Expression of the porcine circovirus type 2 capsid protein subunits and application to an indirect ELISA

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Abstract

Porcine circovirus type 2 (PCV2) is considered to be associated with post-weaning multisystemic wasting syndrome (PMWS), which is a newly emerged economically important swine disease. The entire coding region of open reading frame 2 (ORF2), encoding the viral capsid protein (Cap), of PCV2 was cloned and sequenced from the clinical specimen obtained from PMWS-affected piglets. Six recombinant subunits, A–F, spanning the defined regions of Cap were produced by E. coli expression system and used as antigens for testing their reactivities with swine sera in the indirect ELISA. The recombinant Cap subunit-based ELISA was evaluated by examining a panel of 12 PCV2-negative and 26 PCV2-positive sera. When the positive/negative cut-off value was set at the mean value of negative sera plus 3 standard deviations, all subunits-based ELISA demonstrated 100% specificities. The N-terminal subunits, A and B, revealed poor reactivity with positive swine sera, whereas, greater immunoreactivity was observed for the C-terminal subunits of which subunits C and D demonstrated good sensitivities of 96.2% and 84.6%, respectively. The recombinant Cap subunits possessing defined antigenicity are easy to produce and the subunit-based ELISA was developed with a high specificity and sensitivity that may provide a useful method for routine serodiagnosis of PCV2 infection.

Keywords: Porcine circovirus; Capsid protein; Recombinant subunit; ELISA

1. Introduction

Porcine circovirus (PCV) is a member of the family Circoviridae consisting of a circular single-stranded DNA genome. The virions areicosahedral, nonenveloped, and 17 nm in diameter (Lukert et al., 1995). PCV type 1 (PCV1) was first isolated in 1973 as a persistent contaminant of a porcine kidney cell line and was nonpathogenic in swine (Allan et al., 1995; Tischer et al., 1986). In contrast, type 2 PCV (PCV2) is closely associated with a newly emerged disease called postweaning multisystemic wasting syndrome (PMWS) in growing pigs (Allan et al., 1998; Chae, 2004) and possibly with porcine dermatitis and nephropathy syndrome (PNDS) and porcine respiratory disease complex (PRDC) (Ellis et al., 2004; Kim et al., 2003).

The PCV2 genome has two large open reading frames (ORFs): ORF1 encodes the Rep proteins involved in viral replication, and ORF2 encodes a major capsid protein (Cap) approximately 30 kDa in size (Hamel et al., 1998; Nawagitgul et al., 2000). A comparison of whole genomic sequences of different PCV2 isolates revealed extensive homology between isolates and the structural gene (ORF2) is highly conserved (Kamstrup et al., 2004; Larochelle et al., 2002). The recombinant Cap protein expressed in insect cells could self-assemble to form virus-like particles (Nawagitgul et al., 2000). A comparison of whole genomic sequences of different PCV2 isolates revealed extensive homology between isolates and the structural gene (ORF2) is highly conserved (Kamstrup et al., 2004; Larochelle et al., 2002). The Cap protein contains the type-specific epitopes (Mahe et al., 2000; Truong et al., 2001), and the recombinant Cap protein reacted strongly with serum from PCV2-infected pigs (Liu et al., 2004; Nawagitgul et al., 2002) suggesting the diagnostic potential of Cap protein.

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The most common diagnostic methods for detecting PCV2 antibodies include indirect fluorescent antibody (IFA) and immunoperoxidase monolayer assay (IPMA) (Allan et al., 1998, 1999). These tests are reliable and sensitive but require experienced technicians and are time-consuming. Enzyme-linked immunosorbent assay (ELISA) can be automated and is relatively rapid and suitable for large-scale screening of serum samples. Recently, several ELISA tests with good specificity and sensitivity using baculovirus-expressed recombinant Cap proteins as antigens have been reported (Blanchard et al., 2003; Liu et al., 2004; Nawagitgul et al., 2002). However, the production of recombinant proteins in the eukaryotic expression systems is expensive. In the present study, various recombinant Cap subunits were produced by *E. coli* expression system and their reactivities with specific swine sera to PCV2 were analyzed for further evaluating the diagnostic specificity and sensitivity of Cap subunit-based indirect ELISA.

