Serodiagnostic comparison of enzyme-linked immunosorbent assay and surface plasmon resonance for the detection of antibody to Porcine circovirus type 2

Ho-Seong Cho, Tae-Jung Kim, Jae-Il Lee, Nam-Yong Park

Abstract

This paper describes the cloning and expression of the capsid protein of Porcine circovirus type 2 (PCV2) in an Escherichia coli expression system that was used to produce a fusion protein for subsequent immunologic studies: enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR). Polymerase chain reaction was used to amplify the gene encoding the capsid protein from the DNA of PCV2. The protein was then cloned into a prSET prokaryotic expression vector. Western blot analysis revealed that the recombinant protein gave strong signals on a polyvinylidene difluoride membrane when exposed to the serum from a pig infected with PCV2. The expressed protein was purified and used as an antigen for the ELISA and SPR study. A protein chip based on SPR was developed, and the diagnostic potential of SPR was compared with that of ELISA with the use of 70 serum samples obtained from 6 pig farms. There was a strong positive correlation between the ELISA and SPR titers (r = 0.877, P < 0.01). Therefore, this recombinant capsid protein can be used as an antigen for serologic studies, and the SPR, a label-free method, appears to be a valuable and reproducible tool in the serodiagnosis of a PCV2 infection.

Résumé

Cet article décrit le clonage et l’expression de la protéine de la capside du circovirus porcin de type 2 (PCV2) dans un système d’expression de Escherichia coli qui a été utilisé pour produire une protéine de fusion pour des études immunologiques subséquentes, soit une épreuve immuno-enzymatique (ELISA) et la résonance de surface au plasmon (SPR). La réaction d’amplification en chaîne par la polymérase (PCR) a été utilisée pour amplifier le gène codant la protéine de la capside à partir de l’ADN de PCV2. La protéine a ensuite été clonée dans un vecteur d’expression prokaryote prSET. Une analyse par immunobuvardage a révélé que la protéine recombinante donnait de forts signaux sur une membrane de difluorure de polyvinylidène suite à une exposition à du sérum d’un porc infecté avec PCV2. La protéine exprimée fut purifiée et utilisée comme antigène pour les épreuves ELISA et SPR. Une micropuce protéinique basée sur SPR a été développée et le potentiel diagnostique de SPR a été comparé à celui de l’ELISA en utilisant 70 échantillons de sérum obtenus de six fermes porcinues. Il y avait une forte corrélation positive entre les titres obtenus par ELISA et SPR (r = 0,877, P < 0,01). Ainsi, cette protéine recombinante de la capside peut être utilisée comme antigène dans le cadre d’études sérologiques, et la SPR, une méthode sans marqueur, semble être un outil utile et reproductible pour le séro-diagnostic de l’infection par le PCV2.

(Traduit par Docteur Serge Massier)

Introduction

Postweaning multisystemic wasting syndrome (PMWS) is a disease that affects pigs primarily between the ages of 5 and 12 wk. It has been associated with Porcine circovirus type 2 (PCV2) infection, and its occurrence at this age suggests that infection takes place when the maternal antibodies against PCV2 have declined to subprotective levels (1,2). Generally, a primary or secondary immunodeficiency increases the susceptibility of animals to infectious diseases. Several field and experimental studies have suggested that immunosuppression can develop in PMWS-affected pigs (3). The disease is characterized by weight loss, dyspnea, and jaundice combined with the pathological findings of interstitial pneumonia, generalized enlargement of lymph nodes, hepatitis, and nephritis (4–6). First identified within healthy herds in Western Canada in 1991 (7), PMWS has since been reported in pigs throughout the world (4,5).

A small, nonenveloped, circular DNA virus, PCV2 is only known to cause disease in pigs. Although the precise etiology of PMWS is unclear, PCV2 has been found to be an essential component of this disease. However, cofactors are necessary for the full presentation of PMWS (8). Although experimental infection with PCV2 does not always induce PMWS, the disease has been induced in experimental settings through coinfection with other viral pathogens, such as Porcine parvovirus or Porcine reproductive and respiratory syndrome virus (9–11), or immune stimulation by vaccination (11).

