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Expression and evaluation of porcine circovirus type 2 capsid protein mediated by recombinant adeno-associated virus 8

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ABSTRACT

Background: Porcine circovirus type 2 (PCV2) is an important infectious pathogen implicated in porcine circovirus-associated diseases (PCVAD), which has caused significant economic losses in the pig industry worldwide.

Objectives: A suitable viral vector-mediated gene transfer platform for the expression of the capsid protein (Cap) is an attractive strategy.

Methods: In the present study, a recombinant adeno-associated virus 8 (rAAV8) vector was constructed to encode Cap (Cap-rAAV) *in vitro* and *in vivo* after gene transfer.


Results: The obtained results showed that Cap could be expressed in HEK293T cells and BALB/c mice. The results of lymphocytes proliferative, as well as immunoglobulin G (IgG) 2a and interferon- γ showed strong cellular immune responses induced by Cap-rAAV. The enzyme-linked immunosorbent assay titers obtained and the IgG1 and interleukin-4 levels showed that humoral immune responses were also induced by Cap-rAAV. Altogether, these results demonstrated that the rAAV8 vaccine Cap-rAAV can induce strong cellular and humoral immune responses, indicating a potential rAAV8 vaccine against PCV2.

Conclusions: The injection of rAAV8 encoding PCV2 Cap genes into muscle tissue can ensure long-term, continuous, and systemic expression.

Keywords: Porcine circovirus type 2; recombinant adeno-associated virus 8; capsid protein

INTRODUCTION

Porcine circovirus type 2 (PCV2) is an important infectious pathogen that causes post-weaning pigs multisystemic wasting syndrome, porcine respiratory disease complex, porcine circovirus-associated diseases (PCVAD) [1-3]. PCV2 was first reported in Canada in the 1990s; currently, it is causing significant losses in the pig industry worldwide [4]. At present, no effective drugs are available to control and eliminate PCV2 infection. Vaccination against PCV2 is one of the efficacious strategies that can be employed to protect growing pigs against clinical disease manifestations associated with PCV2 infection, commonly referred to as PCVAD [5-8].

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Li S, Shan Y; Data curation: Li S; Formal analysis: Li S, Qiao Y, Nie J; Funding acquisition: Shan Y; Project administration: Li S, Lan X, Yin Y; Validation: Li S, Wang B, Jiang S; Writing - original draft: Li S; Writing - review & editing: Shan Y, Kong W, Shi Y.

PCV2 is a member of the genus *Circovirus* in the family *Circoviridae*; it has a circular single-stranded DNA genome approximately 1.7 kb in length [9,10]. The viral genome (vg) contains a circular single-strand DNA molecule 1766–1777 nucleotides (nt) in length and encodes at least 11 overlapping open reading frames (ORFs) [11,12]. Among these identified ORFs, ORF2 (702–717 nt) encodes the only virus structural capsid protein (Cap), which consists of 233–236 aa (27.8 kD) [13]. Cap is responsible for imparting the immunogenicity and virulence of PCV2, and is the primary viral immunogenic protein; it plays a crucial role in the process of viral pathogenesis and replication in the host cell [9,14]. Cap is mainly protective antigen of PCV2, and serves as the preferred protein for the clinical development of vaccines used to track PCV2-specific immune responses [15]. However, current PCV2 commercial vaccines could not completely prevent and control PCV2 infection [16]. Therefore, it is still necessary to enhance PCV2 vaccine immune response and efficacy consequently.

Adeno-associated viruses (AAVs) are small nonenveloped viruses that have been widely used as target gene transfer vectors in various tissues and extensively tested in various gene therapy applications approved by the U.S. Food and Drug Administration [17-19]. The AAVs contains a single-stranded DNA genome including 2 viral genes: *cap* and *rep* [20]. The genes can be removed or replaced with a cassette expressing a therapeutic transgene along with the necessary *cap* and *rep* genes [21]. Recombinant adeno-associated virus 8 (rAAV8) has inverted terminal repeats (ITRs) while the *rep* and *cap* genes have been removed. The rAAV8 vectors have advantages over other gene-delivery platforms, such as higher titers and sustainable high-efficiency gene transfer [22]. The rAAV8 vectors possess high affinity toward skeletal muscles; hence, the muscle can be taken as a depot to synthesize the target gene and passively distribute it to the circulatory system [20].

In this study, we aimed to construct a rAAV8 vector that expressed Cap and check its efficacy in providing long-term immunity against PCV2. We additionally investigated the expression of Cap *in vitro* in HEK293T cells and then administered to BALB/c mice with intramuscular injections *in vivo*. Subsequently, enzyme-linked immunosorbent assay (ELISA), antibody isotyping assay, cytokines release assay, lymphocytes proliferation assay were conducted to detect antibody titers, immunoglobulin G (IgG) 1 and IgG2a antibodies levels, cytokines levels, lymphocytes proliferation levels elicited by the fabricated rAAV8.

MATERIALS AND METHODS

Cells and animals

HEK293T cells were obtained from the American Type Culture Collection (USA) and grown adherently in Dulbecco's Modified Eagle's Medium (Gibco, USA) containing 10% fetal bovine serum (Gibco) in a T-flask at 37°C in an atmosphere of 5% CO₂. BALB/c mice (female, 8 weeks old, body weight: 18–20 g) were procured from the Changchun Institute of Biological Products Co., Ltd. (China). The animal trials in this study were carried out in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China (11-14-1988). All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University (permit number: SCXK 2013-0001).

