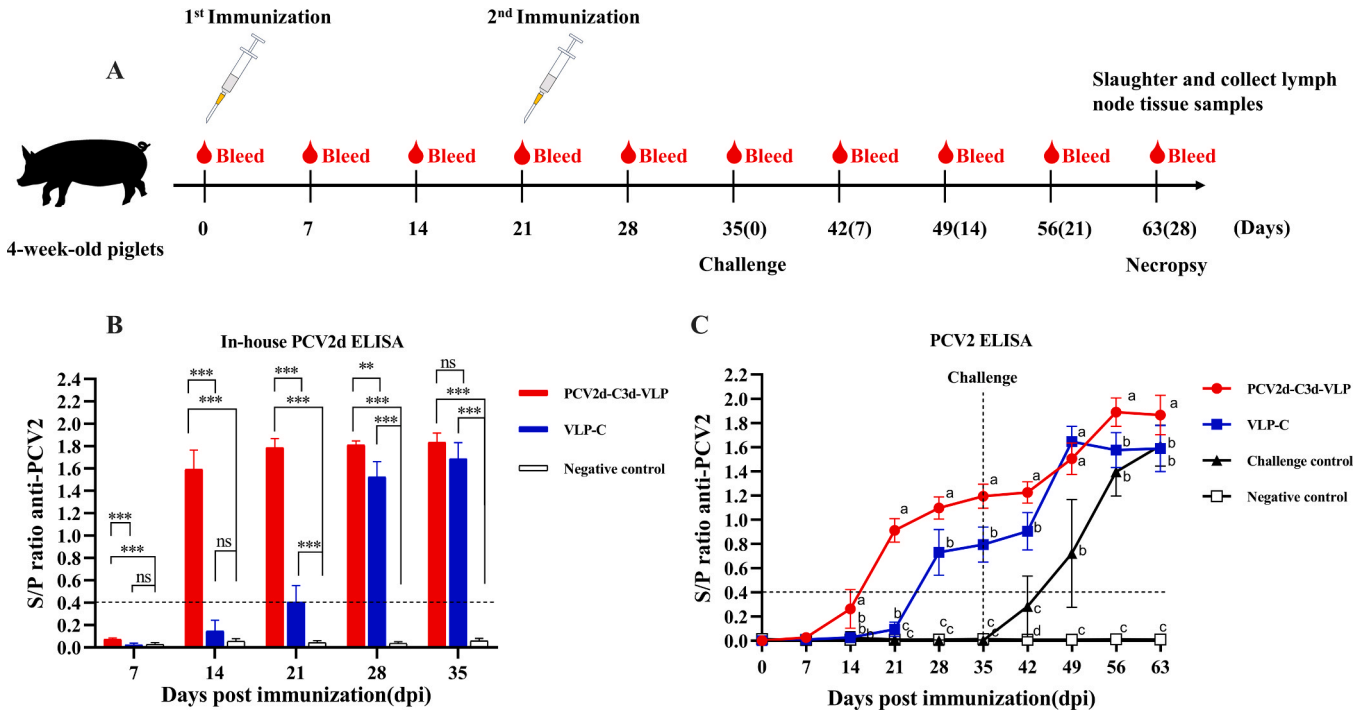

Fig. 4. Immunization strategies and immunogenicity in piglets. (A) Piglets immunization strategy. (B) In-house PCV2d-ELISA for PCV2d-specific antibodies in piglets after immunization. (C) PCV2-ELISA for PCV2-specific antibodies in piglets after immunization with commercial PCV2 antibody detection kit. **: $P < 0.01$, ***: $P < 0.001$, ns: not significant. Different superscripts (a, b, c, d) indicate significant differences among groups ($p < 0.05$).

