Construction and immunogenicity of recombinant adenovirus expressing the capsid protein of porcine circovirus 2 (PCV2) in mice

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Abstract
Porcine circovirus 2 (PCV2) has been implicated as the etiological agent of some diseases, mainly postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS). The capsid (Cap) protein encoded by the PCV2 ORF2 gene may be an excellent candidate for vaccination. In this study, the Cap gene was amplified by PCR, and cloned into the transfer vector pShuttle-CMV. After co-transformation of PmeI-linearized recombinant plasmid pShuttle-CMV-ORF2 and the backbone vector pAdEasy-1 into Escherichia coli bacteria strain BJ5183, recombinant plasmid containing Cap gene (pAd-ORF2) was obtained and identified with PCR. Upon transfection of PacI-linearized plasmid pAd-ORF2 in 293 cell line, a recombinant adenovirus was obtained and named as rAd-Cap with viral titer of 10^13.0 TCID50/ml. The expression of the Cap protein in the 293 cells infected with rAd-Cap was confirmed with specific antibody to PCV2 by Western blotting and IPMA. Mice were inoculated with 10^8, 10^10 and 10^12 TCID50/mouse of rAd-Cap and boosted 2 weeks later, and they could generate antibody against PCV2 detected by indirect ELISA, Western blot and neutralizing activity assay. It indicated that the rAd-Cap was able to express the capsid of PCV2 and could elicit immune responses against the PCV2 in mice. The recombinant adenovirus might be an attractive candidate vaccine for preventing the disease associated with PCV2 infection.

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1. Introduction
Porcine circoviruses (PCV), members of the family circoviridae, are the smallest viruses replicating autonomously in mammalian cells. The virions are icosahedral, nonenveloped, 17 nm in diameter [1]. Currently, there are two recognized types of PCV, porcine circovirus type 1 (PCV1) and porcine circovirus type 2 (PCV2) [2]. PCV1 is non-pathogenic whereas PCV2 is the virus associated with postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), PRD, PNP and congenital tremors [3–5]. PMWS can cause significant levels of mortality in many herds and serve economic losses to porcine industry [6,7]. The diseases caused by PCV2 are now recognized as major disease problems of economic importance in many pig-producing areas of the world [6,8–10], but no commercial vaccine is currently available.

PCV2 contains a single-stranded circular DNA genome of about 1.76 Kb, having two largest open reading frames (ORFs)[11,12]. The capsid protein (Cap protein), encoded by ORF2 of the viral gene, is the major structural protein of virus and has type-specific epitopes [13,14]. Neutralizing monoclonal antibodies have been shown to react with the Capid protein [15,16] and neutralizing swine sera have been shown to recognize this protein as well [17]. An immunorelevant ORF2 epitope of PCV2 has been identified as a serological marker for the virus infection [18]. Therefore, the Cap protein may be a candidate gene of PCV2 for recombinant vaccine [19,20]. In this study we amplified the Cap gene of PCV2 with PCR and constructed a recombinant adenovirus,
which could express the Cap protein and introduce immune responses in mice.

2. Materials and methods

2.1. PCR for amplification of Cap protein gene of PCV2

A pair of PCR primers was designed using computer software (Primer Premier 5.0) according to the published sequence of the PCV2 in GenBank (no. AY686763). The forward primer was 5′-TTC GGT ACC AGC TAT GAC GTA TCC AAG-3′ and the reverse primer was 5′-GCC AAG CTT TCA CTT GTT CCT GTT TTT-3′. The primers contain KpnI and HindIII restriction sites at their 5′-terminal, respectively. The viral DNA was extracted from the cell cultural supernatant of Chinese field isolate of PCV2 (PCVSH). The amplification was performed in a 50 μl reaction mixture containing 1.5 mM MgCl₂, 1× PCR buffer, 0.2 mM of each dNTP, 50 pmol of each primer, 1.5 μl of TaqDNA polymerase (Promega) and 6 μl of extracted DNA. The reaction was run in a thermocycler (PTC-150) with the following program: denaturation at 95 °C for 12 min, 35 cycles composed of denaturation at 95 °C for 40 s, annealing at 58 °C for 40 s and extension at 72 °C for 1 min, and was ended with a final extension step of 10 min at 72 °C.