Table 1. Primers for polymerase chain reaction amplification used in this study

Segment	Primer	Sequence (from 5' to 3')
Cap-rAAV	Cap-rAAV-F	<u>GGGGATCC</u> ATGACGTATCCAAGGAGGCG
	Cap-rAAV-R	AAGGAAAAAAG <u>CGGCCG</u> CTCAGTGGTGGTGGTGGT
Cap-pSec	Cap-pSec-F	<u>CGCGGATCC</u> AATGACGTATCCAAGGAGGCG
	Cap-pSec-R	<u>CCGCTCGAG</u> TTCAGTGGTGGTGGTGGT
CAG	CAG-F	TCATATGCCAAGTACGCCCC
	CAG-R	AGAGTGAAGCAGAACGTGGG

The restriction sites are underlined.

Cap, capsid protein; rAAV, recombinant adeno-associated virus.

Plasmid constructions

The rAAV8 vector, synthesized by Vigene Biosciences Inc. (China), was used as an expression plasmid containing ITRs and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) with the *Kpn* I and *Not* I restriction enzyme sites [20]. According to the genome information of porcine circovirus 2 strain (GenBank accession number KR559724.1), the full-length Cap protein gene of PCV2 and 6-His tag were linked via GGGGS linkers and were constructed into the rAAV8 vector driven by the CAG promoter, termed Cap-rAAV. The same sequences were constructed into pSecTag2 A vector (stored in the laboratory), termed Cap-pSec. The sequences for red fluorescent protein (RFP) gene (GenBank accession number KT452623.1) were constructed into the rAAV8 vector driven by the CAG promoter as a control, termed RFP-rAAV. The primers employed are listed in **Table 1**.

rAAV8 production from HEK293T cells

The rAAV8 vectors were produced as previously described [20]. Briefly, AAV8 vectors were generated by linear polyethylenimine (Sigma, USA)-mediated cotransfection of HEK293T cells with 3 plasmids: an expression plasmid, a helper plasmid encoding the adenovirus helper functions, and a packaging plasmid containing the *rep* and *cap* genes of rAAV8 [20]. The mass ratio of plasmid and transfection reagent is 1:4. The transfected cells and cell culture media were harvested after cotransfection for 72 h. The rAAV8 particles were prepared from the culture supernatant. The culture supernatant added with 10% trichloromethane was shaken at 220 rpm and 37°C for 2 h. The samples were collected by centrifugation at 12,000 × g and 4°C for 30 min after added with sodium chloride (1 mol/L). The culture supernatants were collected, followed by the addition of 10% (w/v) PEG8000, and kept on an ice bath for 2 h. The precipitate was collected and dissolved in 400 µL phosphate-buffered saline (PBS) and then digested by RNase (1 µg/mL) and DNase (1 µg/mL) for 45 min after centrifugation. Subsequently, 50% trichloromethane was added to the culture supernatants, followed by centrifugation at 12,000 × g and 4°C for 10 min. The purified rAAV8 supernatant was stored at -80°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transmission electron microscope (TEM) analysis

The purity of rAAV8 was analyzed by SDS-PAGE using a 12.0% polyacrylamide gel. The samples mixed with 4 × SDS loading buffer were boiled at 100°C for 6 min. Coomassie brilliant blue dye was used to stain the protein. The VP1, VP2, and VP3 Cap bands were analyzed for their molecular weights; the respective molecular weights were predicted by Gene Runner software.

A TEM (JEOL-2000EX; JEOL, Japan) was used to analyze the morphologies of rAAV8. The samples were dropped onto 200-mesh carbon-coated copper grids and stained with 2% (w/v) potassium phosphotungstate, as previously described. The internal morphologies of rAAV8 were then investigated by TEM for 1 min at an acceleration voltage of 100 kV. Images were captured using a CCD camera system.

Purified rAAV8 quantification

Purified rAAV8 was quantified by quantitative polymerase chain reaction (qPCR), which was carried out in the BIO-RAD CFX96 Real-Time System according to the manufacturer's instructions using TransStart Top Green qPCR SuperMix (TransGen Biotech, China). The primers designed against the CAG promoter are listed in **Table 1**. Samples were run in triplicate under the following cycling conditions: 94°C for 30 sec, followed by 42 cycles of 94°C for 5 sec, 60°C for 30 sec and 72°C for 10 sec. The virus titer defined as vg was determined using a standard curve produced by serial dilution of the purified DNA plasmid.

Western blot

After purification of rAAV8, 100 µL Cap-rAAV, RFP-rAAV, and Cap-pSec was added to each well of a 6-well plate to find out whether the purified rAAV8 could infect HEK293T cells. By 72 h post-infection, the supernatant of each well was collected. The expressed protein in HEK293T cells was analyzed under reducing conditions by western blot. The expressed proteins were separated on 12.0% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad, USA), which were blocked with 3% skim milk in PBS at room temperature for 1.5 h and probed with an anti-His tag monoclonal antibody (Invitrogen, USA). Subsequently, the membranes were probed with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Beijing Dingguo Inc., China), and then the immunoreaction was detected using a BeyoECL Moon kit (Beyotime Biotechnology, China). The fluorescence of RFP expressed in HEK293T cells was observed using a fluorescent microscope.

Animal immunization

After a 7-day acclimatization period, specific pathogen-free BALB/c female mice were randomly divided into the following 5 groups (n = 8 mice per group): group 1, controls treated with intramuscular injections of PBS; group 2, immunized intramuscularly with commercial inactivated vaccine (YZ-strain; Yangzhou Uni-bio Pharmaceutical Co. Ltd., China) and booster immunized on 28 days post immunization (dpi); group 3, immunized intramuscularly with Cap-rAAV (1×10^{11} vg/mouse) with a single injection; group 4, immunized intramuscularly with RFP-rAAV (1×10^{11} vg/mouse) with a single injection; group 5, immunized intramuscularly with Cap-pSec (100 ng/mouse) and booster immunized on 28 dpi. Serum samples were collected at 2-week intervals and heat-inactivated at 56°C for 30 min prior to storage at -80°C until analysis.

