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Full Length Article

Construction and Immunogenicity of Recombinant Phage T7 Expressing Capsid of Porcine Circovirus Type 2

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Abstract

Porcine Circovirus type 2 (PCV2) is the causative agent of porcine circovirus disease (PCVD) that is regarded as one of the most important diseases endangering pig industry development. Immunization is one of the most effective ways to prevention and control PCV2 infection. Here, gene encoding capsid protein of a PCV2 from a farm of Chongqing was optimized, synthesized and inserted into the coat protein 10B gene of T7 phage to construct recombinant phage T7-Cap. Capsid protein expressed on the surface of phage T7-Cap was tested using western blot. Anti-PCV2 antibody in the serum was detected after the swine inoculated with T7-Cap. The virus clearance from the serum was assayed by PCR after T-Cap vaccinated pigs were challenged by PCV2. The results showed recombinant phage inserted with the PCV2 capsid gene was successfully constructed. Western blot assay showed that PCV2 capsid was expressed on the surface of phage T7-Cap. Specific anti-PCV2 antibody induced production in the pigs inoculated with T7-Cap, and the PCV2 virus could be cleared from the T7-Cap immunized pigs after they were challenged by PCV2. These findings indicate T7-Cap may be a suitable alternative candidate vaccine of PCVD because it can easily be produced in large scales, cheap and cost-effective. © 2018 Friends Science Publishers

Keywords: PCV2; Capsid protein; T7-Cap; Vaccine

Introduction

Porcine Circovirus is a circular single-stranded DNA virus, there are 2 major genotypes: type 1 and type 2. Porcine Circovirus type 1 (PCV1), originally isolated as a contaminant of porcine kidney cell line PK-15, is considered to be non-pathogenic. Porcine Circovirus type 2 (PCV2) was the essential infectious agent of Post weaning Multisystemic Wasting Syndrome (PMWS) that is regarded as one of the most important swine diseases in the world (Wallgrep *et al.*, 2009). Besides, co-infection with other swine pathogens such as Porcine Reproductive & Respiratory Syndrome virus (PRRSV), Porcine Parvovirus (PPV), Mycoplasma hyopneumoniae (Mhp) may enhance PCV2 replication and porcine circovirusvirus-associated disease (PCVAD) (Opriessnig *et al.*, 2011). Porcine Circovirus disease (PCVD) has become one of the important diseases endangering the healthy development of pig industry in the world (Meng, 2013). Traditional feeding and management have little effect on the prevention and control of the disease.

In the process of pig breeding, vaccination is one of the main measures to prevent and control PCVD. PCV2 vaccines, whether inactivated vaccine or live-attenuated vaccine, depend on cell culture. That requires long production cycle and high production costs, which brings great difficulty for the production, supply and immunization of PCV2 vaccines. So, the development of a new type PCV2 vaccine with protective effect, simple production process and low cost has become an important means of controlling PCVD. While the capsid protein (Cap), encoded by the ORF2 of PCV2, is the major structural and immunogenicity protein (Nawagitgul *et al.*, 2000), which can induce the neutralizing antibody response to PCV2 in swine (Mahe *et al.*, 2000). Cap has now become a common target antigen for the study of PCV2 vaccine.

One problem that has long puzzled researchers is that small peptides are often inadequate to induce immune responses when used as immunogens in animal body. In order to obtain a favorable immune response for the small peptides, an expression vector is needed to express the small peptide (Xu *et al.*, 2013). At the same time, a molecular weight appropriate protein is needed as an immune carrier. Phage display exactly meets the two requirements, because its coat protein gene allows insertion of a certain length of foreign gene fragments, and the foreign gene can be expressed in the form of fusion protein with coat protein displaying on the surface of phage particles. At the same time, the phage itself is the immune carrier. Moreover, phage efficient propagation ability, its stable physical and

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chemical properties can reduce the production cost and simplify the production process.

The aims of the current study were to construct a kind of recombinant phage particle expressing PCV2 capsid protein, and primary evaluate the recombinant particle effect of vaccination against PCV2. It may bring new hope for the prevention and control PCV2 infection.