2. Materials and methods

2.1. Cloning of the PCV2 ORF2 gene

PCV2 genomic DNA was extracted from 25 mg of frozen lymph node that was collected from a severe PMWS-affected piglet provided by the Animal Disease Diagnostic Center, College of Veterinary Medicine, National Chung Hsing University with a commercial DNA extraction kit (QIAamp Tissue kit, Qiagen) according to the manufacturer’s protocol. The full length of PCV2 ORF2 coding sequence was amplified by polymerase chain reaction (PCR) using a primer pair designed according to the Taiwan strain sequence (GenBank no. AF465211). The sequence of forward primer was 5′-ATGACGTATCCAAGGAGG-3′, and the sequence of reverse primer was 5′-TTAGGGTTAAGTGCGGGGTC-3′. The PCR reaction was carried out in a 50 μl mixture containing 75 mM Tris–HCl (pH 8.8), 20 mM (NH₄)₂SO₄, 0.01% Tween 20, 1.5 mM MgCl₂, 200 μM of dNTPs mixture (dATP, dTTP, dCTP, dGTP), 7.5% dimethyl sulfoxide (DMSO), 1.25 units of *Pfu* DNA polymerase (MBI Fermentas), 10 pmol of specific primer pair, and 1 μg of viral DNA. Amplification with a pre-heated thermocycler (GeneAmp PCR System 9700; Perkin-Elmer) consisted of one cycle at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. The PCR reaction was terminated with a final extension step of 7 min at 72 °C. Amplicons were detected by electrophoresing 10 μl aliquots through 1% agarose gel (FMC) in 0.5 × TAE (20 mM Tris-acetate [pH 8.5], 1 mM EDTA). Amplified PCV2 ORF2 product was 3′-end adenylated with 1U *Taq* DNA polymerase (JMR) in the presence of 200 μM dATP at 72 °C for 10 min. The ORF2 DNA fragment was gel-purified and then cloned onto pCRII-TOPO vector (Invitrogen) by TA cloning strategy to generate the recombinant plasmid pTOPO/ORF2. The nucleotide sequences of the ORF2 gene were determined using an automatic DNA sequencer (ABI-377, PE Applied Biosystems). The coding region of ORF2 gene was 702 bp in size and the nucleotide sequence was...
submitted to the GenBank data library under accession no. AY885225.

2.2. Construction of recombinant expression plasmids

The coding region expressing defined Cap subunits were generated by PCR using variant combination of primer pairs, which are listed in Table 1. Each amplified DNA fragment was gel-purified and digested with appropriate restriction enzymes (New England Biolabs) to reveal sticky ends followed by cloning onto the appropriate pET32 expression vector. Six recombinant plasmids (A–F) expressing the capsid protein amino acid residues 1–50 (A), 51–100 (B), 101–150 (C), 151–200 (D), 185–233 (E), and 51–233 (F), respectively were constructed. The accuracy of the open reading frame of ORF2 coding sequences on each recombinant plasmid was confirmed by DNA sequencing.

2.3. Expression of Cap protein subunits in E. coli

Recombinant expression plasmids were transformed respectively into E. coli BL21 (DE3) competent cells according to the manufacturer’s manual. A single colony of transformant was grown in Luria–Bertani (LB) medium containing 50 µg/ml ampicillin at 37 °C until the OD₆₀₀ reached 1.0. Then isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The culture was incubated for an additional 4 h at 37 °C. The cells were harvested by centrifugation and resuspended in 100 mM Tris–HCl (pH 8.0) containing 1 mM EDTA. Cells were broken by sonication, insoluble material was collected by centrifugation at 15,000 × g for 10 min at 4 °C, and solubilized proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis.

2.4. Western blotting analysis of expressed proteins

Expressed products were resuspended in equal volumes of 2 × SDS-PAGE sample buffer (125 mM Tris-Cl [pH6.8], 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.25% bromophenol blue). Proteins were separated by 12% SDS-PAGE according to the method of Laemmli (1970) and transferred by electroblotting onto PolyScreen PVDF transfer membrane (NEN) using anti-His antibody (C). The location of each expressed protein was indicated by an arrow.

Fig. 1. Expression of Cap subunits in E. coli. Schematic diagram of the expressed coding regions of PCV2 Cap recombinant subunits (A). Bars represent expressed coding sequences and the amino acid residue numbers at both termini are indicated. Recombinant plasmids A–F (lanes A–F) transformed E. coli cells were subjected to induction expression with 1 mM IPTG for 4 h and the cellular lysates were harvested for SDS-PAGE (12%) analysis (B) and Western blotting analysis using anti-His antibody (C). The location of each expressed protein was indicated by an arrow.
semi-dry transfer cell (Bio-Rad) according to the manufacturer’s manual. The membrane was then treated sequentially with blocking solution (phosphate-buffered saline [PBS] containing 5% non-fat skim milk), with appropriate dilution of anti-His antibody (Amersham), and with anti-mouse IgG goat antibody conjugated to peroxidase (Zymed). Finally, the membrane was soaked in a chromogen/substrate solution (TMB single solution; Zymed) for color development.