ELISA and antibody isotyping assay

The serum titer was analyzed by ELISA against Cap. Ninety-six well plates were coated with Cap in PBS at 5 µg/mL and then incubated overnight at 4°C. The plates were washed 5 times with PBS containing 0.25% Tween-20 (PBST) and blocked with 5% bovine serum albumin (BSA)/PBS at 37°C for 2 h. Serum samples from individual animals immunized with immunogens were diluted serially with PBS, added to the wells and incubated at 37°C for 2 h. Then the plates were washed 5 times, and HRP-conjugated anti-mouse antibodies (Beijing Dingguo Inc.) were added to the plates. After an incubation at 37°C for 45 min, each well was finally washed with PBST. To visualize the reactions, 3,3',5,5'-tetramethylbenzidine solution was added to the wells, and color development was stopped with H₂SO₄ (2 mol/L) after 15 min. The absorbance of plates was detected at a wavelength of 450 nm using an iMarK™ Microplate Reader. Each sample was tested in triplicate. The reciprocal of the highest serum dilution that yielded an absorbance 2.1-fold over the background value was considered the ELISA end-point titer.

For the antibody isotyping assay, Cap (5 µg/mL) was used to coat ninety-six well plates at 4°C overnight and the plates were blocked with 4% BSA/PBS at 37°C for 2 h. Serum samples were diluted in blocking buffer and incubated at 37°C for 1.5 h. Anti-mouse IgG1 and IgG2a (Sigma) was added to each well incubated at room temperature for 2 h. Anti-sheep IgG-alkaline phosphatase (AP) (Beijing Dingguo Inc.) was added to each well as the secondary antibody at room temperature for 1 h. P-nitrophenyl phosphate substrate diluted with AP buffer at 1 mg/mL was added to each well at room temperature for 45 min. The reaction was terminated with NaOH (0.2 mol/L) and the absorbance in each well was measured at 405 nm using an iMarK™ Microplate Reader. Each sample was tested in triplicate.

Cytokine-release assay

Serum interleukin (IL)-4 and interferon (IFN)-γ levels were detected using a mouse T-helper (Th) 1/Th2 ELISA ready-set-go kit (eBioscience, USA) according to the manufacturer's protocols. The concentrations of IL-4 and IFN-γ were calculated using the standard curves.

Lymphocytes proliferation assay

Lymphocytes were isolated per group in mouse lymphocyte separation medium (Solarbio, China), resuspended at a concentration of 2×10^6 cells/mL in RPMI-1640 medium containing 10% FBS. Lymphocytes were seeded into 96 well plates and stimulated for 48 h with Cap (20 mg/mL) as the stimulant, Concanavalin A (Sigma; 5 mg/mL) as the positive control, and RPMI-1640 as the negative control. Thereafter, MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrasodium bromide tetrazolium; Sigma; 5 mg/mL in PBS) was added to the plates, following a further incubation for 5 h. Dimethyl sulfoxide was added to stop the reactions after 5 h. The stimulation index was measured as the ratio of the average optical density at a wavelength of 570 nm Cap-stimulated wells to that of unstimulated wells.

Statistical analysis

All data are presented as averages \pm standard deviations of the means. Statistical analysis was conducted using GraphPad Prism (v7.0; GraphPad Software Inc., USA). Differences between tested samples were evaluated using analysis of variance test. A *p* value < 0.05 was considered statistically significant.

RESULTS

Construction of rAAV8 for Cap expression

The rAAV8 expression plasmid containing WPRE and the 145-bp AAV2 ITRs was constructed. The CAG promoter in the expression vector of rAAV8 was used to drive and enhance the expression of PCV2 Cap. The PCV2 Cap gene sequences were inserted into the rAAV8 expression vector by *Kpn* I and *Not* I restriction enzyme sites. Schematics of the rAAV8 expression plasmids are shown in **Fig. 1A**.

The constructed rAAV8 expression vectors were digested with *Kpn* I and *Not* I restriction enzyme sites. Two specific bands (0.73 kb and 4.29 kb) were detected using a 1% agarose gel electrophoresis. The molecular weights were completely consistent with theoretical value (**Fig. 1B**), and the sequencing results of the constructed plasmids matched the sequence of PCV2 Cap.

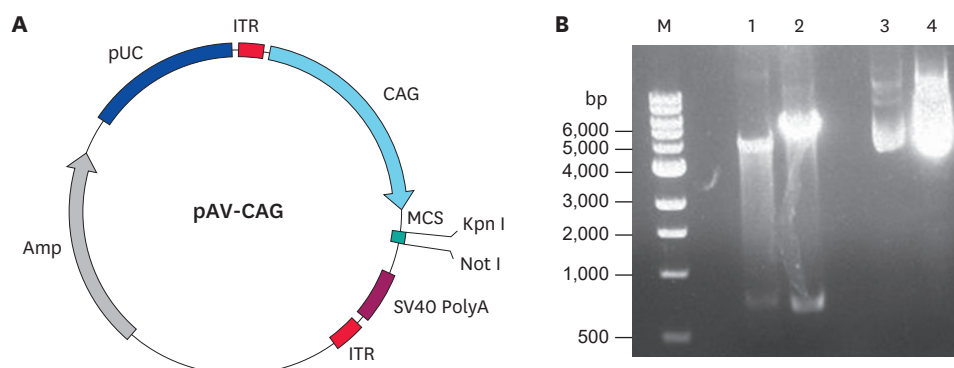


Fig. 1. Construction of Cap-rAAV and Cap-pSec. (A) Schematic illustration of rAAV8. The rAAV8 vector has the basic configuration under the control of CMV, including the AAV-related ITRs and SV40 PolyA, etc. (B) Agarose gel electrophoresis analyses of Cap-rAAV and Cap-pSec. Lane 1: Cap-rAAV digested by *Kpn* I, *Not* I; lane 2: Cap-pSec digested by *Bam*H I, *Xho* I; lane 3: Cap-rAAV plasmid; lane 4: Cap-pSec plasmid. Cap, capsid protein; rAAV, recombinant adeno-associated virus; CMV, cytomegalovirus; AAV, adeno-associated virus; ITR, inverted terminal repeat.

