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Development of an ELISA Based on the Baculovirus-Expressed Capsid Protein of Porcine Circovirus Type 2 as Antigen

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ABSTRACT. The genome of porcine circovirus type 2 (PCV2) contains two major open reading frames, which have been shown to encode the virus capsid and replication-associated proteins. The capsid protein is a major structural protein of the virus; it can be a suitable target antigen for detecting PCV2-specific antibodies to monitor PCV2 infection. To produce the antigen, the capsid protein coding sequence was cloned into a baculovirus transfer vector, and a recombinant capsid (rC) protein of PCV2 was expressed as a combined fusion protein in frame with a C-terminal peptide of six histidines. The affinity-purified rC protein was used as coating antigen to develop an ELISA for detecting the virus-specific antibodies in swine sera. The rC protein-based ELISA (rcELISA) was evaluated by examining a panel of 49 PCV2-positive and 49 PCV2-negative swine sera. In comparative experiments of immunoperoxidase monolayer assay (IPMA) using 102 field sera, there was 89.2% coincidence between data obtained by the rcELISA and IPMA. The rcELISA achieved 88.5% specificity and 89.4% sensitivity for detection of PCV2 antibody in the field sera. The assay showed no cross-re activity with antibodies to PCV type 1, porcine reproductive and respiratory syndrome virus and porcine parvovirus. The results suggest that the rcELISA is suitable for routine serodiagnosis and epidemiological surveys of PCV2-associated diseases.
KEY WORDS: ELISA, PCV2, recombinant capsid protein.

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Porcine circovirus type 2 (PCV2) is the etiological agent of postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) in pigs [2, 8, 13]. In contrast, porcine circovirus type 1 (PCV1) as a PK-15 cell contaminant has been shown to be nonpathogenic in experimental studies [14]. Although PCV2-infected adult pigs may recover from the disease, they can be a virus carrier that allows the infection to persist longer in affected herds [1]. To detect such carrier animals, a simple and reliable diagnostic method for monitoring the status of PCV2 infection in herds has been required. The serological methods reported previously, including immunoperoxidase monolayer assay (IPMA) [3], indirect immunofluorescent antibody (IIF) technique [2] and various enzyme-linked immunosorbent assays (ELISAs) [12, 16], have been developed by means of preparing live virus as a diagnostic antigen in tissue culture. The preparation method is, however, labor-intensive, uneconomical and apt to yield batch variation. In order to minimize the disadvantages, we tried to use a recombinant protein as the antigen in a diagnostic ELISA.

The genomic sequences of a number of PCV2 isolates have been reported to be composed of a single-stranded circular DNA molecule of 1768 nucleotides (nt) [5, 6, 9, 10]. The replicative form of PCV2 genome contains two major open reading frames (ORFs): ORF1 (945 nt) is encoded by the viral strand and involved in virus replication; ORF2 (702 nt) is encoded by the viral complementary strand and suspected to represent the major structural capsid protein [4]. Recently, an approximately 30 kDa of recombinant ORF2 protein of PCV2 is expressed in insect cells and formed virus-like particles by self-assembles [11]. The recombinant ORF2 protein reacts strongly with serum from PCV2-infected swine, suggesting its possible use in diagnostic assays [12]. In order to produce the diagnostic antigen, a recombinant PCV2 capsid protein was expressed as a combined fusion protein in frame with a C-terminal peptide of six histides. The aim of this study is to establish an ELISA based on the baculovirus-expressed and affinity-purified recombinant capsid (rC) protein of PCV2 as a coating antigen for the diagnosis of PCV2-associated diseases.

MATERIALS AND METHODS

Viruses, cells and sera: A Japanese field isolate (J336) of PCV2 from a pig with clinical PMWS was used in the study. PCVs-free swine kidney cells (SK/C1) grown in Eagle's minimum essential medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) were used for PCV2 propagation and passage.

A panel of forty-nine PCV2-negative sera was collected from 30- to 90-day old pigs of a PCVs-free herd. These sera were previously shown to be free of PCV antibodies by IPMA. A panel of forty nine 3-day-old piglets were experimentally infected with PCV2 J336 isolate at a dose of $10^{4.0}$ $TCID_{50}$ by intranasal route. The sera were collected from the pigs from 21 to 49 days after infection. In comparative experiments, 102 sera were collected from 10, 30, 60, 120, and 150-day-old pigs from four commercial herds. Antisera raised against other pig viruses including PCV1, porcine reproductive and respiratory syndrome virus (PRRSV), and porcine parvovirus (PPV) were used in this study to determine the specificity of rcELISA.