Materials and Methods

Pigs and PCV2

All the pigs reported here adhered to the accepted standards (local and national regulations) of humane animal care. Before experiment, polymerase chain reaction (PCR) detection showed all selected pigs were negative for PCV2, indirect enzyme linked immunosorbent assay (ELISA) revealed all experimental animals were also negative for the antibodies to PCV2. A PCV2 isolate CQ1, isolated from a farm of Chongqing, was preserved in Chongqing Academy of Animal Science Veterinary Research Institute. Its genome sequence has been identified, and its open reading frame 2 (ORF2) encoding PCV2 capsid protein was obtained from the genome sequence (Li *et al.*, 2016).

Cloning, Prokaryotic Expression of Gene Encoding PCV2 Capsid Protein

According to codon preference in *E. coli* and the amino acid (aa) sequence of capsid protein from PCV2 isolated CQ1, gene encoding for the capsid protein was optimized and synthesized, which was combined only two encoding sequence of *Eco*R I and *Hin*d III cleavage sites in the sequence at its 5'-end and 3'-end. The optimized DNA sequence was subsequently cloned into expression vector pET-28a (+) via *Eco*R I and *Hin*d III to construct recombinant plasmid pET-28a (+)-ORF2, and then transformed into host BL21 (DE3). Positive clone was selected to induce expression with 0.1 mM Isopropyl β-D-1-thiogalactopyrano side (IPTG).

Construction of Recombinant Phage Particle T7-Cap

The recombinant plasmid pET-28a (+)-ORF2 described above was extracted using plasmid mini kit (Omega Bio-tek, GA, USA) and double digested via *Eco*R I and *Hin*d III. The optimized DNA sequence was then collected using DNA gel extraction kit (TaKaRa Biotech, Dalian, China). Construction of recombinant phage T7-Cap was carried out according to the protocol of T7Selected® system manual (Novagen, Darmstadt and Germany). Collection and purification of T7-Cap were performed as described previously (Sambrook and Russell, 2001). Bacterial endotoxin in the purified T7-Cap solution was removal by using toxin eraser™ endotoxin removal kit (GenScript, Nanjing, China). Phage T7-Cap titer test was done as Liu described (Yang *et al.*, 2016).

Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). After electrophoresis, proteins were transferred onto nitrocellulose membrane using a mini-Trans-Blot transfer cell (Bio-Rad) in transfer buffer (25 mM Tris base, 192 mM glycine, 20% v/v methanol) at 100V for 90 min. Immunoblots were done as harlow described (Harlow and Lane, 1988) with some modification. The nitrocellulose membrane was soaked in blocking solution before incubation with swine anti-PCV2 serum (VMRD, Washington, USA) for 2 h at room temperature (RT). The blots were reacted with horseradish peroxidase -labelled goat anti-pig IgG (HRP) (Sigma-Aldrich, St Louis, USA) for 1 h at room temperature. The membrane was then washed 3 times with Tris buffer saline (50 mM Tris pH 7.5, 200 mM NaCl) containing 0.1% Tween 20 between each incubation. After equilibration in 0.15M Tris pH 9.5, bound antibodies were detected with 3',3'-diaminobenzidine (DAB) substrate peroxidase solution (Zsgb-bio, Beijing, China).

Animal Experimental Protocol

In this study, 6 pigs (21 d of age) were selected as experiment animal. Among them, 3 pigs (numbered 1, 2, 3) were randomly selected as experimental group intramuscularly inoculated with 2 mL emulsified products containing equal volume Freund's adjuvant and phage Cap-T7 (10^{18} pfus/mL), the others (numbered 4, 5, 6) as control group intramuscularly inoculated with 2 mL emulsified products containing equal volume Freund's adjuvant and physiological saline. The i.m. injection site was the nape of the neck. Immunizations were repeated a total of three times at 14 days intervals. The vaccination first day was considered as d 0. The inoculated pigs were maintained in different isolation units in a bio-secure animal building and were fed a commercial diet ad libitum. Blood samples were drawn from 0 d to 1 w, 2 w, 4 w, 6 w post vaccination. Serum was collected from the blood sample by centrifuging at 1,800 × g for 10 min at 4°C. The PCV2 specific antibody (IgG) level in these serum were tested using commercially available ELISA kit porcine circovirus (PCV) type 2 antibody test kit (Combined Biotech, Shenzhen, China).