Purity analysis of rAAV8 and western blot analysis of expressed protein

The purified rAAV8 was assessed via SDS-PAGE using a 12.0% polyacrylamide gel. Three bands corresponding to the VP1 (up to 87 kD), VP2 (up to 72 kD), and VP3 (up to 62 kD) Caps of rAAV8 were obtained (**Fig. 2A**); additionally, no impure protein was detected in the lanes. The morphological analysis showed that rAAV8 was packaged successfully. Both Cap-rAAV8 and RFP-rAAV8 showed icosahedron morphology, with a uniform size distribution, according to TEM (**Fig. 2B and C**). They also showed mean diameters of approximately 20 nm, consistent with the theoretical results.

Proteins in the culture supernatants of the infected HEK293T cells were detected using western blot assay. The protein bands of Cap expressed by Cap-rAAV and Cap-pSec appeared at the molecular weight of up to 26.7 kD; this was consistent with the theoretical values (**Fig. 2D**). No bands appeared in the culture supernatants of the RFP-rAAV8 sample. Furthermore, the results of western blot assay revealed that the expressed Cap contained the His tag.

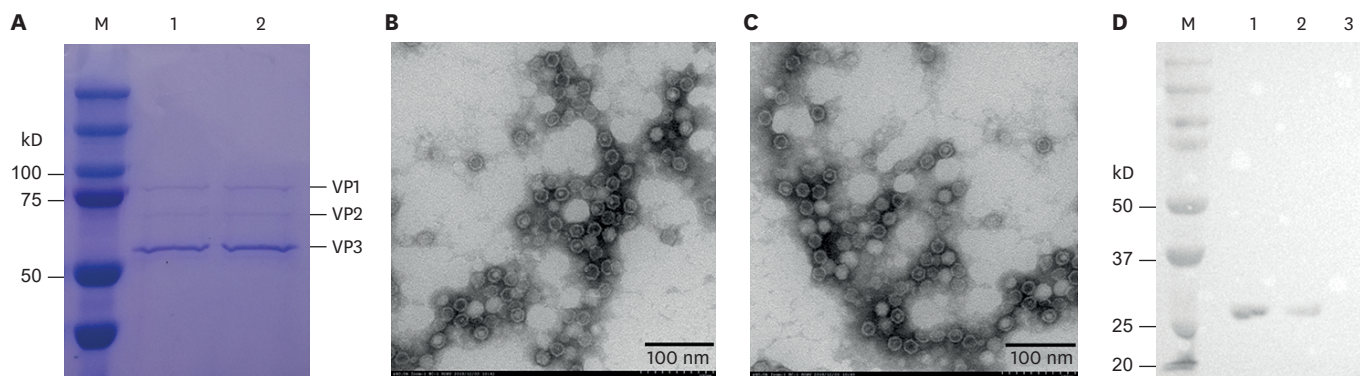


Fig. 2. Characterization of purified rAAV8 and western blot analysis of expressed protein. (A) Analysis of rAAV8 by SDS-PAGE showing the molecular weights of VP1, VP2 and VP3. Lane 1: Cap-rAAV; lane 2: RFP-rAAV. Morphological analysis by TEM of Cap-rAAV (B), RFP-rAAV (C). (D) Proteins expressed by Cap-rAAV (lane 1), Cap-pSec (lane 2), RFP-rAAV (lane 3) in HEK293T cells *in vitro*. rAAV, recombinant adeno-associated virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEM, transmission electron microscope; Cap, capsid protein; RFP, red fluorescent protein.

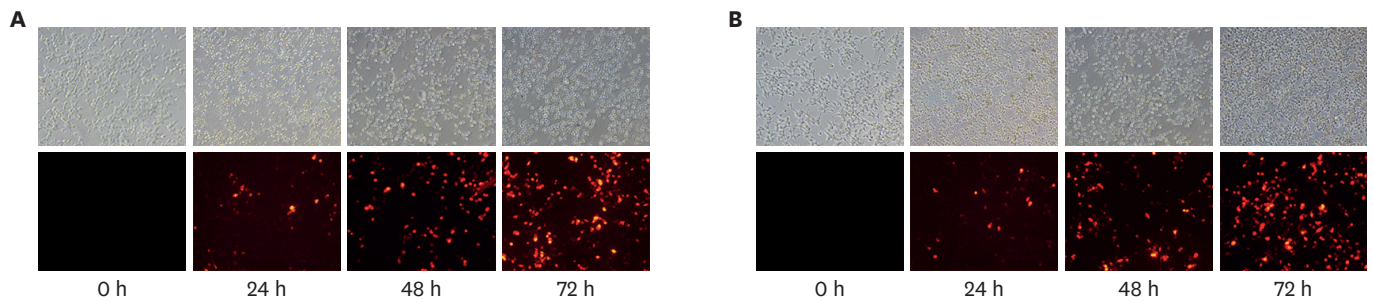


Fig. 3. Fluorescence imaging analysis of RFP expression. (A) Transfection process of RFP-rAAV plasmid in HEK293T cells. The RFP expression was detected by fluorescence imaging at 0, 24, 48, 72 h. The first row of pictures were taken in brightfield conditions. (B) Infection process of HEK293T cells by RFP-rAAV detected at 0, 24, 48, 72 h under a fluorescent microscope. The first row of pictures were taken in brightfield conditions. RFP, red fluorescent protein; rAAV, recombinant adeno-associated virus.