Spodoptera frugiperda (Sf-21) cells were used for the expression of rC protein, which was propagated in Grace's media (Gibco, BRL) supplemented with 0.26% of Bacto

tryptose broth, Kanamycin (0.05 mg/m*l*), and 10% FBS.

Cloning and sequencing of PCV2 capsid protein gene: The viral DNA was prepared from a 100 μl of 10% lymphoid tissue homogenates of PCV2-infected piglets by adding 4 μl of 20 mg/m*l* proteinase-K treatment (37°C, 1 hr), followed by boiling 5 min. The DNA was used as template for amplification of the virus capsid protein gene by polymerase chain reaction (PCR). A pair of synthetic oligonucleotide primers, P1 (forward: 5'-ATGACGTATCCAGGAGGCG-3') and P2 (reverse: 5'-GGGTTTAAGTGGGGGGTCT-3'), was designed based on the reference sequences registered in the GenBank (Accession numbers: AF16652, AF027217, AF118097, AF264043, AF201897). The PCR was performed using a kit of TaKaRa Ex Taq (Takara, Biomedicals, Japan) under the condition of denaturing 94°C for 2 min; 35 cycles at 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min; a prolonged elongation time up to 7 min at 72°C. The PCR product was directly cloned into a baculovirus transfer vector of pBlueBac4.5/V5-His-TOPO (Invitrogen, Leek, The Nethelands) in frame with a C-terminal peptide encoding a 6 × His-tag sequence so that the expressed recombinant protein could be purified by chromatography on metal-chelating resin. The obtained plasmid was subjected to sequence analysis using a 373A DNA sequencer (Perkin-Elmer, Applied Biosystems). The nucleotide and amino acid sequences were analyzed with the aid of the software of GENETYX-MAC ver.10.1 (SDC, Tokyo, Japan).

Generation of recombinant baculovirus: Sf-21 cells were transfected with a mixture of the purified transfer vector and the linearized baculovirus DNA provided by a kit of transfection with a reagent of Cellfectin according to the manufacturer's protocols (Invitrogen). After 3 days of incubation, the culture media of transfected cells was serially diluted, and plated on a monolayer of the cells and incubated for 1 hr. The inoculum was removed, and then the monolayer was overlayed with a final concentration of 1% low melting point agarose (SeaPlaque, Rockland, U.S.A.) containing 150 μg/m*l* X-gal and 10% FCS in Grace's media. After 4 days of incubation, the deep blue plaques were screened as the recombinant baculoviruses. An individual viral plaque was identified by PCR analysis, and then the positive clones were selected by plaque assay again. A representative strain in the recombinant viruses was used for expression studies.

Recombinant protein expression analysis: For the rC protein expression, a high-titer seed virus stock was prepared by three passages of infecting Sf-21 cells with a multiplicity of infection (MOI) of 0.1 plaque forming units (PFU)/cell. A crude extract of the expressed proteins were produced from the cells infected with a MOI of 5 PFU/cell and harvested at 0, 24, 48, 72, 96, and 120 hr post-infection (p.i.). Total proteins from 10^5 cells were analyzed on a 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue (CBB). For Western blotting, the separated proteins on the gel were blotted onto a polyvinylidene difluoride membrane (Millipore, Yonezawa, Japan). The membrane was blocked with Tris buffered saline-Tween20 (TBS-T: 10 mM Tris-HCl pH 8.0, 15 mM NaCl, 0.05% Tween 20) containing 5% (w/v) skim milk (Difco, Detroit, U.S.A.) overnight at 4°C, and then reacted with swine anti-PCV2 serum (1:100) and goat anti-pig IgG conjugated with horse radish peroxidase (1:2500) (Bethyl, Montgomery, TX) for 1 hr at room temperature, respectively. The membrane was washed with TBS-T three times each for 5 min between each incubation. After equilibration in TBS solution, the reacted patterns were visualized with 3,3'-diaminobenzidine (DAB) substrate (Sigma, St. Loius, MO).