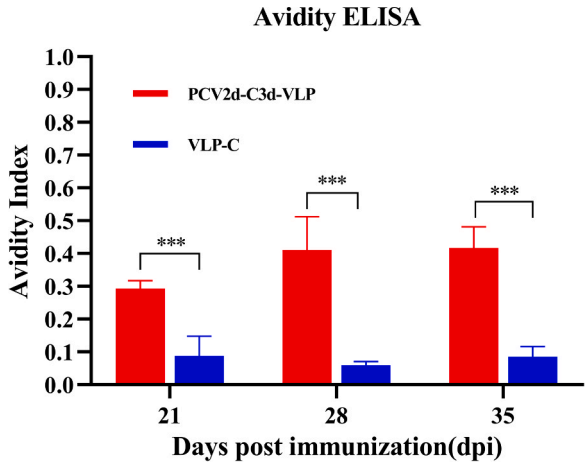

Fig. 5. Avidity ELISA was modified with urea for PCV2 high-avidity antibodies in piglets after immunization using a commercial PCV2 antibody detection kit. ***: $P < 0.001$.

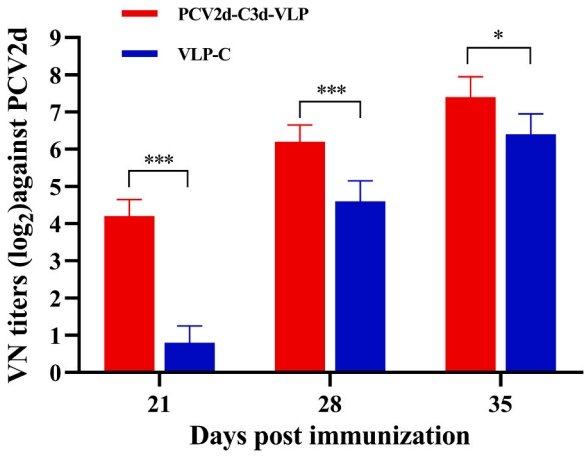

Fig. 6. Detection of PCV2d-specific viral neutralization (VN) levels. The VN titers against PCV2d were detected by immunofluorescence assay and were determined as the highest serum dilution that exhibited 70 % neutralization of the well. *: $P < 0.05$, ***: $P < 0.001$.

observed in the tested tissues from the PCV2d-C3d-VLP group or the VLP-C group, indicating that viral proteins are absent in the lymph nodes of these two groups. Thus, PCV2d-C3d-VLP protected piglets from PCV2d challenge.

4. Discussion

The size and conformation of VLP closely resemble those of natural virions, and it can be an excellent mimic of pathogens with good immunogenicity. Meanwhile, VLP lacks viral nucleic acids. Thus, VLP has been proven as an ideal vaccine in many studies (Janitzek et al., 2019, Hirschberg et al., 2023, Li et al., 2023). VLP has been recently used as a subunit vaccine to control PCV2 infection. Most current PCV2 subunit vaccines provide immune protection largely through humoral immune response (Chae, 2012, Guo et al., 2022). Thus, an effective adjuvant is essential for enhancing the efficacy of subunit vaccines. Studies have shown that C3d is a potent molecular adjuvant to enhance the adaptive immune response against immunogens (Zhang et al., 2011, Zhao et al., 2017, Hou et al., 2019, Lee et al., 2022). For example, C3d as an adjuvant in the HIV-1 subunit vaccine improved antibody response against HIV (Bale et al., 2023). In this study, recombinant PCV2d-Cap protein fused with the C3d epitope was successfully expressed in *E. coli* expression system. The introduction of C3d did not affect the assembly of PCV2d-VLP, which enhanced the humoral immune response after vaccination in mice and pigs and provided protection against PCV2d challenge.