2.2. Construction of recombinant plasmid

Amplificon was digested with KpnI and HindIII and cloned into the transfer vector pShuttle-CMV according to the introduction of pAdEasy-1 system (Qbiogene). The recombinant plasmid, named pShuttle-CMV-ORF2, was identified by PCR. KpnI and HindIII enzymes digestion and sequence. The PnuI-linearized plasmid pShuttle-CMV-ORF2 was co-transformed into Escherichia coli bacteria strain BJ5183 with pAdEasy-1 vector by electroporation with a Bio-Rad Gene Pulser at 2.5 kV, 25 μF and 200 V. The transformed bacteria were plated onto LB plate with 50 μg/ml kanamycin. The recombinant plasmid, named pAd-ORF2, was obtained and identified by PCR.

2.3. Transfection and isolation of recombinant adenovirus

To produce the recombinant virus, 293 cell monolayers in a 24-well plate were transfected with 2–4 μg per well of plasmid pAd-ORF2 digested with restriction enzyme PstI by using TransFast™ Transfection Reagent (Promega). The virus was collected upon the appearance of cytopathic effect, propagated in 293 cells and purified with plaque test by three times. The titer of the virus passaged at five times was determined by the method of tissue culture infectious dose 50 (TCID₅₀).

2.4. Identification of expression of Cap protein

Two hundred ninety-three cells were seeded into 6- or 96-well plates and cultured for 24 h to reach 80% confluence. Cells were then infected with rAd-Cap at 20 MOIs for 24–48 h. The expression of Cap protein in the cells was identified by Western blot and immunoperoxidase monolayer assay with PCV2-specific antiserum (swine antibody to PCV2, kept in our laboratory) as following.

2.4.1. Western blot

Cells lysates (rAg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Pall Corporation). 293 cells non-infected with rAd-Cap were used as control. Membrane was placed in blocking solution (10% fat-free milk in PBS, PBS-M) at room temperature overnight and incubated with PCV2-specific antiserum in PBS-M for 2 h at room temperature, followed by incubation for 1 h with rabbit anti-pig IgG conjugated with horseradish peroxidase (Boshide, Wuhan, China), diluted 1/2000 in PBS-M. Detection was performed using chemiluminescence luminol reagents (SuperSignal West Pico Trial Kit, PIERCE).

2.4.2. Immunoperoxidase monolayer assay (IPMA)

The cells in 96-well culture plate were rinsed with PBS and fixed with cold ethanol for 45 min at 4 °C. The cells were washed and then incubated with PCV2-specific antiserum (1:100 diluted in PBS containing 0.5% Tween 80, PBS-T) for 1 h at 37 °C. After washing with PBS-T, the cells were incubated with rabbit anti-pig IgG conjugated with horseradish peroxidase (1:100 in PBS-T) for 1 h at 37 °C. After rinsing for three times, the color cells were developed with 3-amin-9-ethylcarbazole (AEC) and hydrogen peroxide in 0.05 M acetate buffer (pH 5.0).

2.5. Expression and purification of GST-Cap protein of PCV2 in E. coli

A recombinant plasmid pGEX-ORF2, containing 570 bp Cap protein gene without nuclear localization signal of PCV2, was constructed and provided by Dr. Feng in Nanjing Agriculture University. The recombinant GST-Cap protein was expressed and purified as described by Zhou et al. [21]. Briefly, E. coli containing the plasmid was incubated in 2× YT medium for 3 h, and then was induced with the final concentration of 0.1 mM IPTG at 37 °C for 2 h. After centrifugation, the pellet cells were washed and lysed by sonication on ice. Then, Triton X-100 was added to a final concentration of 1% and mixed gently for 30 min, and centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was loaded to GSTrap FF affinity column (Amersham) according to the instruction of the manufacturer. The protein concentration eluted was determined by the Bradford assay. Meanwhile, a pekaryotic expression vector pGEX-4T-1 (Pharmacia) was used as control. The purified recombinant GST-Cap and GST protein were used as antigen in Western blot and iELISA for detection of Cap protein.
detecting the antibody to PCV2 in the sera of mice inoculated with rAd-Cap.