Recombinant protein purification: To purify the protein, the harvested cells were resuspended in lysis buffer (6 M guanidine hydrochloride in 20 mM sodium phosphate and 500 mM sodium chloride, pH 7.8), and the lysed cells were passed through an 18-gauge needle to shear the nucleic acids. After centrifugation (3000 × g for 15 min), cleared lysate was collected and applied to ProBond resin affinity column chromatography (Invitrogen) under denaturing condition according to the manufacturer's instructions. Finally, the purified rC protein was released with an elution buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 4.0). The eluted fractions containing the purified rC protein were pooled and dialyzed against 10 mM Tris-HCl, pH 8.0, 0.1% Triton-X100 overnight at 4°C for the removal of urea and refolding of the protein. After dialysis, the aliquot samples were stored at –70°C until use. The concentration of rC protein was measured using an advance protein assay reagent (Cytoskeleton, Denver, U.S.A.), and the purity was estimated on a CBB-stained polyacrylamide gel by a densitometer, PDSI (Molecular Dynamics, Sunnyvale, U.S.A.).

An ELISA using the rC protein as coating antigen (rcELISA): A volume of 100 μl purified rC protein which was diluted to the concentration of 4.8 μg/m*l* with 50 mM sodium carbonate buffer (pH 9.6) was added into each well of 96-well microtiter plates (MaxiSorp, Nunc, Denmark), and incubated overnight at 4°C. The antigen-coated plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBS-T) and blocked with PBS-T containing 5% (w/v) skim milk for 1 hr at 37°C. After washing, diluted pig sera with the blocking solution were added, and then incubated for 1 hr at 37°C. After washing, 100 μl/well of the diluted goat anti-pig IgG conjugated with horse radish peroxidase (Bethyl) in the blocking solution was added, and then incubated for 1 hr at 37°C. Thereafter, reaction products were washed and 100 μl/well of substrate solution (FAST *o*-phenylenediamine dihydrochloride, Sigma) was added. After incubation for 30 min at room temperature in the dark, the reaction was stopped by adding 50 μl of 4 N H_2SO_4. Extinctions were measured at 490 nm and 600 nm as a reference using a kinetic microplate reader (Molecular Devices, Vmax, U.S.A.).

IPMA as a comparative assay: IPMA was used as the reference method to detect the presence of antibodies to PCV2. Ninety-six-well plates containing PCV2-and mock-infected cells were fixed in 33.3% acetone for 20 min at room tem-

perature and dried (stored at –80°C). The cells were blocked with PBS containing 3% (w/v) skim milk for 1 hr at 37°C, and then testing serum samples (50 μl) were added to PCV2-infected and noninfected SK/C1 cells, and then incubated at 37°C for 1 hr. After the unbound antibodies were washed three times with PBS, an optimum dilution (1:3000) of horseradish peroxidase—conjugated Protein A (Zymed, U.S.A.) was added and incubated for 30 min at 37°C. After washing, color development was carried out with 3-amino-9-ethylcarbazole (AEC) and hydrogen peroxide in 0.05 M acetate buffer (pH 5.0) for 30 min at room temperature. The reaction was terminated by removal of the substrate. After washing once with distilled water, the cells were dried again and then examined under an inverted light microscope. Serum samples that gave a positive signal at a serum dilution of 1:10 and higher were judged to be positive.

RESULTS

Cloning and sequencing of the gene encoding capsid protein and construction of recombinant baculovirus: A genomic fragment representing PCV2 capsid protein gene was obtained by PCR amplification. The amplified product of 699 bp containing the first ATG codon but omitting its stop codon was cloned directionally into a baculovirus expression vector. Three individual plasmid colonies were sequenced. The results demonstrated that there were no differences present between the three sequences. The obtained sequence appeared to be more than 96.7% identical with those of other reported strains. A representative colony was used for constructing a recombinant baculovirus by cotransfection with linearized baculovirus DNA into Sf-21 cells. Three individual virus plaques were confirmed to be positive by PCR, and then screened throughout another cycle of virus plaque assay. A resulting recombinant baculovirus termed as baculo-PCV2Cap with a virus titer of 1.28×10^8 PFU/m*l* was prepared for the protein expression.