Maternal-derived antibodies (MDA) interfere with the efficacy of

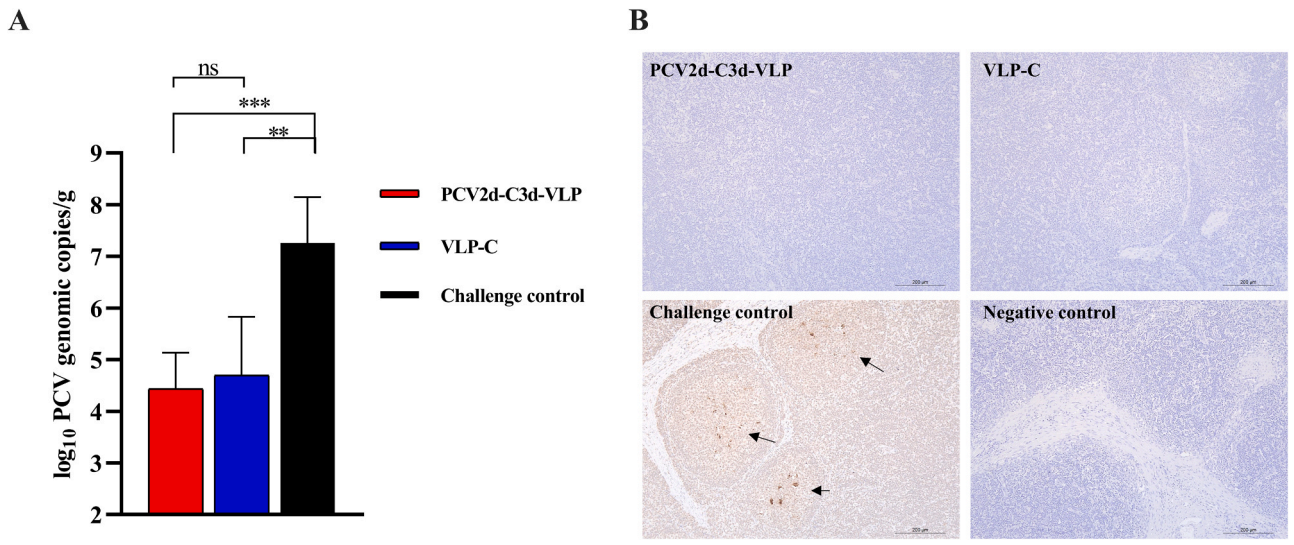

Fig. 7. Comparison of protection of two different VLPs against PCV2d challenge. (A) Inguinal lymph node virus load in piglets at autopsy. **: $P < 0.01$, ***: $P < 0.001$, ns: not significant. (B) IHC of inguinal lymph node tissue in piglets. Black arrows indicate tan-colored cells in inguinal lymph node tissue.

PCV2 vaccines. Many studies have shown that high MDA titers during vaccination interfered with the development of the humoral immune response against the PCV2 vaccine (Fraile et al., 2012, Oh et al., 2014). However, few studies have proposed strategies for inducing a robust immune response against PCV2 by effectively overcoming MDA. Recently, a C3d-fused FMD vaccine induced a robust cellular and humoral immune response, effectively overcoming MDA interference. In the MDA (+) and MDA (−) pigs, the virus-neutralizing antibody titers were significantly higher in pigs that administered the O PA2-C3d + A22-C3d bivalent vaccine than those administered the vaccine with O PA2 + A22 (Lee et al., 2022). Thus, the PCV2d-C3d-VLP vaccine is expected to overcome MDA interference in pig populations, and further validation in herds under field conditions is required.

PCV2d-C3d-VLP induced PCV2d-specific humoral immune response. After vaccination in mice, the IgG titers in the PCV2d-C3d-VLP group were significantly higher than those in the VLP-C group ($P < 0.001$). Similarly, pigs immunized with PCV2d-C3d-VLP showed significantly higher titers of PCV2-Cap-specific antibodies than those of the VLP-C group ($P < 0.05$). A study suggested that increases in neutralizing antibody titers corresponded with decreases in PCV2 viral loads (Fort et al., 2007). Similarly, our results showed that PCV2d-C3d-VLP vaccination induced high levels of neutralization antibodies, effectively decreasing PCV2d viral load in pigs. Moreover, PCV2d-C3d-VLP markedly enhanced the avidity of PCV2-Cap-specific antibodies compared to the commercial PCV2d subunit vaccine, reflected by the increased avidity index in PCV2-C3d-VLP vaccinated pigs. The high-affinity antibodies are secreted by differentiated B cells undergoing germinal center (GC) reactions (Maclennan and Gray, 1986). The fusion of C3d to Env enhanced germinal center formation and the affinity maturation of Env-specific antibodies (Bale et al., 2023). Therefore, the improved PCV2-specific antibody quantity and avidity could be due to the enhancement of germinal center formation and affinity maturation by C3d. Besides, the increased PCV2-specific antibody quantity and avidity by vaccination appear to induce the protective immune response and contribute to viral clearance in tissues after a PCV2d challenge.