2.5. Swine serum and indirect fluorescence antibody (IFA) assay

Thirty-eight swine sera collected from the specific pathogen free (SPF) pigs or field pigs were obtained from Animal Technology Institute Taiwan (ATIT). The serum samples at 100-fold dilution were subjected to detect the presence of antibodies to PCV2 by indirect fluorescence antibody (IFA) assay with Porcine Circovirus FA Substrate Slide (VMRD) according to the manufacturer’s protocol.

2.6. Indirect ELISA

ELISA plates (Nunc) were coated at 4°C overnight with 50 μl volume of 10 μg/ml purified Cap subunit in coating buffer (carbonate buffer, pH 9.6). Each well was then thoroughly washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST) and blocked with blocking buffer (PBS containing 1% bovine serum albumin) at 37°C for 1 h. After washing, each well received 50 μl volume of 200-fold dilution of tested swine serum in blocking buffer containing 5% E. coli lysate and incubated at 37°C for 1 h. Subsequently, the plate was washed with PBST thoroughly and each well received 50 μl volume of 2000-fold dilution of goat anti-swine IgG conjugated to peroxidase (Zymed) in blocking buffer at 37°C for 45 min. Finally, the plate was washed with PBST three times followed by with PBS twice and 50 μl of freshly prepared chromogen/substrate solution (ABTS single solution, Zymed) was added into each well and the plate was incubated at room temperature for 30 min. All OD values were measured at 405 nm.

Fig. 2. Reactivities of swine serum to different recombinant Cap subunits in the indirect ELISA. Two IFA-negative and four IFA-positive pig serum samples were analyzed for reactivity (optical density at 405 nm) with immobilized Cap subunits A–F (A–F) at 10 μg/ml each.
15 min. Optical density of each well was read at 405 nm using a microplate reader (MRXII, Dynex). Each sample was analyzed in duplicate, and the OD of duplicates was averaged.

3. Results

3.1. Expression of PCV2 Cap subunits in E. coli

Six recombinant plasmids containing various coding regions of PCV2 ORF2 were constructed in order to overexpress different Cap subunits for utilization as ELISA antigens. Schematic diagrams of the expressed Cap subunits are shown in Fig. 1A. Six subunits including the amino acids residues 1–50 (A), 51–100 (B), 101–150 (C), 151–200 (D), 185–233 (E), and 51–233 (F) respectively were expressed successfully in E. coli. All these expressed fusion proteins were analyzed by SDS-PAGE (Fig. 1B) and confirmed further by Western blotting analysis with anti-His antibody (Fig. 1C).

3.2. Indirect ELISA

Expressed Cap subunits A–F were purified, followed by testing for their reactivities to two IFA-negative and four IFA-positive specific swine serum samples against PCV2 in a preliminary indirect ELISA test. Both N-terminal subunits A and B showed poor reactions to the IFA-positive sera (Fig. 2A and B). In contrast, the C-terminal subunits C–E all demonstrated highly antigenic and significant difference of OD values between IFA negative and positive serum samples (Fig. 2C–E). Thus, those C-terminal subunits C–E and the near full length lacking the N-terminal portion subunit F were further tested with 38 PCV2-specific swine serum samples including 12 IFA-negative and 26 IFA-positive samples in the indirect ELISA.

<table>
<thead>
<tr>
<th>Serum no.</th>
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<tbody>
<tr>
<td></td>
<td>C</td>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>0.092</td>
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<tr>
<td>7</td>
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</tr>
<tr>
<td>8</td>
<td>0.101</td>
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<tr>
<td>9</td>
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<tr>
<td>10</td>
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</tr>
<tr>
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</tr>
<tr>
<td>S.D.</td>
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</tr>
<tr>
<td>Mean + 2 S.D.</td>
<td>0.148</td>
</tr>
<tr>
<td>Mean + 3 S.D.</td>
<td>0.169</td>
</tr>
</tbody>
</table>

* Sera 1–12 were obtained from colostrum-deprived SPF piglets which were all identified as negative for IFA test and were provided by Dr. Yang at ATIT. The OD values at 405 nm (OD405) of 12 IFA-negative swine sera reacted to the C-terminal subunits are shown individually in Table 2 and were averaged to define a positive threshold. When the positive/negative cut-off value was set at mean plus 3 standard deviations (mean + 3 S.D.), all the subunits C to F-based ELISA were demonstrated 100% (12/12 × 100%) specificity. The specificities of subunits D and F-based ELISA decreased to 91.7% (11/12 × 100%) when the cut-off value set at mean plus 2 S.D. Among 26 IFA-positive field serum samples, the subunit C and D-based ELISA revealed better sensitivities of 96.2% (25/26 × 100%) and 84.6% (22/26 × 100%) respectively no matter if the cut-off value was set at mean plus 2 or 3 S.D.