The PCV genome has 2 major open reading frames (ORFs): ORF1, which is essential for viral replication, and ORF2, which encodes a major capsid protein (12). The ORF2 protein contains the type-specific epitopes (13), which suggests that ORF2 contributes to...
development of PMWS, vaccine potential (14), and type-specific diagnostic potential (15). Epidemiologic data suggest that the virulence of PCV2 is strongly related to the presence of the capsid protein.

Surface plasmon resonance (SPR) systems are sensitive to changes in the thickness or refractive index of biomaterials at the interface between a thin gold film and an ambient medium. Therefore, they can characterize the biomolecular interactions in real time without the need for labeling (16,17). Briefly, a light incident on a metal surface at a given angle can excite a surface-bound electromagnetic wave, a surface plasmon, which propagates along the interface between the metal and the ambient medium. Associated with the surface plasmon is an evanescent field that probes local changes in the refractive index of the ambient medium that are induced, for example, by binding a biomolecule to the surface. A change in refractive index will shift the angle of incidence at which SPR excitation occurs. This shift is tracked by monitoring the movement of the intensity minima of the reflected light as a function of time, with use of the Kretschmann configuration, and the binding event is presented as a sensorgram (18).

In this study, recombinant ORF2 protein was expressed and purified and then used to develop a protein chip based on SPR for measuring the antibody titers of PCV2 in swine serum. The diagnostic efficacy of use of the chip was compared with that of the conventional enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Strains, plasmids, and serum

*Escherichia coli* strains JM109 and BL21(DE3)pLysS were purchased from Invitrogen (Carlsbad, California, USA). The pRSET vector (Invitrogen), which has been described previously (19), was used to produce the 6X histidine-tagged protein. The *E. coli* manipulations were performed according to the manufacturer’s instructions. The standard DNA and protein manipulations were carried out as previously described (20,21). A total of 70 pig serum samples were obtained randomly (various breeds and ages; both sexes) at 6 pig farms (an average of 12 samples per farm) and diluted 1:100 for the ELISA and SPR studies.

Construction of bacterial expression vector

As described elsewhere (14), DNA was extracted from viruses in the lymph nodes of pigs on a farm with PCV2 infection. The region coding for the PCV2 capsid protein was amplified by polymerase chain reaction (PCR), with the use of PCV2 genomic DNA as a template, a 5’-primer (5’-ATAT GGATCC ATG TAT CCA AGG AGG C-3’) containing a BamHI restriction site (underlined), and a 3’-primer (5’-TGTG AAGCTT TTA GGG TT T AAG TGG GGG G-3’) containing a HindIII restriction site (underlined). For PCR we used 2 μL (50 ng/μL) of DNA and 1 μL (50 pM) of each primer in 5 μL of a 10X reaction buffer, 5 μL of a 10-mM deoxyribonucleotide triphosphate (dNTP) mixture (2.5 mM of each dNTP), and 1 μL of 5U Ex Taq DNA polymerase (TaKaRa, Shiga, Japan), for a final volume of 50 μL, in a thermal cycler (PTC-100; MJ Research, Waltham, Massachusetts, USA). An incubation step (95°C for 5 min) was followed by 30 cycles of PCR at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension step at 72°C for 7 min. The amplified DNA products were separated by electrophoresis in a 1.0% agarose gel, purified with a PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, digested with 2 restriction enzymes (BamHI and HindIII), and cloned into BamHI+ HindIII-digested pRSET, generating a pRSET-circo-capsid plasmid.

The recombinant DNA was transformed into chemically competent *E. coli* JM109 cells, which were used to propagate the plasmid construct. The transformants were selected on Luria–Bertani (LB) plates containing 100 μg/mL of ampicillin. A miniscale isolation of the plasmid DNA was used to prepare the recombinant plasmid for sequencing with the use of primers corresponding to the capsid gene. The sequencing reactions were performed in an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, California, USA). The sequencing data were assembled and edited by the BLAST method. In addition to the previously described Korean isolate, AF544024 (sequence submitted to GenBank), 2 new isolates were identified by their different sequences coding for the capsid protein (Table I: GenBank accession numbers AY672600 and AY672601). A clone containing the capsid gene corresponding to AY672600 was chosen for further analysis.