RFP expression *in vitro*

RFP-rAAV, Cap-rAAV, and Cap-pSec were transfected simultaneously to detect transfection efficiency, and RFP-rAAV was used as a positive control. The transfected HEK293T showed apparent RFP expression at predetermined intervals (0, 24, 48, and 72 h) using a fluorescence imaging analysis. RFP expression showed a gradually increase over time, which showed that plasmids were successfully transfected into HEK293T cells (**Fig. 3A**).

The infectivity of RFP-rAAV was also detected at predetermined intervals (0, 24, 48, and 72 h) using a fluorescence imaging analysis. RFP expression also showed a gradual increase over time, which indicated the purified rAAV8 still reserved the ability to infect HEK293T cells (**Fig. 3B**).

Cap-specific antibody titers in immunized mice

Five groups of BALB/c mice were immunized intramuscularly with PBS, commercial vaccine, Cap-rAAV, RFP-rAAV, and Cap-pSec, respectively. PCV2-specific antibody titers of antisera were measured by ELISA. Commercial vaccine elicited the highest antibody response at 14–56 days, with a peak IgG titer of $8,605.24 \pm 411.41$ (**Fig. 4A**). Statistically, the Cap-specific IgG titer in the Cap-rAAV group was significantly higher than that in the PBS group at 28–56 days ($p < 0.001$); furthermore, IgG titers in the Cap-pSec group were significantly higher than those in the control group at 42–56 days ($p < 0.001$). These data indicated that the administration of Cap-rAAV, and Cap-pSec elicits the Cap-specific antibody responses, and that Cap-rAAV elicits strong antibody responses that closely resemble the responses elicited by commercial vaccines.

For further evaluation of the expressing protein *in vivo*, sera samples from BALB/c mice were analyzed using the same methodology by western blot. The protein bands of Cap from the sera samples of group 2, 3, 5 appeared at the molecular weight of up to 26.7 kD (**Fig. 4B**). No bands were detected from the sera samples of group 4.

Subsequently, we analyzed the IgG1/IgG2a isotypes against Cap protein to confirm the state of antibody responses and to determine the main type of immune response elicited by the immunogen. Cap-rAAV stimulated significantly higher IgG2a antibody response in comparison to the IgG1 response from 42 days to 56 days ($p < 0.05$). However, the IgG2a and IgG1 antibody responses elicited by Cap-pSec, RFP-rAAV, and PBS did not demonstrate any significant differences (**Fig. 4C–G**). The IgG2a:IgG1 ratio in response to Cap-rAAV and Cap-pSec at 56 days was 1.18 ± 0.01 and 1.13 ± 0.08 , respectively. The results demonstrated that Cap-rAAV elicited a stronger IgG2a response than Cap-pSec.