Fig. 3. Reactivities of swine serum to four Cap C-terminal subunits in the indirect ELISA. Twelve IFA-negative and 26 IFA-positive pig serum samples were tested for the reactivity with the C-terminal subunits C–F respectively (A–D) as described in Section 2.
Reactivities (OD_{405} values) of 12 IFA-negative and 26 IFA-positive swine sera to the C-terminal subunits C to F-based ELISA are shown in Fig. 3. Summary of cut-off values and sensitivities of the indirect ELISA to the subunits C–F are shown in Table 3.

4. Discussion

For the purpose of producing recombinant Cap proteins as ELISA antigens, the complete coding region of PCV2 ORF2 gene was cloned and sequenced (GenBank no. AY885225). Six PCV2 Cap recombinant subunits with the defined regions including the amino acid residues 1–50 (subunit A), 51–100 (subunit B), 101–150 (subunit C), 151–200 (subunit D), 185–233 (subunit E) and 51–233 (subunit F) were successfully expressed using E. coli pET32 expression system. Full length of Cap failed to express in E. coli (data not shown), deletion of first 50 amino acids residues (subunit F) circumvented this dilemma. Liu et al. (2001) have demonstrated that the N-terminal 41 amino acid residues of the PCV2 Cap protein possess a nuclear localization signal (NLS). Sequence analysis reveals that this region exhibits possession of functional antigenic sites within the C-terminal region. Two antigenic domains spanning residues 69–83 and 117–131 of Cap protein have been identified to be specific to PCV2 (Mahe et al., 2000). Antibodies to the 117–131 epitope (B-133) were detected in all the experimentally inoculated pigs suggesting that epitope B-133 is a serological marker of PCV2 infection that could be used for the detection of PCV2 antibody response (Truong et al., 2001). In addition, a synthetic peptide-derived polyclonal antibody against the residues 132–146 of PCV2 Cap has been demonstrated to be specific for the detection of PCV2 and not to react with PCV1 or other swine viruses in immunohistochemistry (Ha et al., 2005). Subunit C was demonstrated to have the highest specificity and sensitivity in the indirect ELISA (Table 3). This result is consistent with the properties of B-133 epitope; indeed, both the B–133 epitope and the synthetic peptide localized at subunit C. Further comparison of the amino acid sequences of Cap subunit C region among geographically disparate PCV2 isolates available in GenBank, Taiwan (AY885225.1), Canada (DQ870484.1), Netherlands (AF201897.1), South Africa (AY325495.1), and South Korea (AY672601.1), revealed extremely high identity (96–100%) between isolates. The subunit C is possessed of discriminating immunorelevant epitopes and the high degree of conservation among different isolates should prove this subunit-based ELISA test helpful in the detection of PCV2 specific antibodies.

Nevertheless, the near full length subunit F without the NLS but containing subunit C sequences did not show a compatible sensitivity with subunit C in the indirect ELISA. Since subunit F region may be important for capsid formation of PCV2 virion and the expressed subunit F proteins had a tendency to form insoluble products that may interfere with the binding activity to antibody and the coating efficiency on the ELISA plate. Lekcharoensuk et al. (2004) have demonstrated that first 47 amino acid residues at the N terminus of PCV2 Cap are not involved in the formation of conformational epitope(s), deletion of NLS did not reduce the antigenicity of Cap protein for PCV2. Thus, subunit F appears to be a potential candidate for developing a subunit vaccine of PCV2. Currently, an animal test of immunization of subunit F in swine is undertaken for evaluating its vaccine efficacy. In addition, since the subunit C and D-based ELISA revealed better results, a longer subunit C–D is under development for further improving the sensitivity in the indirect ELISA.

In summary, several recombinant subunits spanning the defined regions of PCV2 Cap were produced and tested their reactivities for the usage as ELISA antigens. A C-terminal near
central Cap subunit-based ELISA is developed with a high specificity and sensitivity that may provide a useful method for routine serodiagnosis of PCV2 infection.

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