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Original article

Development of a capsid protein-based ELISA for the detection of PCV2 antibodies in swine serum

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Abstract

Porcine circovirus type 2 (PCV2) is the major causative agent of postweaning multisystemic wasting syndrome which leads to significant economic losses in the global swine industry. In China, there is a widespread dissemination of PCV2 infection in the pig population. Serological diagnosis of the disease is considered as an effective control measure. Here, we developed a capsid protein (Cap)-based enzyme-linked immunosorbent assay (Cap-ELISA) for the detection of PCV2 antibodies in swine serum using a nuclear localization signal-truncated capsid protein produced in *Escherichia coli*. The Cap protein was expressed as water-soluble and purified using nickel-nitrilotriacetic acid (Ni-NTA) chromatography. After the optimization of the working conditions of the Cap-ELISA using chessboard titrations, a total of 649 serum samples were tested using the Cap-ELISA and a commercial ELISA kit. The diagnostic sensitivity (DSN), diagnostic specificity (DSP) and accuracy of the Cap-ELISA were determined to be 96.7%, 94.1% and 99.5%, respectively. Cross-reactivity analysis indicated that the Cap-ELISA was PCV2-specific and possessed no cross-reactions with antibodies against other common swine pathogens including porcine circovirus type 1 (PCV1), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), porcine parvovirus (PPV), foot and mouth disease virus (FMDV), porcine epidemic diarrhea virus (PEDV) and pseudorabies virus (PRV). Repeatability of the experiment showed that Cap-ELISA was highly repeatable with the intra- and inter-plate coefficients of variation less than 10%. Hence, the Cap-ELISA has the potential for the swine industry to monitor PCV2 epidemiology and to evaluate PCV2 vaccine efficacy.

Keywords: porcine circovirus type 2, capsid protein-based enzyme-linked immunosorbent assay, PCV2 antibody detection

Introduction

Porcine circovirus type 2 (PCV2), a member of the family *Circoviridae*, is a small non-enveloped virus containing a single-strand circular DNA genome (Allan and Ellis 2000). It is the primary causative pathogen of porcine circovirus associated diseases (PCVADs), which include postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis, nephropathy syndrome and porcine respiratory disease (Segalés et al. 2008, Segalés 2012). PCVADs were first described in Canada in 1991 and are now found abundantly in major swine-rearing countries (Huang et al. 2021). Clinically, PCV2 co-circulates with PCV1 which is a persistent non-cytopathic contaminant of the pig kidney (PK-15) cells, yet the presence of PCV1 has not been associated with any recognized clinical signs or lesions (Tischer et al. 1982, Bucarey et al. 2009). In PCV2-affected farms, due to the co-infections with classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* (*M. hyo*) and secondary infections caused by *Streptococcus suis*, *Escherichia coli* and *Salmonella*, clinical diagnosis of PCVADs is usually hard to confirm (Ouyang et al. 2019, Zhu et al. 2021). Therefore, laboratory-based etiological or serological detections are urgently needed for confirming PCV2 infection and epidemiology.

The genome of PCV2 contains two primary open reading frames (ORFs): ORF1 and ORF2 (Hamel et al. 1998). ORF1 encodes Rep proteins which are essentially involved in viral replication, while ORF2 encodes a major capsid protein (Cap protein) (Nawagitgul et al. 2000, Truong et al. 2001). It has been demonstrated that the ORF1-encoded proteins of PCV1 and PCV2 are antigenically related, whereas the Cap protein of PCV2 contains immunorelevant epitopes essential for virus discrimination (Mahé et al. 2000). Epitope mapping revealed that the Cap protein of PCV2 contains type--specific linear and conformational epitopes (Liu et al. 2001, Ge et al. 2013). Amino acid residues 195-202 and 231-233 on Cap protein have been reported to be PCV2-specific epitopes (Shang et al. 2009). Hence, Cap protein is considered as a valuable diagnostic marker for detecting PCV2-specific antibodies and monitoring PCV2 epidemiology.