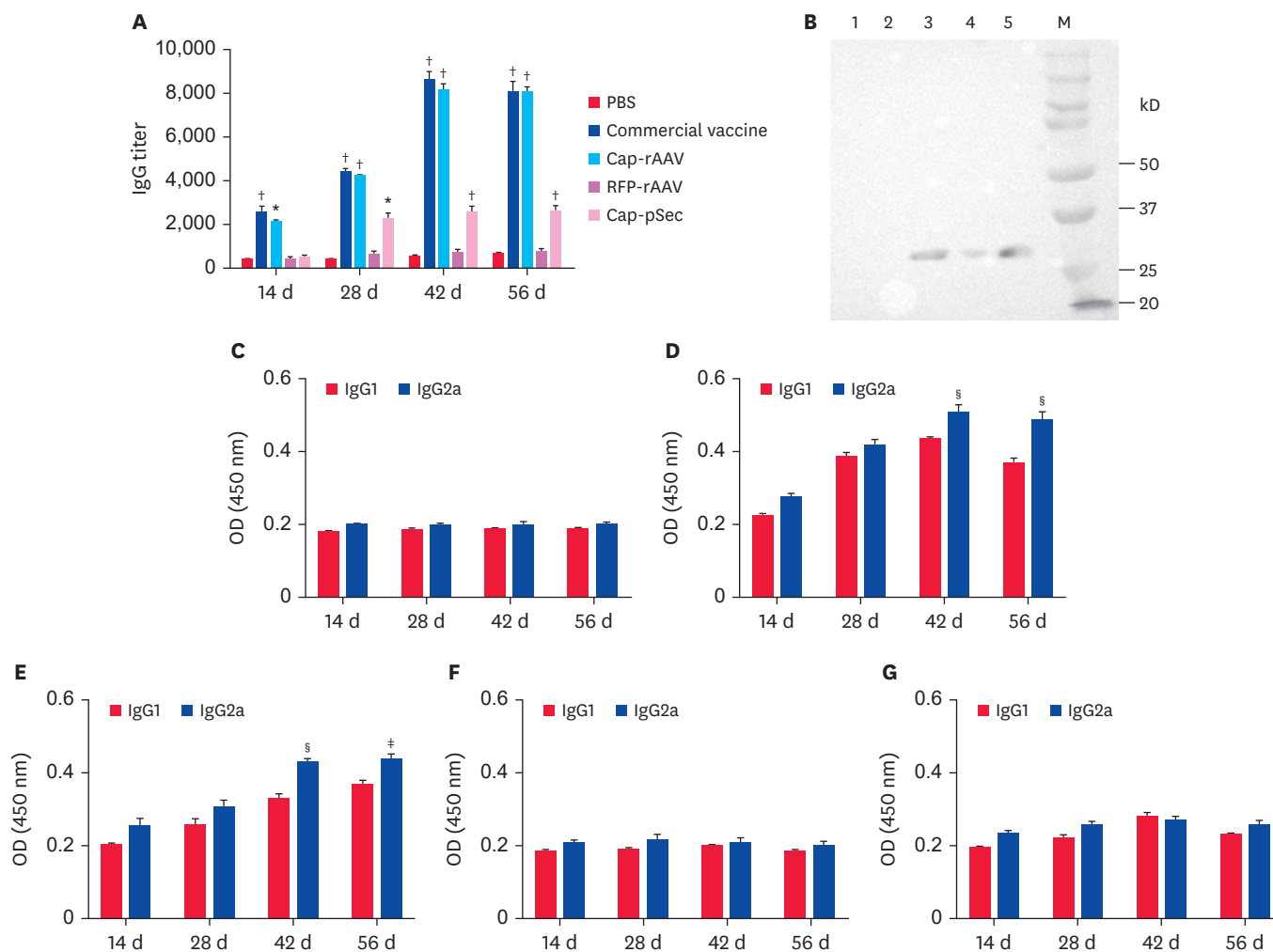


Fig. 4. Antisera IgG titers and antibody isotyping assay. (A) Detection of antisera IgG titers induced by PBS, commercial vaccine, Cap-rAAV, RFP-rAAV, Cap-pSec by ELISA. (B) Proteins expressed by PBS (lane 1), RFP-rAAV (lane 2), Cap-rAAV (lane 3), Cap-pSec (lane 4), commercial vaccine (lane 5) in mice *in vivo*. Antibody isotyping assay of IgG1/IgG2a isotypes elicited by PBS (C), commercial vaccine (D), Cap-rAAV (E), RFP-rAAV (F), Cap-pSec (G). IgG, immunoglobulin G; PBS, fetal bovine serum; Cap, capsid protein; rAAV, recombinant adeno-associated virus; ELISA, enzyme-linked immunosorbent assay; RFP, red fluorescent protein.

* $p < 0.01$, $^{\dagger}p < 0.001$ compared to sera of PBS group. $^{\ddagger}p < 0.05$, $^{\S}p < 0.01$ showed significantly difference between IgG2a antibody and IgG1 in each group.

Cytokine detection and lymphocyte proliferation response

IL-4 and IFN- γ secretion levels were measured to evaluate immune responses. Commercial vaccine, Cap-rAAV, and Cap-pSec elicited significantly increased levels of IL-4 than the levels measured in the control group ($p < 0.001$) (Fig. 5A). Identically, significantly higher levels of IFN- γ were observed in the commercial vaccine, Cap-rAAV, and Cap-pSec groups compared to the levels observed in the control group ($p < 0.001$) (Fig. 5B).

To evaluate lymphocyte proliferation activity in the mice of experimental groups, lymphocyte proliferation assays were detected at 28, 42, and 56 days. Immunization with commercial vaccine and Cap-rAAV demonstrated a significantly increased splenocyte proliferative response than that elicited by PBS ($p < 0.001$) (Fig. 5C). Simultaneously, Cap-pSec elicited higher lymphocyte proliferation level than that elicited by PBS ($p < 0.01$). The data indicated that both Cap-rAAV and Cap-pSec can elicit a cap-specific splenocyte proliferative response. Nevertheless, Cap-rAAV has a stronger effect on spleen lymphocyte proliferation activity.

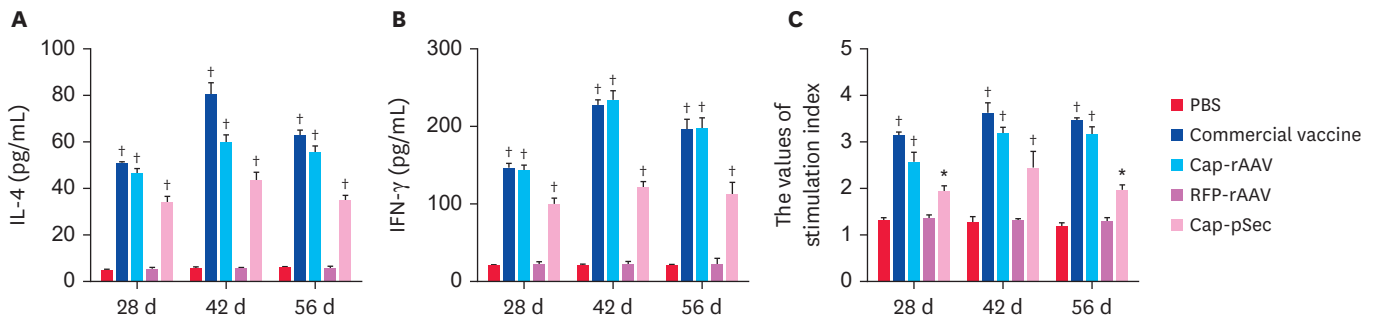


Fig. 5. IL-4, IFN- γ and lymphocyte proliferation levels of antisera. Detection of IL-4 (A) and IFN- γ (B) levels of antisera elicited by PBS, commercial vaccine, Cap-rAAV, RFP-rAAV, Cap-pSec. (C) Lymphocyte proliferation levels of antisera elicited by PBS, commercial vaccine, Cap-rAAV, RFP-rAAV, Cap-pSec. IL, interleukin; IFN, interferon; PBS, fetal bovine serum; Cap, capsid protein; rAAV, recombinant adeno-associated virus; RFP, red fluorescent protein. * $p < 0.01$, † $p < 0.001$ compared to sera of PBS group.

DISCUSSION

PCV2 is the main infectious pathogen of PCVAD, causing significant economic losses in the pig industry worldwide. Cap protein is a major target for anti-PCV2 vaccination, since it is responsible for imparting immunogenicity and virulence. Consequently, the rAAV8 vector was used for gene delivery to achieve sustainable high-efficiency gene transfer in the present study. The rAAV8 vector possesses high affinity toward skeletal muscles; consequently, the muscle can be used as a depot for synthesizing the target gene and secreting it into the blood circulation passively.