Expression of capsid protein

Once the sequence of pRSET-circo-cap had been verified by sequencing, the construct was transformed into BL21(DE3)pLysS host cells (Invitrogen), generating BL21(DE3)pLysS(pRSET-circo-cap). The transformants were grown on LB plates with 100 μg/mL of ampicillin and 35 μg/mL of chloramphenicol at 37°C for 24 h. A single colony was grown in 25 mL of LB and containing ampicillin and chloramphenicol to an optical density (OD) of 0.5. Protein expression was induced with the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration

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Table I. Comparison of the nucleotide sequence of the capsid protein genes of Korean isolates of *Porcine circovirus type 2* (PCV2)

<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Sequence alignment of nucleotides</th>
</tr>
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<tr>
<td>AF544024</td>
<td>G C T A A G T T A C A C G G</td>
</tr>
<tr>
<td>AY672600</td>
<td>A A G C A A G A T A A G A T A</td>
</tr>
<tr>
<td>AY672601</td>
<td>A A G C A A G A T C G A</td>
</tr>
</tbody>
</table>

* Numbering from the ATG codon
The recombinant capsid protein was purified by centrifuging 25 mL of the bacterial culture at 1000 × g and washing the pellet twice in phosphate-buffered saline (PBS: 150 mM NaCl and 10 mM NaH₂PO₄, pH 8.0). The pellet was lysed in a 6 M guanidine lysis buffer with 0.1 mg/mL of lysozyme at room temperature for 60 min. The lysate was then sonicated 30 times for 2 s with a 2-s interval by means of an Ultrasonic VC-100 (Vibracell, Danbury, Connecticut, USA). Next they were heat-shocked 3 times (frozen at −80°C for 30 min, then incubated at 37°C for 10 min). The recombinant protein was purified under denaturing conditions by means of a 6X histidine affinity column (Probond; Invitrogen), according to the manufacturer’s instructions, and then eluted by addition of the appropriate volume of an elution buffer (500 mM NaCl, 8 M urea, and 20 mM sodium phosphate, pH 4.0).

**Purification of capsid protein expressed by E. coli**

The recombinant capsid protein was purified by centrifuging 25 mL of the bacterial culture at 1000 × g and washing the pellet twice in phosphate-buffered saline (PBS: 150 mM NaCl and 10 mM NaH₂PO₄, pH 8.0). The pellet was lysed in a 6 M guanidine lysis buffer with 0.1 mg/mL of lysozyme at room temperature for 60 min. The lysate was then sonicated 30 times for 2 s with a 2-s interval by means of an Ultrasonic VC-100 (Vibracell, Danbury, Connecticut, USA). Next they were heat-shocked 3 times (frozen at −80°C for 30 min, then incubated at 37°C for 10 min). The recombinant protein was purified under denaturing conditions by means of a 6X histidine affinity column (Probond; Invitrogen), according to the manufacturer’s instructions, and then eluted by addition of the appropriate volume of an elution buffer (500 mM NaCl, 8 M urea, and 20 mM sodium phosphate, pH 4.0).

**Titration of IgG with ELISA**

The titer of PCV2-specific antibodies in pig serum was determined by ELISA as described elsewhere (22). Briefly, 96-well plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with the recombinant capsid protein (1 μg/well/90 μL). The plates were then blocked with PBS containing 1% skim milk and washed with PBS plus 0.05% Tween-20, pH 7.4 (PBST). The serum samples in 90 μL of PBST (prepared with 1:100 dilutions) were incubated for 1 h at 37°C. The plates were then emptied and washed 3 times with PBST. The bound antibody was detected with the use of HRP-conjugated goat antibody against pig IgG (SeraC, Oxford, England) according to the manufacturer’s instructions.