Various serological tests have been developed for the detection of PCV2 antibodies, including immunoperoxidase monolayer assay (Dulac and Afshar 1989, Pileri et al. 2014), immunohistochemistry (Szczotka et al. 2011), immunofluorescence assay (Allan et al. 1998, Racine et al. 2004) and enzyme-linked immunosorbent assays (Blanchard et al. 2003, Liu et al. 2004, Ge et al. 2012a). However, the diagnostic antigens applied in these assays require the propagation of PCV2 in PK-15 cell lines or eukaryotic expression of Cap protein in insect cells, which is time-consuming, expensive and labor-intensive. Considering the high-yield and low-cost, the *Escherichia coli* (*E. coli)* system is the ideal option to produce recombinant Cap protein on a large scale.

In this study, a nuclear localization signal-truncated Cap protein of PCV2 was expressed in *E. coli* as water-soluble after optimization of expression conditions and purified using Ni-NTA affinity chromatography. With an overall yield of more than 70 mg per liter LB media, the Cap protein was used as the coating antigen in an indirect ELISA system. A Cap protein--based ELISA (Cap-ELISA) was established, optimized and validated for the detection of PCV2-specific antibodies and screening of PCV2 prevalence.

Materials and Methods

Ethics statement

All the collection and operation of viruses and serum samples were approved by the Ethics Committee of Xinxiang Medical University (XYLL-2020018).

Virus and sera

PCV2 was previously isolated in central China's Henan province and stored in our laboratory. PCV1-free PK-15 cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. Reference PCV2 positive and negative swine sera and reference positive sera against porcine circovirus type 1 (PCV1), porcine reproductive and respiratory syndrome virus (PRRSV), classic swine fever virus (CSFV), porcine parvovirus (PPV), foot and mouth disease virus (FMDV), porcine epidemic diarrhea virus (PEDV) and pseudorabies virus (PRV) were purchased from the China Institute of Veterinary Drug Control. 649 field sera were collected in different regions of Henan province from 2010 to 2022 and stored at -20°C.

Preparation of recombinant Cap protein

The preparation of PCV2 Cap protein was carried out as previously described with some modifications (Jin et al. 2012). In brief, *E. coli* BL21 cells harboring recombinant plasmid pET28a-ORF2 were grown at 37°C in Luria Bertani (LB) media supplemented with 50 μg/mL kanamycin to an optical density of 0.6 at a wavelength of 600 nm (OD_{600}). Isopropyl-β-D--thiogalactopyranoside (IPTG) was then added to a final concentration of 1.0 mM to induce the expres-

sion of Cap protein at 37°C for 4 h. The cells were then harvested by centrifugation at 8,000 rpm for 30 min and the pellet was resuspended in 100 mM Tris–HCl (pH 8.0). The suspension was disrupted on ice by sonication before centrifugation at 12,000 rpm for 25 min. The supernatant was collected and filtered through a 0.45 μm filter, and recombinant Cap protein was purified using Ni-NTA affinity chromatography following the manufacturer's protocol. The concentration of Cap protein was measured under its predicted extinction coefficient and molecular weight using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA).

SDS-PAGE and Western blot

The purity of Cap protein was examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and its antigenicity was analyzed using Western blot as previously described (Wang et al. 2016). Briefly, Cap protein was subjected to 12% SDS-PAGE and then stained with 0.25% Coomassie brilliant blue R250 or transferred onto a PVDF membrane. After blocking with 5% skimmed milk at 37°C for 1 h, the transferred membrane was incubated with 1:100 diluted swine polyclonal antibodies against PCV2 at 37°C for 1 h. Horseradish peroxidase (HRP)-conjugated goat-anti-pig IgG was then allowed to incubate with the membrane at 37°C for 1 h. Finally, enhanced chemiluminescence (ECL) substrate was used to visualize the specific bands. The membrane was washed with phosphate buffered saline containing 0.05% Tween-20 (PBST) during the steps.