Cap protein expression using the rAAV8 vector was achieved *in vitro* and *in vivo* successfully. These data showed that, following the intramuscular immunization of rAAV8 vectors carrying the Cap genes, the mice muscle cells express bioactive Cap protein, which is distributed into the blood circulatory system. Peak Cap serum IgG titer levels elicited by Cap-rAAV were as high as $8,104.07 \pm 346.93$, suggesting higher humoral immune response than that elicited by the Cap-pSec DNA vaccine group ($2,551.37 \pm 307.01$). The immune response elicited by Cap-rAAV showed a continuous trend compared with that generated by traditional vaccination [6].

We evaluated the IgG1/IgG2a isotypes against PCV2 Cap protein to confirm the main type of immune response elicited by various immunogens. IgG2a antibody is generated as a result of Th1-cell activation in mice, while Th2-cell activation increases the generation of IgG1 and inhibits that of IgG2a [23]. Generally, immunized mice developed higher IgG2a titers in comparison to IgG1 titers. Cap-rAAV elicited significantly higher levels of IgG2a than IgG1 from 42 days to 56 days, indicating a Th1 immune response in the corresponding period after immunization. The strong Cap-specific Th1 immune response is essential for the protective immunization against PCV2 infection [24]. However, no significant difference between IgG1 and IgG2a demonstrated a non-biased Th response in the DNA vaccine group of Cap-pSec.

The signature cytokines for Th1 and Th2 cells are interferon IFN- γ and interleukin IL-4, respectively [3,25]. Th1 cells mediate the killing of intracellular organisms through IFN- γ production, while Th2 cells mediate IL-4 production. Cap-rAAV and Cap-pSec elicited significantly higher levels of IFN- γ and IL-4 compared to the corresponding levels in the control group, implying the application of both Th responses in immunotherapy against pathogenic infections. The immune response elicited by Cap-rAAV indicated a continuous trend compared with that generated by traditional vaccination [6].

The lymphocyte-proliferative level is an important index of cellular immune response. These data demonstrated that lymphocyte-proliferative levels elicited by Cap-rAAV were significantly higher than those of the control group, thus indicating that the specific T lymphocyte proliferative response was augmented in immunized mice. These results indicated that Cap-rAAV has the potential to improve the continuous cellular immune responses in mice.

According to the above analysis, Cap-rAAV induces a strong Th1-biased immune response through increased T lymphocyte proliferation, IgG2a and IFN- γ production. Cap-rAAV elicits a significantly higher degree of T lymphocyte proliferation, IgG2a/IgG1 ratio, and IFN- γ level, indicating that Cap-rAAV enhances specific immune responses in mice. The commercial inactivated vaccine requires another booster immunization on 28 dpi. In contrast, the AAV vector vaccine was immunized intramuscularly with a single injection, which could reduce the number of administrations and increase the compliance of swine. Due to its heredity, the rAAV8 vector-based vaccines have the potential as alternative systems to protect the offspring pigs from PCV2 infection for the delivery of PCV2 antigens to the host. The long-term, systemic, and continuous expression of Cap protein can be achieved with an intramuscular injection of rAAV8 containing Cap genes into muscle, which would overcome the challenges of traditional vaccination and life-term repetitive passive transfer of such biological agents for therapy.

In conclusion, we investigated the influences of Cap-rAAV on immunogenicity by comparing the Cap-specific humoral and cellular immune responses. Overall, the rAAV8 vector vaccine constructed in the present study induces stronger immune responses than those in the control and Cap-pSec groups, which would render Cap-rAAV an attractive candidate vaccine to prevent and control PCV2 infection. The Cap protein can be continuously produced at a therapeutic level through rAAV8 gene transfer, because viral rebound only occurs after the antibody concentration drops to a low concentration. In addition, AAV gene transfer therapy may protect the offspring pigs from PCV2 infection due to heredity. In addition, further studies should be performed to determine the immunogenicity of Cap-rAAV in pigs.

REFERENCES

1. Zhang Z, Luo Y, Zhang Y, Guo K. Enhanced protective immune response to PCV2 adenovirus vaccine by fusion expression of Cap protein with InvC in pigs. *J Vet Sci.* 2019;20(4):e35.
[PUBMED](#) | [CROSSREF](#)
2. Huang L, Sun Z, Xia D, Wei Y, Sun E, Liu C, et al. Neutralization mechanism of a monoclonal antibody targeting a porcine circovirus type 2 Cap protein conformational epitope. *J Virol.* 2020;94(9):e01836-19.
[PUBMED](#) | [CROSSREF](#)
3. Kim HK, Lyoo KS, Huynh TM, Moon HJ, Nguyen VG, Park BK. Duplex nested reverse transcriptase polymerase chain reaction for simultaneous detection of type 2 porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 from tissue samples. *J Vet Sci.* 2017;18(2):253-256.
[PUBMED](#) | [CROSSREF](#)
4. Cao W, Cao H, Yi X, Zhuang Y. Development of a simple and high-yielding fed-batch process for the production of porcine circovirus type 2 virus-like particle subunit vaccine. *AMB Express.* 2019;9(1):164.
[PUBMED](#) | [CROSSREF](#)
5. Karuppanan AK, Opriessnig T. Porcine circovirus type 2 (PCV2) vaccines in the context of current molecular epidemiology. *Viruses.* 2017;9(5):99.
[PUBMED](#) | [CROSSREF](#)
6. Segalés J. Best practice and future challenges for vaccination against porcine circovirus type 2. *Expert Rev Vaccines.* 2015;14(3):473-487.
[PUBMED](#) | [CROSSREF](#)