Expression, purification and analysis of recombinant protein: The expression of the recombinant proteins in baculo-PCV2Cap-infected Sf-21 cells was confirmed by SDS-PAGE analysis. The results revealed a band of increasing intensity in the expected size range for the protein. It was easy to distinguish the recombinant proteins from the background proteins when compared with the Sf-21 cells at 0 hr p.i. (Fig. 1). The expression of the rC protein was first detected at 48 hr and reached a maximum level (more than 17.2% of total cellular proteins) from 96 to 120 hr p.i. (Fig. 1). The molecular weight of the rC protein was determined to be 32.8 kDa by SDS-PAGE with CBB staining. The size of the rC protein was close to the calculated one (31.8 kDa), which included addition of the V5 epitope and histidine tag sequence (encoding about 4 kDa peptides) in the C-terminus of the rC protein.

By Western blotting analysis, the virus-specific antibodies could react with the rC protein from 48 to 120 hr p.i. (Fig. 2), but did not react with the crude of Sf-21 cells infected with a wild type (wt) parental baculovirus (Fig. 2).

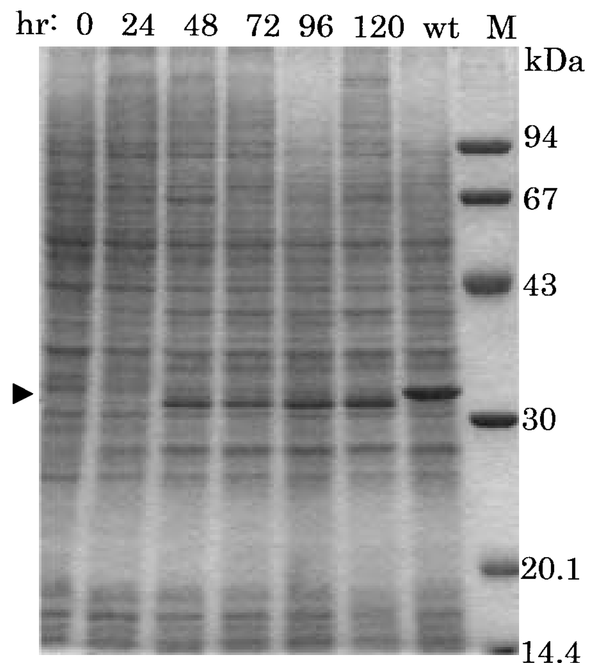

Fig. 1. SDS-PAGE analysis (12.5%) of baculovirus-expressed PCV2 rC protein. The expressed crude products were collected at 0, 24, 48, 72, 96 and 120 hr after the recombinant baculovirus infection. The samples were electrophoresed in SDS-PAGE, and stained using Coomassie brilliant blue (lane 0 to 120). The cells infected with the parental baculovirus wild type (wt) at 96 hr were used as comparison (lane wt). Molecular weight markers were marked on the right side of the panel (lane M). An arrow on the left of the panel indicated the migrating position of the rC protein.

No reactivity was observed in normal pig sera (data not shown).

The rC protein was produced in Sf-21 cells infected with baculo-PCV2Cap at a MOI of 5. The expressed crude products from total 10^8 Sf-21 cells were harvested at 96 hr p.i., and used for purification of the rC protein. The yield of purified rC protein varied from 1.7–1.9 mg /10^8 Sf-21 cells among three batches, and the purity reached 92.7% (Fig. 3).

Evaluation of rcELISA using purified rC protein as antigen: The optimal dilutions of the antigen and the test serum in the rcELISA were determined by checker board titration. Using the homologous porcine hyperimmune serum, the optimal dilution of the testing sera was found to be 1:50 or more. A final rC protein concentration of 4.8 μg/m*l* for coating antigen and a dilution at 1:2500 for the goat anti-pig IgG conjugate were determined. A good positive/negative (P/N) ratio was obtained with PBS containing 0.05% Tween20 and 5% (w/v) skim milk as the blocking and dilution buffer. All sera were tested in duplicates.