The improved T cell responses seem to be another reason for the increased antibody response in PCV2d-C3d-VLP vaccinated mice. C3d enhanced immune responses by internalization and processing of C3d into peptides that activate CD4+ T helper cells (De Groot et al., 2015). The CD4/CD8 ratio is an important indicator for evaluating immune states. Previous studies used the CD4/CD8 ratio to determine immunity states in several immunosuppressive diseases such as HIV (Serrano-Villar and Deeks, 2015). Lower CD4/CD8 ratios were detected in PCV2 infected pigs (Guo et al., 2012). In this study, the CD4/CD8 ratio was significantly ($P < 0.01$) increased in the peripheral blood of PCV2d-C3d-VLP vaccinated mice (Fig. 3C). This indicated that the PCV2d-C3d-VLP stimulated the CD4+T cell immune defense state in mice. CD4+ T cells play an essential role in promoting the B-cell response and GC reaction (Qi et al., 2014). Thus, the enhanced humoral response could be related to the increased CD4/CD8 ratio.

This study has several limitations regarding exploring the protective efficacy of PCV2d-C3d-VLP against PCV2 infection. First, the sample size was relatively small, which may have limitations in the statistical analysis. Second, this study focused solely on the protective effect of the PCV2d-C3d-VLP against PCV2d infection instead of PCV2a and PCV2b. Additionally, this study did not detect cellular immune responses in pigs. Despite these limitations, the study provides an initial understanding of the immunoprotective effect of PCV2d-C3d-VLP against PCV2d infection and establishes a foundation for future larger-scale investigations.

Our results showed that C3d-fused PCV2d-VLP induced a strong humoral immune response in mice and piglets and protected pigs against the PCV2d challenge. Compared to the baculovirus/insect cell expression system, the novel PCV2d-C3d-VLP vaccine prepared in *E. coli* expression system is cost-effective and efficient in production. Further, we will evaluate the efficacy of the PCV2d-C3d-VLP vaccine in pigs under field conditions.

Declarations

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Institutional review board statement

Not applicable.

Informed consent statement

Not applicable.

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CRediT authorship contribution statement

Haiwei Wang: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Xinyu Qi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Tongqing An:** Resources, Funding acquisition. **Mingxia Sun:** Writing – review & editing, Supervision, Resources, Project administration, Methodology. **Yan Ju:** Investigation. **Xuehui Cai:** Resources, Funding acquisition. **Xuyan Xiang:** Writing – original draft, Validation. **Zheng Fang:** Writing – review & editing, Methodology, Investigation, Data curation. **Liang Meng:** Writing – review & editing, Investigation.

Declaration of Competing Interest

H.W., M.S., T.A., and X.C. are listed as inventors on a patent application (Patent application No. 202410370650.9) filed by Harbin Veterinary Research Institute describing the preparation of C3d-fused PCV2d-VLP vaccine. Other authors have no conflict of interest to declare.

Data availability

The datasets generated and analyzed in the current study are available from the corresponding author upon reasonable request.

References

De Groot, A.S., Ross, T.M., Levitz, L., Messitt, T.J., Tassone, R., Boyle, C.M., Vincelli, A.J., Moise, L., Martin, W., Knopf, P.M., 2015. C3d adjuvant effects are mediated through the activation of C3d-specific autoreactive T cells. J. Immunol. Cell Biol. 93, 189–197. https://doi.org/10.1038/icb.2014.89.