Cap-ELISA protocol

Ninety-six well microtiter plates manufactured from polyvinyl chloride were coated with 100 μL Cap protein (2 μg/mL) in 0.1 M carbonate-bicarbonate buffer (CBS, pH 9.6) and incubated at 4°C overnight. After three washes with PBST, the plates were incubated with 5% skimmed milk at 37°C for 1 h. After washing with PBST, the plates were incubated with 100 μL serum samples diluted in PBS at 37°C for 30 min, with each sample in duplicate. After washing six times with PBST, the plates were incubated with 100 μL HRP-conjugated goat anti-pig IgG at 37°C for 30 min. The plates were then washed again and finally incubated with 100 μL 3,3',5,5'-tetramethylbenzidine (TMB) for color development. After 10 min of incubation at room temperature, the enzymatic reaction was stopped with 100 μL 2 M H_2SO_4. The horseradish peroxidase product was quantified at A450 (OD_{450}) with an automatic microplate reader (Bio-Rad, USA). All data were measured in triplicate, and the mean OD_{450} value and standard deviation of each sample were calculated.

Optimization of ELISA conditions

According to the procedure described above, the working conditions of Cap-ELISA were optimized by means of chessboard titrations (Crowther 2000). Briefly, the Cap protein was immobilized in the column of a 96-well microtiter plate using a twofold dilution from 10.00 μg/mL to 0.15 μg/mL at 4°C overnight. Reference PCV2 positive and negative swine sera were then diluted in the row from 1:100 to 1:1600 and incubated with the plates at 37°C for 30 min. After the determination of the optimal antigen concentration and serum dilution, the optimal conjugate dilution was determined by adding HRP-conjugated goat anti-pig IgG at dilutions of 1:500, 1:1000, 1:1500, 1:2000 and 1:2500 onto the plates. With the OD_{450} value of the positive serum close to 1.0, the conditions exhibiting the highest ratio between the OD_{450} values of positive serum and negative serum (P/N value) were scored as the optimal ELISA conditions.

After the above conditions were fixed, the coating buffer in Cap-ELISA was optimized from 0.1 M NaOH (pH 13.0), 0.1 M HCl (pH 3.0), 0.05 M Tris-HCl buffer at pH 8.5 (TBS), double distilled water at pH 6.7 (DDW), 0.05 M phosphate-buffered saline at pH 7.4 (PBS) to 0.1 M carbonate-bicarbonate buffer at pH 9.6 (CBS). Subsequently, to determine the optimal reaction time of the serum samples and the conjugate, the reaction was stopped by washing with PBST after the plates were incubated at 37°C for 15 min, 30 min, 45 min, 60 min and 75 min, respectively. The optimal working conditions were selected according to the same standard as above.

Validation of Cap-ELISA

To determine a cut-off value for the Cap-ELISA, 30 PCV2-negative sera were tested and the cut-off value was determined as the mean OD_{450} value of the negative sera (X) plus three times of the standard deviation (X+3SD).

The Cap-ELISA was validated by testing 649 serum samples in parallel with a commercial ELISA kit (Jeno Biotech Inc., Republic of Korea). The diagnostic sensitivity (DSN), diagnostic specificity (DSP) and accuracy of the Cap-ELISA were calculated according to the formula: DSN = TP/(TP + FN) × 100; DSP = TN/(TN + + FP) × 100 and accuracy = (TP + TN)/total number of serum samples tested × 100, where TP, FP, TN and FN represented true-positive, false-positive, true-negative and false-negative, respectively.

The specificity of the Cap-ELISA was examined by testing the cross-reactions of the Cap protein of PCV2 with antibodies against other swine viruses

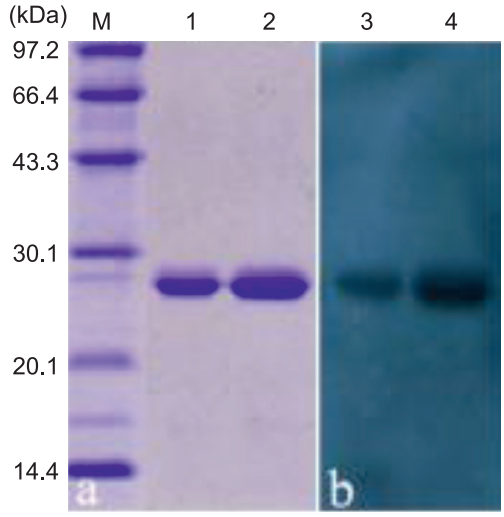

Fig. 1. Preparation of recombinant Cap protein of PCV2. Purified Cap protein (26 kDa) was examined by 12% SDS-PAGE (a) and Western blot (b). Lane M, low molecular weight protein marker (kDa). Lanes 1-2: purified Cap protein. Lanes 3-4: the reaction of Cap protein and swine polyclonal antibodies against PCV2.