A panel of reference swine sera collected from 49 normal and 49 PCV2-infected pigs was examined by the rcELISA and IPMA. The averaged OD value of 49 normal pig sera in the rcELISA was 0.039 ± 0.020 (mean ± SD), which gave

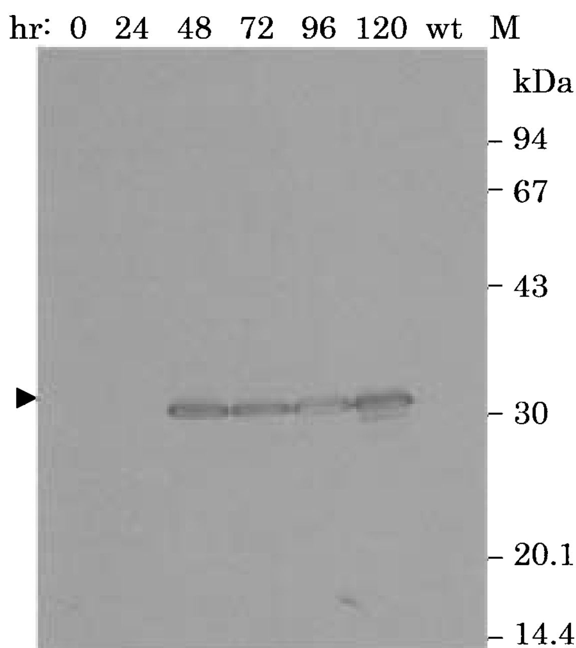

Fig. 2. Western blotting analysis of baculovirus-expressed PCV2 rC protein. As shown in the Fig. 1A, the separated proteins in the SDS-PAGE were transferred into a membrane and then reacted with anti-PCV2 pig serum. The expressed samples were collected at 0, 24, 48, 72, 96 and 120 hr after the recombinant baculovirus infection (lane 0 to 120), and the cells infected with the parental baculovirus wild type (lane wt) at 96 hr as a control. Molecular weight markers were marked on the right side of the panel. An arrow on the left side of the panel indicated the migrating position of the rC protein.

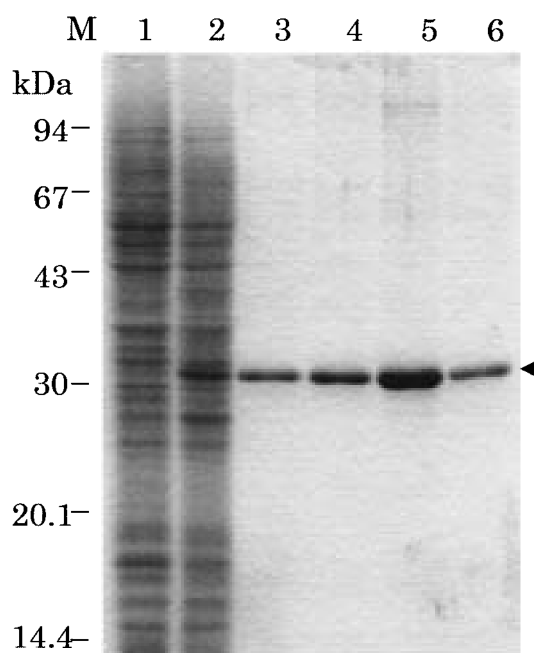

Fig. 3. The baculovirus-expressed PCV2 rC protein was purified by nickel chelate affinity chromatography, and analyzed by SDS-PAGE followed by staining with CBB. The affinity purified rC protein in the eluting fractions were showed in the picture (lane 3 to 6), and Sf-21 cells with mock expression and original crude expressed products were used as comparison (lane 1 to 2). An arrow on right of the panel indicated the migrating position of the purified rC protein. Molecular weight markers were marked on the left side of the panel.

suitable cut-off OD value of 0.100 (mean ± 3SD) in this assay. Based on this criterion, all samples of the 49 normal pig sera were negative, and 48 samples of 49 PCV2-infected swine sera were positive in the rcELISA. The IPMA showed 47 samples of 49 PCV2-infected swine sera were positive for detection of the same samples (Table 1).

In comparative experiments using 102 sera from commercial pig herds, there was 89.2% coincidence between data obtained by the ELISA and previously developed IPMA (Table 2). When the IPMA was used as a reference, specificity and sensitivity of the rcELISA were calculated as 88.5% and 89.4% respectively, based on the following formula: specificity = 100 × (number of rcELISA negative) / (total number of IPMA negative); sensitivity = 100 × (number of rcELISA positive) / (total number of IPMA positive). No cross-reactions with antisera of PCV1, PRRSV, and PPV were detected in the ELISA (Table 3).