After the last blood samples, all the pigs were intramuscular injection inoculated with 2 mL PCV2 ($10^{4.75}TCID_{50}$/mL) (determined by the Reed-Muench method). The challenged first day was considered as d 0. The clinical signs and mortality of the pigs challenged PCV2 were recorded daily for 7 d. The serum were sampled at 0 w, 1 w, 2 w, 3 w, 4 w, 5 w post challenged PCV2, and PCR was performed to detect the PCV2 described above.

Results

The Optimized DNA Sequence and its Prokaryotic Expression Product Analysis

Seq. 1 was the optimized and synthesized DNA sequence encoding the capsid protein of PCV2 isolate CQ1, its prokaryotic expression was detected by using SDS-PAGE assay (Fig. 1). It can be find that there has a more and more thick band (as arrow pointed) with the culture time prolonged, and the percents of the expressed target proteins from each band were 21.4%, 30.2%, 30.1%, 25.0% and 23.6% at 2 h, 4 h, 6 h, 8 h, and 10 h respectively according to the result of protein gray scanning. The molecular weight (MW) of expressed protein is approximately 30 kDa, which is consistent with the MW of PCV2 capsid protein described before (Nawagitgul *et al.*, 2000). This result verified that the optimized DNA sequence encoding PCV2 capsid protein is correct.

Identification of Recombinant Phage Particle Expressing PCV2 Cap

Sequencing analysis for PCR production of positive recombinant plaques showed that optimized DNA sequence was successfully inserted the genome of phage T7 (data not shown). Further, in order to determine the Cap antigenic authenticity expressed on the surface of recombinant phage particles, samples of anti-PCV2 hyper-immune serum (VMRD, Washington, USA) were used for subsequent western blotting. Blots were prepared from the lysate of *E. coli* BLT5403 cells infected with recombinant phage particles T7-Cap, and incubated with the swine hyper-immune serum containing the antibodies to PCV2. Results indicated there was an obvious single band, its molecular weight (MW) is approximately 72 kDa in lane 2, while no band in the lane 1 (Fig. 2), which is in line with the expected results. All that suggesting PCV2 capsid protein was successfully displayed on the surface of recombinant phage particle T7-Cap.

Monitoring of PCV2 Neutralizing Antibody Induced by Phage T7-Cap

Sera were obtained from blood samples of anterior vena cava at periodic intervals before and after the swine inoculated with recombinant phage particle T7-cap, the neutralizing antibody against PCV2 was monitored. Table 1 showed all the optical density (OD_{450}) values of anti-PCV2 antibody response on the 6 pigs' serum post-vaccination according to the test results of porcine circovirus (PCV) type 2 antibody test kit, and their corresponding fluctuation curves of OD_{450} values to each immunized pig with the extension of time were demonstrated in Fig. 3. The results showed the recombinant phage particle has certain immunogenicity to PCV2, and the change of