Chae, C., 2012. Commercial porcine circovirus type 2 vaccines: efficacy and clinical application. J. Vet. J. 194, 151–157. https://doi.org/10.1016/j.tvjl.2012.06.031.

Yu, C., Cao, M., Wei, Y., Liu, J., Zhang, H., Liu, C., Feng, L., Huang, L., 2023. Evaluation of cross-immunity among major porcine circovirus type 2 genotypes by infection with PCV2b and PCV2d circulating strains. J. Vet. Microbiol. 283, 109796. https://doi.org/10.1016/j.vetmic.2023.109796.

Gagnon, C.A., Music, N., Fontaine, G., Tremblay, D., Harel, J., 2010. Emergence of a new type of porcine circovirus in swine (PCV): a type 1 and type 2 PCV recombinant. J. Vet. Microbiol. 144, 18–23. https://doi.org/10.1016/j.vetmic.2009.09.072.

Janitzek, C.M., Peabody, J., Thrane, S., H, R.C.P., G, T.T., Salanti, A., Chackerian, B., A, N.M., Sander, A.F., 2019. A proof-of-concept study for the design of a VLP-based combinatorial HPV and placental malaria vaccine. J. Sci. Rep. -Uk 9, 5260. https://doi.org/10.1038/s41598-019-41522-5.

Xiao, C.T., Harmon, K.M., Halbur, P.G., Opriessnig, T., 2016. PCV2d-2 is the predominant type of PCV2 DNA in pig samples collected in the U.S. During 2014-2016. J. Vet. Microbiol. 197, 72–77. https://doi.org/10.1016/j.vetmic.2016.11.009.

Zhang, D., Xia, Q., Wu, J., Liu, D., Wang, X., Niu, Z., 2011. Construction and immunogenicity of DNA vaccines encoding fusion protein of murine complement C3d-p28 and GP5 gene of porcine reproductive and respiratory syndrome virus. J. Vaccin. 29, 629–635. https://doi.org/10.1016/j.vaccine.2010.11.046.

Hemmati, F., Hemmati-Dinarvand, M., Karimzade, M., Rutkowska, D., Eskandari, M.H., Khanizadeh, S., Afsharifar, A., 2022. Plant-derived VLP: a worthy platform to produce vaccine against SARS-CoV-2. J. Biotechnol. Lett. 44, 45–57. https://doi.org/10.1007/s10529-021-03211-0.

Franzo, G., Segales, J., 2020. Porcine circovirus 2 genotypes, immunity and vaccines: multiple genotypes but one single serotype. J. Pathog. 9. https://doi.org/10.3390/pathogens9121049.

Shen, G., Jin, N., Ma, M., Jin, K., Zheng, M., Zhuang, T., Lu, H., Zhu, G., Jin, H., Jin, M., Huo, X., Qin, X., Yin, R., Li, C., Li, H., Li, Y., Han, Z., Chen, Y., Jin, M., 2007. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. J. Vaccin. 25, 4193–4202. https://doi.org/10.1016/j.vaccine.2007.03.010.

Qi, H., Chen, X., Chu, C., Lu, P., Xu, H., Yan, J., 2014. Follicular T-helper cells: controlled localization and cellular interactions. J. Immunol. Cell Biol. 92, 28–33. https://doi.org/10.1038/icb.2013.59.

Maity, H.K., Samanta, K., Deb, R., Gupta, V.K., 2023. Revisiting porcine circovirus infection: recent insights and its significance in the piggery sector. J. Vaccin. -Basel. 11. https://doi.org/10.3390/vaccines11081308.

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. J. Nat. 295, 64–66. https://doi.org/10.1038/295064a0.

Maclennan, I.C., Gray, D., 1986. Antigen-driven selection of virgin and memory B cells. J. Immunol. Rev. 91, 61–85. https://doi.org/10.1111/j.1600-065x.1986.tb01484.x.