Table 1. Evaluation of the rcELISA for normal and PCV2-infected pig sera

Reference sera	rcELISA		IPMA	
	Positive	Negative	Positive	Negative
Noninfected (49)[a)]	0	49	0	49
PCV2-infected (49)[b)]	48	1	47	2

a) Noninfected sera were collected from 30- to 90-day old pigs of a PCVs-free herd (ELISA titers: < 50; IPMA titers: < 10).
b) PCV2-infected sera were collected from pigs at 21 to 49 days after PCV2 infection (ELISA titers: 50 to 12,800; IPMA titers: 10 to 12,800).

Table 2. Comparison between the rcELISA and IPMA for 102 field sera

Assays		IPMA	
	Reactions	Positives	Negatives
rcELISA	Positives	74.5% (76/102)*	1.9% (2/102)
	Negatives	8.8% (9/102)	14.7% (15/102)*

* Agreement rate: 74.5% + 14.7% = 89.2%.

DISCUSSION

In order to assess the suitability of the rC protein of PCV2 as an ELISA antigen for the detection of specific anti-PCV2 antibodies, we constructed a recombinant baculovirus which

Table 3. Specificity of the rcELISA to the antisera against other viruses of pig

Antisera to[a)]	Number of samples	OD value (mean ± SD)
PCV2	3	1.205 ± 0.176
PCV1	3	0.020 ± 0.003
PRRSV	3	0.024 ± 0.004
PPV	3	0.025 ± 0.005
Noninfected	3	0.023 ± 0.009

a) PCV2 antiserum titers: IPMA ≥ 10240, PCV1 antiserum titers: IPMA ≥ 2560, PRRSV antiserum titers: ELISA ≥ 12800, and PPV antiserum titers: HI ≥ 640.

could express the rC protein efficiently in Sf-21 cells. A strong specific signal with low background was observed in Western blotting confirming the specific immunoreactivity of the rC protein. The affinity-purified rC protein was used as the rcELISA antigen to make a more consistent binding pattern of the coating antigen to the ELISA plate and to avoid the need to use a mock antigen which is required if the protein is not purified. The reference sera as well as all field sera were tested in duplicates and the average value was used for validation, resulting in an improved reproducibility of the results.

The IPMA has been considered as a sensitive method for the detection of seropositive animals of PCV2 with a disadvantage of needing the virus-infected cells that must be prepared in cells free from PCVs [2, 3]. The technique also requires experienced technicians for examination of the stained plates, which is laborious and time-consuming. Therefore, IPMA is not suitable for large-scale surveys. The ELISA based on a recombinant protein as a coating antigen is a simple and specific method especially for large-scale surveys. In this study, the rcELISA was less expensive and easier to perform than the routine IPMA, without risk for pathogenetic contamination. The data from the rcELISA showed a good agreement with the IPMA for assay of the field sera. Although the results of rcELISA were comparable with that of IPMA, the cut-off dilutions for IPMA-positive sera were different. In this study, sera with an IPMA titer of 10 or more were defined as positive, while for the rcELISA, sera with an ELISA titer of 50 or more were considered positive. When the IPMA was used as reference, there were about 8.8% false negatives and 1.9 % false positives for the rcELISA. Therefore, it was considered that the IPMA might result in a considerable number of false positives due to the low dilution of serum samples.

Recently, an ELISA based on cell-culture-propagated PCV2 and another ELISA based on recombinant major capsid protein have been described by Nawagitgul *et al.* [12]. The two ELISAs are performed by partial purified antigen with the necessity of a negative antigen as a control. Their results also demonstrate that the ELISA based on recombinant major capsid protein is more effective than the ELISA based on cell-culture-propagated virus as a coating antigen. In contrast of this study, PCV2 capsid gene was constructed into the frame with a C-terminal peptide of six histides. The C-terminal peptide of six histides allows the baculovirus-expressed capsid protein to be easily purified by a nickel-chelating resin column to chromatography. The affinity-purified rC protein was used as antigen to improve the sensitivity and specificity of ELISA.

Sequence comparison of PCV1 and PCV2 reveals that the ORF1-encoded protein is more conserved in both viruses than the ORF2-encoded protein (86% and 56% homologies, respectively) [6, 10]. The existence of common immunoreactivity epitope on the ORF1-encoded protein, but the lack of cross-reactivity between the ORF2-encoded proteins in both types of PCV has been described previously [7, 15]. In fact, no cross-reactivity was found in the antisera against PCV1 by the rcELISA. The results suggest that the rcELISA has the capacity of distinguishing PCV2-infected pig sera from PCV1-infected ones.

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