including PCV1, PRRSV, CSFV, PPV, FMDV, PEDV and PRV.

The repeatability of the Cap-ELISA within and between runs was evaluated as described (Jacobson 1998). 6 PCV2-positive sera and 4 PCV2-negative sera were included in the repeatability assay using three batches of Cap-ELISA. Intraplate variation was obtained by testing three replicates of each serum sample in the same plate. Interplate variation was obtained by testing the same serum sample in different plates within a run and between runs. The mean OD_{450} value, standard deviation (SD) and coefficient of variation (CV) were calculated.

Results

Expression and purification of Cap protein

The Cap protein of PCV2 was expressed with a histidine tag in *E. coli* BL21(DE3) cells as a water-soluble protein when the cells were grown to an OD_{600} value of 0.6 and induced with a final IPTG concentration of 1.0 mM at 37°C for 4 h. After purification by Ni-NTA chromatography, the purity of Cap protein was shown to be over 90% by SDS-PAGE (Fig. 1). Western blot indicated that recombinant Cap protein could react specifically with swine polyclonal antibody against PCV2 (Fig. 1). It was estimated that the overall yield of purified Cap protein was more than 70 mg per liter of cell culture.

Optimization of Cap-ELISA conditions

In chessboard titrations, the conditions exhibiting the highest P/N value with the OD_{450} value of the positive serum close to 1.0 were considered as optimal. Using this standard, the optimal Cap protein concentration was determined to be 2.5 μg/mL (Fig. 2a). The optimal serum and conjugate dilutions were determined to be 1:400 and 1:1500, respectively (Fig. 2b and 2c). 0.1 M carbonate-bicarbonate buffer (pH 9.6) was found to be the optimal coating buffer (Fig. 2d). The optimal reaction time of serum samples and the conjugate were 45 min and 30 min, respectively (Fig. 2e and 2f).

Validation of Cap-ELISA

The cut-off value of Cap-ELISA was determined by testing 30 PCV2-negative swine sera (Table 1). The mean OD_{450} value was 0.185 and the standard deviation was 0.077. The cut-off value was calculated to be 0.416 according to the formula cut-off value=X+3SD. Therefore, if the OD_{450} value of the sample is greater than or equal to 0.416, it is judged as positive for PCV2 antibody. If the OD_{450} value of the sample is less than 0.416, it is judged as negative for PCV2 antibody.

The specificity of the Cap-ELISA was examined by testing the serological cross-reactions of antibodies against other swine viruses with Cap protein. The mean OD_{450} value of the reference positive sera against PCV1, PRRSV, CSFV, PPV, FMDV, PEDV and PRV were significantly less than 0.416 (Table 2), indicating that there was no cross-reactivity between Cap protein and other swine sera.

Repeatability analysis with 10 serum samples showed that the intraplate CV ranged from 0.32% to 7.68% and the interplate CV was between 3.03% and 9.91%, indicating that the assay was highly repeatable.