Guo, J., Hou, L., Zhou, J., Wang, D., Cui, Y., Feng, X., Liu, J., 2022. Porcine circovirus type 2 vaccines: commercial application and research advances. J. Virus -Basel. 14. https://doi.org/10.3390/v14092005.

Kim, K., Shin, M., Hahn, T.W., 2020. Deletion of a decoy epitope in porcine circovirus 2 (PCV2) capsid protein affects the protective immune response in mice. J. Arch. Virol. 165, 2829–2835. https://doi.org/10.1007/s00705-020-04831-z.

Zhao, K., Duan, X., Hao, L., Wang, X., Wang, Y., 2017. Immune effect of newcastle disease virus DNA vaccine with C3d as a molecular adjuvant. J. J. Microbiol. Biotechn. 27, 2060–2069. https://doi.org/10.4014/jmb.1708.08017.

Fraile, L., Grau-Roma, L., Sarasola, P., Sinovas, N., Nofrarias, M., Lopez-Jimenez, R., Lopez-Soria, S., Sibila, M., Segales, J., 2012. Inactivated PCV2 one shot vaccine applied in 3-week-old piglets: improvement of production parameters and interaction with maternally derived immunity. J. Vaccin. 30, 1986–1992. https://doi.org/10.1016/j.vaccine.2012.01.008.

Guo, L., Fu, Y., Wang, Y., Lu, Y., Wei, Y., Tang, Q., Fan, P., Liu, J., Zhang, L., Zhang, F., Huang, L., Liu, D., Li, S., Wu, H., Liu, C., 2012. A porcine circovirus type 2 (PCV2) mutant with 234 amino acids in capsid protein showed more virulence in vivo, compared with classical PCV2a/b strain. J. Plos One 7, e41463. https://doi.org/10.1371/journal.pone.0041463.

Fort, M., Olvera, A., Sibila, M., Segales, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. J. Vet. Microbiol. 125, 244–255. https://doi.org/10.1016/j.vetmic.2007.06.004.

Sun, M., Wang, S., Fang, Z., Zhao, M., Gao, Y., An, T., Tu, Y., Wang, H., Cai, X., 2022. A Sandwich ELISA for quality control of PCV2 virus-like particles vaccine. J. Vaccin. -Basel. 10. https://doi.org/10.3390/vaccines10122175.

Lee, M.J., Kim, H.M., Shin, S., Jo, H., Park, S.H., Kim, S.M., Park, J.H., 2022. The C3d-fused foot-and-mouth disease vaccine platform overcomes maternally-derived antibody interference by inducing a potent adaptive immunity. J. Npj Vaccin. 7, 70. https://doi.org/10.1038/s41541-022-00496-8.

Bale, S., Yang, L., Alirezaei, M., Wilson, R., Ota, T., Doyle, E.D., Cottrell, C.A., Guenaga, J., Tran, K., Li, W., Stamatatos, L., Nemazee, D., Ward, A.B., Wyatt, R.T., 2023. Fusion of the molecular adjuvant C3d to cleavage-independent native-like HIV-1 Env trimers improves the elicited antibody response. J. Front Immunol. 14, 1180959. https://doi.org/10.3389/fimmu.2023.1180959.

Hirschberg, S., Ghazaani, F., Ben, A.G., Pydde, M., Nagel, A., Germani, S., Monica, L., Schlor, A., Bauer, H., Hornung, J., Voetz, M., Dwai, Y., Scheer, B., Ringel, F., Kamal-Eddin, O., Harms, C., Funer, J., Adrian, L., Pruss, A., Schulze-Forster, K., Hanack, K., Kamhieh-Milz, J., 2023. An efficient and scalable method for the production of immunogenic SARS-CoV-2 virus-like particles (VLP) from a mammalian suspension cell line. J. Vaccin. -Basel. 11. https://doi.org/10.3390/vaccines11091469.

Li, S., Zhao, R., Song, H., Pan, S., Zhang, Y., Dong, H., Bai, M., Sun, S., Guo, H., Yin, S., 2023. Local and systemic immune responses induced by intranasal immunization with biomineralized foot-and-mouth disease virus-like particles. J. Front Microbiol. 14, 1112641. https://doi.org/10.3389/fmicb.2023.1112641.