GAATTCATGACTTATCCGCGTCGTCGCTATCGTCGTCGCCGTCACCGCCCACGTTCCC
ATCTGGGTCAGATCCTGCGTCGTCGTCCGTGGCTGGTACATCCGCGCCACCGCTACC
GTTGGCGCCGTAAAAACGGTATTTTCAACACCCGTCTGTCTCGTACCTTCGGTTACAC
TATCAAACGTACCACCGTGAAGACCCCTTCTTGGGCAGTTGACATGATGCGCTTCAAC
ATCAACGACTTCCTGCCGCCAGGCGGTGGTTCCAACCCGCGTTCCGTTCCGTTTGAA
TACTACCGCATTCGTAAAGTGAAAGTTGAGTTCTGGCCGTGCTCTCCGATTACGCAGG
GCGACCGTGGTGTTGGCTCTTCTGCGGTTATCCTGGATGATAATTTCGTAACGAAAGC
TACTGCTCTGACCTACGACCCGTACGTCAATTACTCTAGCCGCCACACCATTACCCAA
CCGTTCTCTTACCACAGCCGTTACTTCACGCCGAAACCGGTACTGGATTCCACCATCG
ACTATTTTCAGCCGAACAATAAGCGTAACCAGCTGTGGCTGCGTCTGCAAACTGCCG
GTAACGTGGATCACGTTGGCCTGGGCACTGCGTTTGAGAACAGCATCTATGACCAGG
AATATAACATCCGTGTGACCATGTACGTCCAGTTCCGCGAATTTAACCTGAAAGATCC
TCCACTGAACCCGTAAAAGCTT

>Seq. 1:

Note: the optimized DNA sequence encoding PCV2 capsid protein. In the sequence, only *Eco*R I and *Hin*d III cleavage sites were designed at its 5' end and 3' end, and were underlined

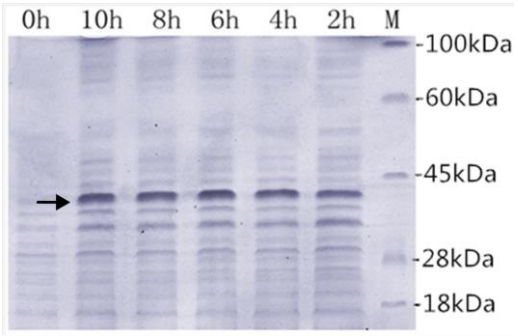

Fig. 1: The expression of the recombinant bacterium pET28a(+)-ORF2 /BL21 using SDS-PAGE assay with culture time prolonged at 0 h, 2 h, 4 h, 6 h, 8 h, and 10 h. The arrow pointed was the prokaryotic expression

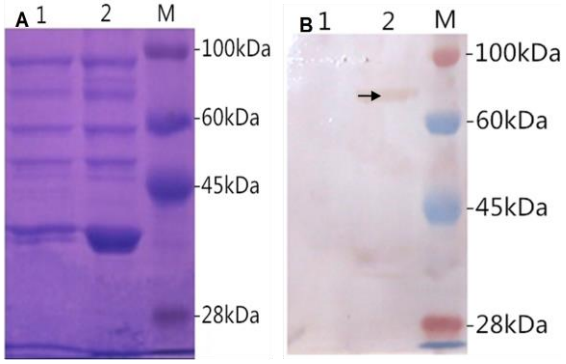

Fig. 2: The SDS-PAGE assay and Western blot assay of recombinant phage particle T7-Cap. A obvious single band, ~72kD in size (as arrow pointed), was showed in lane 2, while no band in lane 1 on the Western blot

A. SAS-PAGE assay of phage particles (Coomassie brilliant blue) B. Recombinant T7 phage coat protein 10B fusion PCV2 capsid antigenicity (Western Blot assay). 1. Control T7Select415-3b phage, 2. Recombinant phage particle T7-Cap, M. Marker

specific antibodies level against PCV2 is closely related to recombinant phage particle immunization. It can be observed that initially the antibodies level in both

experiment group and control group were negative before intramuscular injection, while the antibodies level from experiment group increase gradually with the increase of the swine immunization times using recombinant phage particle from 0 to 6 weeks. On the contrary, the specific neutralizing antibodies (IgG) level against PCV2 from control group swine immunized with physiological saline almost has no obvious change and still showed negative.

The Dynamic of Viraemia of Vaccinated Pigs after PCV2 Challenge

Animal experimental results showed that all challenged pigs were alive during the test, no obvious changes in the health status and clinical signs of these pigs were observed. The clearance percentages of PCV2 isolate CQ1 in the serum of the test swine after challenge, as determined by using PCV2 PCR assay, are shown in Table 2. It can be observed the pigs with viraemia in experimental group become less and less till disappear with the time extending, while the pigs with viraemia in the control group almost no change, which means that the pigs inoculated with T7-Cap can anti-PCV2 infection.