The Cap-ELISA was validated by detecting 649 clinical serum samples in parallel with a commercial

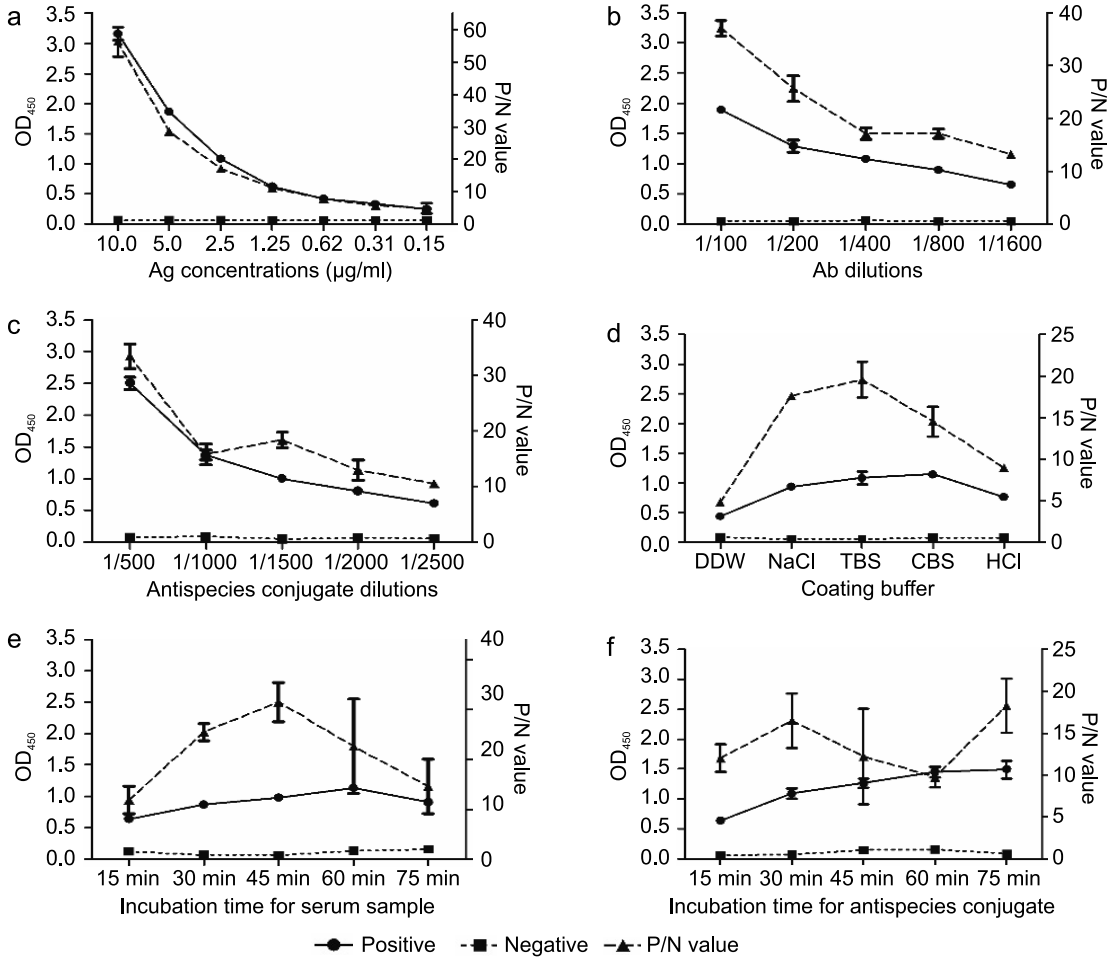

Fig. 2. Optimization of the working conditions of Cap-ELISA. The antigen concentration (a), serum dilution (b), antispecies conjugate dilution (c), coating buffer (d) and the incubation time of serum samples and the conjugate (e and f) were optimized using chessboard titrations. The conditions exhibiting the highest P/N value with the OD_{450} value of the positive serum close to 1.0 were considered as optimal.

Table 1. Determination of cut-off value from swine sera

Serum	Mean OD_{450} value	Serum	Mean OD_{450} value	Serum	Mean OD_{450} value
1	0.202	11	0.128	21	0.267
2	0.067	12	0.132	22	0.273
3	0.068	13	0.143	23	0.274
4	0.068	14	0.166	24	0.281
5	0.078	15	0.174	25	0.284
6	0.1	16	0.175	26	0.284
7	0.107	17	0.181	27	0.287
8	0.116	18	0.205	28	0.295
9	0.119	19	0.232	29	0.234
10	0.126	20	0.25	30	0.067

ELISA kit. Compared with the commercial ELISA kit, the DSN, DSP and accuracy of the Cap-ELISA were 96.7%, 94.1% and 99.5%, respectively (Table 3).