2.6. Inoculation of mice with rAd-Cap

One hundred mice (6-week-old) were randomly divided into five groups with twenty each. In groups 1, 2, 3 and 4 the mice were inoculated subcutaneously with $10^8$, $10^{10}$ and $10^{12}$ TCID$_{50}$/mouse of rAd-Cap and boosted with same dose of rAd-Cap 2 weeks later. In group 4 the mice were vaccinated with the parental (wild) adenovirus ($10^9$ TCID$_{50}$/mouse kept in our laboratory) and boosted 2 weeks later. In group 5 the mice were inoculated with PBS and served as sham-inoculated negative controls. Before the second immunization, the sera of the mice in each group were collected. At days 10, 20, 30 and 40 after the second immunization, five mice of each group were euthanized and the serum samples were collected from mice for detection of antibody to PCV2 by indirect enzyme-linked immunosorbent assay (iELISA), Western blot and virus neutralization assay as following.

2.6.1. iELISA

The GST-Cap protein of PCV2 purified as above was used as iELISA antigen and coated in 96-well plates at the concentration of 5 g/ml. The plates were blocked with 0.15% BSA in PBS. The sera were diluted 1/2 in PBS-T and added into the plates. After incubation for 90 min at 37 °C, the wells were washed for three times and goat anti-mouse IgG conjugated with horseradish peroxidase (Boshide, Wuhan, China) diluted 1:10,000 in PBS was added and incubated for 60 min at 37 °C. The absorbance of each well was read in a spectrophotometer at 490 nm. Meanwhile, the mouse sera sham-inoculated with rAd-Cap were used as negative control. The results were expressed as the ratio of OD$_{490}$ produced by the serum samples compared to negative control serum. Sera, giving a ratio value higher than 2.1 were considered to be positive sera. The titers were expressed as the highest dilution of antibody producing 2.1 ratio value.

In order to detect the antibody to wild type adenovirus in rAd-Cap-inoculated mice, the purified and concentrated wild adenovirus (kept in our laboratory) was used as ELISA antigen and the procedures were same, as above.

2.6.2. Western blot

This experiment was done with the method as above. The recombinant Cap protein of PCV2 expressed and purified from E. coli was used as the PCV2 antigen.

2.6.3. Viral neutralization assay

PCV2 neutralizing activity in serum from rAd-Cap-inoculated mice was investigated using a fluorescent focus neutralizing assay. Briefly, an equal volume of PCV2 (1000 TCID$_{50}$/ml) was added to the serum samples and incubated for 1 h at 37 °C. The mixture was then inoculated to a 96-well plate containing confluent PK15 cells as previously described [19]. After 48 h, the culture plate was fixed with 90% acetone, dried and incubated with antibody to PCV2 and then stained with FITC-labeled Staphylococcus protein A (SPA) (Boshide, Wuhan, China). The serum titers were determined as the reciprocal of the last serum dilution at 70% or greater fluorescent focus reduction in the infected cell cultures under a fluorescent microscope.

2.7. Statistics analysis

Serological responses and lymphocyte proliferation of the inoculated mice were compared using analysis of variance and t-test. A $P$-value <0.05 was considered statistically significant.