Discussion

T7 phage is a small virulent phage infecting *Escherichia coli*. Its p10B gene encodes the phage's minor head protein, 41.5 kD in size, which is not required in the construction of phage capsid. After the moderate modification of p10B gene, the foreign protein can be displayed at the carboxyl end of the p10B protein in the form of fusion protein.

In this study, based on bioinformatics analysis of the amino acid sequence encoding PCV2 CQ1 capsid proteins, T7Select 415-3b cloning system (Novagen, Darmstadt, Germany) was selected to construct recombinant phage T7-Cap. This system is a novel display system that takes advantage of the properties of T7 phage, and it is easy to use and has the capacity to display all kinds of peptides from 50 aa to 1200 aa in size. The coding sequence for the PCV2 Cap was cloned in multiple cloning site following aa 346 of the 10B protein, and Cap protein was verified to successfully display on the surface of the T7 phage.

Due to unable to replicate in mammalian cells, phage is a safe for vertebrate cells and can be applied in animals and human beings (Clark and March, 2006; Wright *et al.*, 2009). As particle antigens, phage also can be rapidly taken up by antigen-presenting cells (APCs) (Clark *et al.*, 2011), cleared from the circulation, and targeted to the Kuppfer cells of spleen and liver. As one of phages, T7-Cap realizes the organic combination between expression vector and immune carrier, the whole viral particles is a kind of ideal antigen. Animal experiments showed that phage T7-Cap really induce specific antibody against PCV2. Although the antibody level is low, it has a certain protective effect on the test animal, which can clearance the PCV2 from the blood of the inoculated pigs after PCV2 challenge.

Table 1: The optical density (OD_{450}) values of the specific anti-PCV2 antibody response of the 6 pigs' serum post-vaccination measured by using ELISA kit porcine circovirus (PCV) type 2 antibody test kitS

No.	The time of vaccination (weeks)				
	0	1	2	4	6
1	0.041	0.065	0.110	0.209	0.297
2	0.038	0.063	0.125	0.215	0.289
3	0.044	0.093	0.163	0.251	0.444
4	0.040	0.040	0.039	0.038	0.040
5	0.038	0.041	0.038	0.039	0.037
6	0.045	0.038	0.037	0.041	0.042

Table 2: Dynamic of viraemia of the vaccinated pigs after challenge PCV2 by using PCR assay

Group	The time of post challenge (weeks)						Positive number	Positive rate (%)
	0	1	2	3	4	5		
Control	0	3/3	3/3	3/3	3/3	3/3	15	100%
T7-Cap	0	2/3	1/3	0/3	0/3	0/3	3	20%

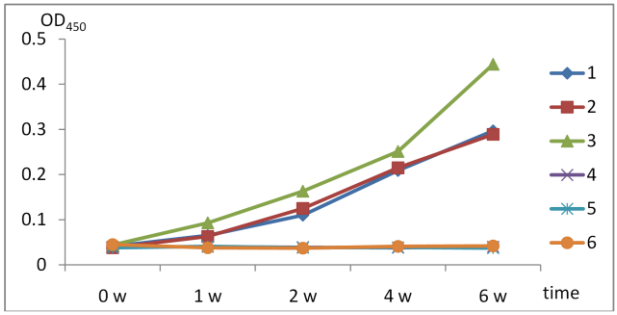

Fig. 3: The corresponding fluctuation curves of OD_{450} values of specific anti-PCV2 antibody response in the serum of 6 pigs post-vaccination measured porcine circovirus (PCV) type 2 antibody test kit. Number 1 - 3 are for experimental animals, number 4 - 6 are for blank control pigs

The results were similar to the reports that recombinant bacteriophage whole particles expressing peptides had been used for vaccination of animal (Manoutcharian *et al.*, 2004; Xu *et al.*, 2013). Therefore, T7-Cap may be considered as suitable alternative candidate vaccines of PCVD because of simple to produce in large scales, cheap and cost-effective.

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