Serrano-Villar, S., Deeks, S.G., 2015. CD4/CD8 ratio: an emerging biomarker for HIV. J. Lancet Hiv. 2, e76–e77. https://doi.org/10.1016/S2352-3018(15)00018-1.

Kang, S.J., Kang, H., You, S.H., Lee, H.J., Lee, N., Hyun, B.H., Cha, S.H., 2020. Genetic diversity and different cross-neutralization capability of porcine circovirus type 2 isolates recently circulating in South Korea. J. Bmc Vet. Res. 16, 334. https://doi.org/10.1186/s12917-020-02549-3.

Opriessnig, T., Xiao, C.T., Gerber, P.F., Halbur, P.G., Matzinger, S.R., Meng, X.J., 2014. Mutant USA strain of porcine circovirus type 2 (mPCV2) exhibits similar virulence to the classical PCV2a and PCV2b strains in caesarean-derived, colostrum-deprived pigs. J. J. Gen. Virol. 95, 2495–2503. https://doi.org/10.1099/vir.0.066423-0.

Gong, W., Du, H., Wang, T., Sun, H., Peng, P., Qin, S., Geng, H., Zeng, Z., Liang, W., Ling, H., Tu, C., Tu, Z., 2023. Epizootiological surveillance of porcine circoviruses in free-ranging wild boars in China. J. Virol. Sin. 38, 663–670. https://doi.org/10.1016/j.virs.2023.08.008.

Chang, X., Krenger, P., Krueger, C.C., Zha, L., Han, J., Yermanos, A., Roongta, S., Mohsen, M.O., Oxenius, A., Vogel, M., Bachmann, M.F., 2021. TLR7 signaling shapes and maintains antibody diversity upon virus-like particle immunization. J. Front Immunol. 12, 827256. https://doi.org/10.3389/fimmu.2021.827256.

Mo, X., Li, X., Yin, B., Deng, J., Tian, K., Yuan, A., 2019. Structural roles of PCV2 capsid protein N-terminus in PCV2 particle assembly and identification of PCV2 type-specific neutralizing epitope. J. Plos Pathog. 15, e1007562. https://doi.org/10.1371/journal.ppat.1007562.

Liu, Y., Gong, Q.L., Nie, L.B., Wang, Q., Ge, G.Y., Li, D.L., Ma, B.Y., Sheng, C.Y., Su, N., Zong, Y., Li, J.M., Shi, K., Leng, X., Du, R., 2020. Prevalence of porcine circovirus 2 throughout China in 2015-2019: a systematic review and meta-analysis. J. Microb. Pathog. 149, 104490. https://doi.org/10.1016/j.micpath.2020.104490.

Oh, Y., Seo, H.W., Park, C., Chae, C., 2014. Comparison of sow and/or piglet vaccination of 3 commercial porcine circovirus type 2 (PCV2) single-dose vaccines on pigs under experimental PCV2 challenge. J. Vet. Microbiol. 172, 371–380. https://doi.org/10.1016/j.vetmic.2014.05.028.

Hou, Z., Wang, H., Feng, Y., Li, Q., Li, J., 2019. A candidate DNA vaccine encoding a fusion protein of porcine complement C3d-P28 and ORF2 of porcine circovirus type 2 induces cross-protective immunity against PCV2b and PCV2d in pigs. J. Virol. J. 16, 57. https://doi.org/10.1186/s12985-019-1156-2.

Ma, Z., Liu, M., Liu, Z., Meng, F., Wang, H., Cao, L., Li, Y., Jiao, Q., Han, Z., Liu, S., 2021. Epidemiological investigation of porcine circovirus type 2 and its coinfection rate in Shandong province in China from 2015 to 2018. J. Bmc Vet. Res. 17, 17. https://doi.org/10.1186/s12917-020-02718-4.