7. Wang QH, Kuang N, Hu WY, Yin D, Wei YY, Hu TJ. The effect of *Panax notoginseng* saponins on oxidative stress induced by PCV2 infection in immune cells: *in vitro* and *in vivo* studies. *J Vet Sci.* 2020;21(4):e61.
[PUBMED](#) | [CROSSREF](#)
8. Shin MK, Yoon SH, Kim MH, Lyoo YS, Suh SW, Yoo HS. Assessing PCV2 antibodies in field pigs vaccinated with different porcine circovirus 2 vaccines using two commercial ELISA systems. *J Vet Sci.* 2015;16(1):25-29.
[PUBMED](#) | [CROSSREF](#)
9. Huang L, Wei Y, Xia D, Liu D, Zhu H, Wu H, et al. A broad spectrum monoclonal antibody against porcine circovirus type 2 for antigen and antibody detection. *Appl Microbiol Biotechnol.* 2019;103(8):3453-3464.
[PUBMED](#) | [CROSSREF](#)
10. Eclercy J, Larcher T, Andraud M, Renson P, Bernard C, Bigault L, et al. PCV2 co-infection does not impact PRRSV MLV1 safety but enhances virulence of a PRRSV MLV1-like strain in infected SPF pigs. *Vet Microbiol.* 2020;244:108656.
[PUBMED](#) | [CROSSREF](#)
11. Wang X, Lv C, Ji X, Wang B, Qiu L, Yang Z. Ivermectin treatment inhibits the replication of porcine circovirus 2 (PCV2) *in vitro* and mitigates the impact of viral infection in piglets. *Virus Res.* 2019;263:80-86.
[PUBMED](#) | [CROSSREF](#)
12. Wei C, Lin Z, Dai A, Chen H, Ma Y, Li N, et al. Emergence of a novel recombinant porcine circovirus type 2 in China: PCV2c and PCV2d recombinant. *Transbound Emerg Dis.* 2019;66(6):2496-2506.
[PUBMED](#) | [CROSSREF](#)
13. Duan J, Yang D, Chen L, Yu Y, Zhou J, Lu H. Efficient production of porcine circovirus virus-like particles using the nonconventional yeast *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol.* 2019;103(2):833-842.
[PUBMED](#) | [CROSSREF](#)
14. Wang T, Du Q, Wu X, Niu Y, Guan L, Wang Z, et al. Porcine MKRN1 modulates the replication and pathogenesis of porcine circovirus type 2 by inducing capsid protein ubiquitination and degradation. *J Virol.* 2018;92(11):e00100-18.
[PUBMED](#) | [CROSSREF](#)
15. Wang L, Zhao D, Sun B, Yu M, Wang Y, Ru Y, et al. Oral vaccination with the porcine circovirus type 2 (PCV-2) capsid protein expressed by *Lactococcus lactis* induces a specific immune response against PCV-2 in mice. *J Appl Microbiol.* 2020;128(1):74-87.
[PUBMED](#) | [CROSSREF](#)
16. Li D, Du Q, Wu B, Li J, Chang L, Zhao X, et al. Immunogenicity of adenovirus vaccines expressing the PCV2 capsid protein in pigs. *Vaccine.* 2017;35(36):4722-4729.
[PUBMED](#) | [CROSSREF](#)
17. Bryant LM, Christopher DM, Giles AR, Hinderer C, Rodriguez JL, Smith JB, et al. Lessons learned from the clinical development and market authorization of Glybera. *Hum Gene Ther Clin Dev.* 2013;24(2):55-64.
[PUBMED](#) | [CROSSREF](#)
18. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, et al. Single-dose gene-replacement therapy for spinal muscular atrophy. *N Engl J Med.* 2017;377(18):1713-1722.
[PUBMED](#) | [CROSSREF](#)
19. Smalley E. First AAV gene therapy poised for landmark approval. *Nat Biotechnol.* 2017;35(11):998-999.
[PUBMED](#) | [CROSSREF](#)
20. Yu Y, Fu L, Jiang X, Guan S, Kuai Z, Kong W, et al. Expression of HIV-1 broadly neutralizing antibodies mediated by recombinant adeno-associated virus 8 *in vitro* and *in vivo*. *Mol Immunol.* 2016;80:68-77.
[PUBMED](#) | [CROSSREF](#)
21. Giles AR, Govindasamy L, Somanathan S, Wilson JM. Mapping an adeno-associated virus 9-specific neutralizing epitope to develop next-generation gene delivery vectors. *J Virol.* 2018;92(20):e0101148.
[PUBMED](#) | [CROSSREF](#)
22. Penaud-Budloo M, Le Guiner C, Nowrouzi A, Toromanoff A, Chérel Y, Chenuaud P, et al. Adeno-associated virus vector genomes persist as episomal chromatin in primate muscle. *J Virol.* 2008;82(16):7875-7885.
[PUBMED](#) | [CROSSREF](#)
23. Shen HG, Zhou JY, Zhang X, Huang ZY, He JL, Yan Y. Interference of porcine circovirus type 2 ORF2 immunogenicity by ORF1 and ORF3 mixed DNA immunizations in mice. *Virology.* 2009;393(1):104-111.
[PUBMED](#) | [CROSSREF](#)
24. Shen HG, Zhou JY, Huang ZY, Guo JQ, Xing G, He JL, et al. Protective immunity against porcine circovirus 2 by vaccination with ORF2-based DNA and subunit vaccines in mice. *J Gen Virol.* 2008;89(Pt 8):1857-1865.
[PUBMED](#) | [CROSSREF](#)
25. Seder RA, Hill AV. Vaccines against intracellular infections requiring cellular immunity. *Nature.* 2000;406(6797):793-798.
[PUBMED](#) | [CROSSREF](#)