**Surface plasmon resonance assay**

The surface of a gold chip was modified for the specific binding of antigens as described elsewhere (23). Briefly, a patterned glass slide with a gold film (K-Mac, Daejeon, Korea) was used to prepare the gold chip. The slides were cleaned in freshly prepared piranha solution (3:1 mixture of concentrated H₂SO₄ and 30% H₂O₂), coated with ProLinker B (Proteogen, Seoul, Korea), washed with DW, and dried under a stream of N₂ gas. Next they were soaked in 3 mM ProLinker B solution for 1 h and rinsed sequentially with CHCl₃, acetone, ethanol, and DW. Each chip was inserted into an Autolab ESPRIT SPR system (Eco Chemie, Utrecht, The Netherlands), which was operated at a constant flow rate of 1 μL/min and a temperature of 25°C. The ORF2 gene of PCV2 was immobilized on the activated chip surface by injecting a 150 ng/μL solution of the antigen in 0.1 M acetate buffer, pH 4.5, for 30 min. After injection of 10 mM PBS for washing and 1% bovine serum albumin (BSA) in PBS for 10 min to block the chip surface, a solution of the antibody or serum sample in a PBS buffer containing 1 mg/mL BSA was allowed to flow onto the chip for 30 min. Subsequently, a solution of 1 mM H₂O₂ and 1 mM cyanide in 50 mM Tris buffer, pH 8.0, containing 10% ethylene glycol was injected onto the chip for 10 min. Each step of the immunoassay was monitored by the Autolab SPR system. The monoclonal antibody against PCV2 ORF2 was prepared by serial 10-fold dilution. The concentrations used were from 0.1 mg/mL to 0.001 μg/mL. All the angle-shift values for the serum samples were calculated by subtracting the BSA angle-shift value.

The Pearson correlation coefficient and SPSS Base 12.0 (SPSS, Chicago, Illinois, USA) were used to analyze the relationship between the titers of PCV2 antibodies in the 70 swine serum samples as determined by SPR and ELISA.

### Results

**Construction of bacterial expression vector**

The pSET-circo-capsid plasmid was constructed by cloning a BamHI-HindIII PCR fragment of PCV2 ORF2 (702 base pairs [bp])

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**Table II. Comparison of the deduced amino acid sequence of PCV2 capsid protein between Korean isolates**

<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Sequence alignment of amino acids (numbering from methionine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF544024</td>
<td>T I F T T</td>
</tr>
<tr>
<td>AY672600</td>
<td>K L V A</td>
</tr>
<tr>
<td>AY672601</td>
<td>K L V A</td>
</tr>
</tbody>
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1 mM) and incubation at 37°C for 4 h. Time-course and expression studies were performed, and 1-mL aliquots were taken at 0, 1, 2, 3, and 4 h after IPTG induction.

The samples were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. For SDS-PAGE, performed under denaturing conditions (21), 10% gels were stained with Coomassie blue. For Western blot analysis, the proteins were first separated in a 10% gel and transferred onto a polyvinylidene difluoride (PVDF) membrane, which was incubated with the monoclonal antibody against PCV2 ORF2 (produced with the viral capsid protein used as antigen; Jenobiotech, Daejeon, Korea), diluted 1:500, and a goat antibody (diluted 1:1000) conjugated with horseradish peroxidase (HRP) against mouse IgG (Pierce Biotechnology, Rockfield, Illinois, USA). The proteins were visualized with use of a diaminobenzidine (DAB) substrate buffer and a DAB concentrate (Serotec, Oxford, England) according to the manufacturer’s instructions.

The pRSET-circo-capsid plasmid was constructed by cloning a 2000;64:0–00 the manufacturer’s instructions.

The titer of PCV2-specific antibodies in pig serum was determined by ELISA as described elsewhere (22). Briefly, 96-well plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with the recombinant capsid protein (1 μg/well/90 μL). The plates were then blocked with PBS containing 1% skim milk and washed with PBS plus 0.05% Tween-20, pH 7.4 (PBST). The serum samples in 90 μL of PBST (prepared with 1:100 dilutions) were incubated for 1 h at 37°C. The plates were then emptied and washed 3 times with PBST. The bound antibody was detected with the use of HRP-conjugated goat antibody against pig IgG (SeraC) diluted 1:500, with incubation for 1 h at 37°C, emptying, and washing 3 times with PBST. A
into the BamHI-HindIII site of the prokaryotic expression vector pRSET. A clone containing the insert was selected, and the plasmid was digested with BamHI and HindIII. By nucleotide sequencing, the capsid protein gene of the clone was shown to be 98.3% homologous with that of isolate AF544024, previously reported from Korea: 690 of the 702 bp were in identical sequence (Table I). The 2 isolates were 97.2% homologous in terms of the deduced amino acid sequence of the protein, with 211 of 217 amino acids in identical sequence (Table II).