Discussion

Since the outbreak of PMWS in 2002, pig production in China has suffered major economic losses

Table 2. Cross-reaction of the Cap-ELISA

Antisera	Mean OD_{450} value
PCV1	0.069
PRRSV	0.065
CSFV	0.077
PPV	0.089
FMDV	0.118
PEDV	0.043
PRV	0.109
PCV2	1.255

Table 3. Comparison of the Cap-ELISA with a commercial ELISA kit

	Commercial ELISA kit		
Cap-ELISA	Positive	Negative	Total
Positive	614	2	616
Negative	21	32	43
Total	635	34	649

(Ge et al. 2012b). Nowadays, PCV2 infection continues to be widespread, though the positive rates of PCV2 antibody varies in different areas across China (Zheng et al. 2020, Chen et al. 2023, Lv et al. 2023). One of the main obstacles in controlling PCV2 infection is that PCV2-infected pigs might show no clinical signs but still carry viruses (carrier animals), allowing the infection to persist longer in affected herds (Patterson et al. 2011). Moreover, PCV2 infection may cause a loss of the connection between the innate and acquired immune systems, rendering the infected pigs more susceptible to secondary or concomitant microbial infections (Vincent et al. 2007, Kekarainen and Segalés 2015, Rakibuzzaman and Ramamoorthy 2021). It has been demonstrated that PCV2 can act synergistically with PPV, PRRSV and *M. hyo* to enhance the severity of PMWS (Ellis et al. 2004, Chen et al. 2016, Zhang et al. 2022). Specifically, PCV2 exhibits more variable strains and higher infection rates in China than in North America and Europe (Lv et al. 2023).

To contain the spread of PCV2 infections, vaccines have been applied extensively in controlling PCV2 infection in pig health programs (Segalés 2012, Afghah et al. 2017). PCV2 commercial vaccines in use are non-replicative (subunit or inactivated) and most contain the PCV2 Cap protein as the immunogenic antigen (Bucarey et al. 2009, Yuan et al. 2022). Widespread vaccination against PCV2 requires the development of an effective evaluation system for assessing vaccine efficacy. Hence, developing a useful diagnostic method based on Cap protein is of significance for screening PCV2 prevalence, monitoring PCV2 carrier animals and evaluating PCV2 vaccine efficacy.

In this study, *E. coli* cells were used to produce recombinant Cap protein, due to the high-yield and low-cost of this prokaryotic expression system. Through the optimization of expression conditions, recombinant Cap protein was expressed as water-soluble protein and the final amount of purified Cap protein was estimated to be over 70 mg per liter cell culture. The preparation of Cap protein was simple and easy to operate, which greatly facilitated the development of immunoassays including an indirect ELISA.

The development of an indirect ELISA is influenced by several factors that include the purity of the antigen, the coating concentration of the antigen and the dilution of serum (Wang et al. 2016). In this study, recombinant Cap protein was purified using Ni-NTA chromatography and the purity was estimated to be over 90%. After optimization of the working conditions and the determination of a cut-off value, 649 clinical swine sera were tested using a Cap-ELISA and a commercial ELISA kit. Considering the commercial ELISA kit as a gold standard, the DSN, DSP and accuracy of the Cap-ELISA were 96.7%, 94.1% and 99.5%, respectively. Additionally, the Cap-ELISA developed in this study was found to possess high specificity for the detection of PCV2 antibodies and have no cross-reactivity with antibodies against PCV1, PRRSV, CSFV, PPV, FMDV, PEDV and PRV. Thus, it has the potential to be used in field prac-

tices to monitor PCV2 epidemiology and evaluate PCV2 vaccine efficacy. However, the Cap-ELISA fails to discriminate vaccine-induced antibodies from antibodies derived from natural exposure. Future studies should focus on identifying a discriminating candidate in PCV2 or inserting a marker in PCV2 vaccine to help differentiate infected animals from vaccinated animals.

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