3. Results

3.1. Construction of recombinant adenovirus containing Cap protein gene of PCV2

The Cap protein gene of PCV2 was amplified with PCR with the length of 750bp. The nucleotide sequence of the protein gene in recombinant plasmid pAd-ORF2 was the same as the original sequence in GenBank (no. AY686763) and maintained in correct open reading frame. Cap protein gene in the palasmid pAd-ORF2 was confirmed by PCR (Fig. 1). At 14 days post-transfection of plasmid rAd-ORF2 into 293 cells, cytopathic effects were observed. Recombinant adenovirus rAd-Cap was obtained after plaque purification for three times, and the presence of the Cap protein gene in the viral DNA was confirmed by PCR. The viral titer was $10^{13.0}$ TCID$_{50}$/ml.
3.2. Expression of Cap protein by rAd-Cap in cell culture

For detecting the expression of Cap protein in vitro, 293 cells were infected with rAd-Cap at 20 MOIs for 24–48 h and infected cells were collected. Western blotting was performed to detect Cap protein. A specific ~30kDa protein band, consistent with the predicted size of the Cap protein of PCV2, was clearly observed in rAd-Cap infected cells as visualized by PCV2-specific antibody, whereas no Cap protein band was found in non-infected 293 cells (Fig. 2a). IPMA was used to detect Cap protein in rAd-Cap-infected 293 cells. The results showed that the infected 293 cells could be stained with antibody to PCV2 and anti-pig IgG conjugated with horseradish peroxidase, while the non-infected cells could not be stained with antibody (Fig. 3).

3.3. Immunization and detection of PCV2-specific antibody elicited by rAd-Cap in mice

ELISA analysis showed that PCV2-specific antibody response was detected in mice subjected to two subcutaneous immunizations with $10^8$, $10^{10}$ and $10^{12}$ TCID$_{50}$/mouse of rAd-Cap, whereas no immune response was found in animals treated with wild adenovirus or PBS. The antibody to PCV2 was detected in rAd-Cap-treated mice 14 days after the first immunization, with the levels gradually increasing to maximum titers of 1:2200, 1:4800 and 1:6400 in the $10^8$, $10^{10}$
Fig. 4. ELISA assay for PCV2 specific antibodies (a) and analysis of neutralization activity (b) of sera from in the mouse immunized with rAd-Cap at different doses. Arrows (↑) indicate time of initial immunization and boost. Wild Ad means wild adenovirus.

3.4. Neutralizing activity assay

For detecting the neutralizing activity of sera from immunized mice against PCV2, PK15 cells were challenged with PCV2. As expected, the sera of mice inoculated with rAd-Cap protected PK15 cells from PCV2 challenge in vitro, with neutralization titters up to 1:8, 1:14 and 1:17 in the $10^8$, $10^9$ and $10^{12}$ TCID50 groups, respectively, 30 days after second immunization, whereas sera from the wild adenovirus or PBS-treated mice could not (Fig. 4b).
[20] injected the plasmid expressing Cap protein into the mice for three times and the titer of the antibody against PCV2 was 1,400–3,200. To validate the immunogenicity of recombinant adenovirus in this study, mice were inoculated with rAd-Cap at different doses. The results showed that the rAd-Cap by 10^{10} to 10^{12} TCID50/ml could induce a strong humoral immune response against PCV2 in mice (Fig. 4a), and the sera from immunized mice could effectively protect PK15 cells against PCV2 infection in vitro (Fig. 4b). In order to detect the protection of rAd-Cap-immunized mice from PCV2 infection, the rAd-Cap-immunized and non-immunized mice were challenged with PCV2 (PCV5SH strain) 30 days after second immunization. No evident pathological changes (e.g. inflammatory response) were observed in lung, liver, heart, spleen and kidney tissues of all mice, which is different from the finding of Kupel et al. [33] (data not shown). It may be due to the difference of experimental animals, protocols and the PCV2 isolates. But the protective efficacy of rAd-Cap should be further detected in pigs.

In summary, a recombinant adenovirus expressed the Cap protein of PCV2 was firstly constructed and the immunization against PCV2 was confirmed in mice in this study. It might be an attractive candidate vaccine for preventing the disease associated with PCV2 infection.

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References