**Small-scale expression and time-course studies**

A small-scale (25 mL) expression study of one of the clones (AY672600) was used to confirm expression of the recombinant protein. The presence of the capsid protein in the cell fractions was analyzed by SDS-PAGE and Western blotting with the use of serum from pigs infected with PCV2. In the bands containing the cultured cell samples, there was clear evidence of the production of a 27-kDa protein from pigs infected with PCV2. In the bands containing the cultured cell samples, there was clear evidence of the production of a 27-kDa protein on the SDS-PAGE gel (Figure 1). The protein was recognized by the serum from a pig with a PCV2 infection when blotted onto a PVDF membrane (Figure 2). Analysis of gel stained with Coomassie blue showed that production of the recombinant protein began 1 h after the addition of IPTG. The predicted 27-kDa band was observed with the aliquots taken at 1 through 4 h after IPTG induction. Figure 1 shows the expression pattern of the PCV2 capsid protein generated in the *E. coli* BL21(DE3)pLysS host cells. Figure 2 shows that the recombinant protein reacted with the monoclonal antibody against PCV2 ORF2 and showed a strong signal on the PVDF membrane, indicating that it shared antigenicity with the viral capsid protein.
Studies with ELISA and SPR

In determining the presence of PCV2-specific antibodies in pig serum by means of ELISA, an absorbance at least 0.3 OD above that of the negative controls was considered positive, and 45 (64%) of the 70 samples were positive. An SPR angle shift of at least 50°m was considered positive for PCV2-specific antibodies because the lowest antibody concentration detectable by the protein chip was 0.001 μg/mL, which corresponds to 50°m; 61 (87%) of the 70 samples tested positive by SPR.

Figure 3 shows that the shift in the SPR angle increased in proportion to the amount of PCV2 ORF2 protein, in a linear relationship. Figure 4 shows sensorgrams of a serum sample (sample no. 5; thick line) and the control (BSA binding; thin line).

The titer of PCV2 antibodies in the 70 swine serum samples as determined by SPR showed a good correlation \( r = 0.877, P < 0.01 \) with the levels measured by ELISA (Figure 5).

Discussion

The SPR-based assay was used for the first time to determine PCV2 antibody concentrations in pig serum. The lowest antibody concentration detectable with the protein chip and SPR was 0.001 μg/mL. The assay was more sensitive than standard ELISA, as shown previously (24,25). Although ELISA is very reliable, its methodology is quite laborious; in contrast, use of the SPR method took just 1.5 h in total. We found a good correlation between the antibody levels measured by SPR and ELISA, as did previous investigators.

Until now, most studies using the SPR assay have detected antigens or pathogens such as Foot and mouth disease virus (26), Vaccinia virus, Poliovirus, Cowpea mosaic virus, Tobacco mosaic virus, and Legionella pneumophila (17). However, using the SPR system to detect antibodies has been rare except in studies of tumor antigens (24).

Compared with ELISA, the SPR method with a protein chip appears to be a fast, sensitive, and reproducible tool in the serodiagnosis of PCV2 infection.

Eradication of PCV2 is difficult because the virus is very stable, persisting in the environment of infected herds. The high degree of conservation in the structural genes among the different isolates of PCV2 indicates that a monotypic vaccine might be possible (1). Unfortunately, the gene encoding the capsid protein of the PCV2 isolate used in this study (AY672600) was only 98.3% homologous in nucleotide sequence with that of the previously described Korean isolate (AF544024), and the deduced amino acid sequence of the protein was only 97.2% homologous. In addition, Wen, Guo, and Yang (28) reported that the ORF2 genes of the PCV2 isolated in China showed some variation, with 90.5% to 99.5% and 88% to 100% homology in the nucleotide and amino acid sequences, respectively. Furthermore, there were 2 genetic variants on the same pig farm (AY672600 and AY672601). This indicates that there may be many antigenic variants in the same area, which could hinder a vaccination program that is based on a single reference strain.


