Development and Application of a Double-Antigen Sandwich Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to Porcine Circovirus 2

Meng Ge, Wei Luo, Daliang Jiang, Runcheng Li, Wenwei Zhao, Guoliang Chen, Xingdong Yang, and Xinglong Yu

College of Veterinary Medicine, Hunan Agricultural University, Changsha, China

A double-antigen sandwich enzyme-linked immunosorbent assay (ELISA) is described for detection of porcine circovirus 2 (PCV2) antibodies using the well-characterized recombinant PCV2 capsid protein. In a comparative test of 394 pig sera against an indirect immunofluorescence (IIF) test and a commercial ELISA kit (also based on the recombinant PCV2 capsid protein), the results showed that the diagnostic sensitivity, specificity, and accuracy of the assay were, respectively, 90.61, 94.02, and 91.62% compared with IIF and 94.38, 95.28, and 94.67% compared with the commercial ELISA kit. Assay of 12 PCV-free pigs over a 5-week period produced only PCV2-negative titers by all 3 methods. These results and the sero-profiles of 4 pig farms obtained by both the commercial ELISA kit and the double-antigen sandwich ELISA indicate that the sandwich ELISA is a reliable method for detection of antibodies to PCV2. Additionally, the method described here permits the use of undiluted test serum samples simultaneously loaded with horseradish peroxidase (HRP)-conjugated antigen into the test well, and the complete test procedure can be performed in less than 90 min. This double-antigen sandwich ELISA should be a useful tool to aid swine industry professionals in deciding the intervention strategies for the control of PCV2-associated diseases.

Porcine circovirus (PCV) is a small nonenveloped virus with a diameter of approximately 17 nm and a circular single-stranded DNA genome (36). There are two known PCV species: PCV1 and PCV2 (35). PCV1 has been identified as a persistent noncytopathic contaminant of the continuous PK15 porcine kidney cell line (37), while PCV2 is often associated with many severe diseases, such as postweaning multisystemic wasting syndrome (PMWS) (1, 8), porcine dermatitis and nephritic syndrome (PDNS) (33), porcine respiratory disease complex (PRDC) (16), reproductive disorders (42), enteritis (17), and proliferative and necrotizing pneumonia (PNP) (7). In coinfections, PCV2 also enhances the severity of the diseases caused by porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), swine mycoplasma, Haemophilus parasuis, Streptococcus suis, and other common pathogens of pigs (2, 29, 30) due to its invasion of the immune system. Since PCV2-associated disease is generally recognized as having a significant economic impact on the swine industry worldwide (9), detection and surveillance of PCV2 infection have become important for swine producers.

The PCV2 genome contains two major open reading frames (ORFs), ORF1 and ORF2. ORF1 is essential for viral DNA replication (6), whereas ORF2 encodes a capsid protein involved in the host immune response (3, 15, 18). Capsid protein (Cap) is the only structural protein of PCV2 with good immunogenicity and with a molecular mass of approximately 30 kDa (21). The amino acid sequence identity of Cap between PCV1 and PCV2 is only 65% (27); consequently, although there is some antigenic relationship, only PCV2 Cap protein is recognized by PCV2 polyclonal antibodies, which is the basis for the establishment of methods for specific serological detection of PCV2 (24). Thus, besides indirect immunofluorescence (IIF) (1), immunoperoxidase monolayer assay (IPMA) (8), and some enzyme-linked immunosorbent assays (ELISAs) based on the PCV2 virion (38, 40), more and more ELISAs, mostly indirect, have been developed using recombinant PCV2 Cap protein (4, 20, 25, 28, 34, 44). Here, we report a double-antigen sandwich ELISA, which has certain advantages over current methods for detection of antibodies to PCV2.

MATERIALS AND METHODS

Cells, viruses, and test pig sera. The porcine PK15 cell line, tested as free of PCV, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 6% newborn calf serum (NBCS) (Gibco BRL) and 1% antibiotics (100 U of penicillin G per ml, 100 μg of streptomycin sulfate per ml; Gibco BRL). The PCV2 strain YZH (GenBank accession number AY943819) used in the study was isolated from a superficial inguinal lymph node from a pig with naturally occurring PMWS. Three hundred ninety-four serum samples constituted two sets of samples in the research. First, a total of 60 serum samples from 12 PCV-free pigs were collected weekly for 5 weeks (all the serum samples tested PCV negative by PCR detection). Second, 334 serum samples were obtained from four conventional pig farms (A, B, C, and D) with PMWS (farms A and B were the most severely affected, followed by farms C and D). The origins of the serum samples were as follows; 96 serum samples were from farm A (12 serum samples each from 2-, 4-, 6-, 8-, 10-, 12-, and 14-week-old piglets and 12 samples from sows); 108 serum samples were from farm B (12 serum samples each from 0-, 2-, 4-, 6-, 8-, 10-, 12-, and 14-week-old piglets and 12 samples from sows); 70 serum samples were from farm C (10 samples each from 2-, 5-, 8-, 11-,
Another reference method. Based on the determination criteria, the PCV2 antibody was used according to the manufacturer's instructions as recombinant Cap proteins immobilized on ELISA plates for detection of commercial indirect ELISA kit (Jeno Biotech Inc., South Korea) based on serum samples. PAGE and Western blotting. The Trx protein expressed by pET-32a(123, the Trx-Cap protein. The PCR products were double digested with EcoRI and Sall and cloned into the prokaryotic expression vectors pET-28a(+) and pET-32a(+) (containing a thioredoxin [Trx] coding sequences), and the resulting recombinant expression plasmids, pET28a-ORF2Δ123 and pET32a-Δ123, were used to transform competent DH5α cells. Clones containing the recombinant plasmids were identified by restriction enzyme digestion and DNA sequencing. For expression of the cloned gene, Rosetta(DE3) pLysS Escherichia coli cells (Novagen, Madison, WI) were transformed with pET28a-ORF2Δ123 and pET32a-ORF2Δ123. The cells were also transformed with plasmid pET-32a(+) for analysis of the Trx protein. Single colonies of transformants were grown in Luria-Bertani (LB) medium at 37°C (with shaking) to an optical density at 600 nm (OD600) of about 0.6, and isopropyl-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After induction at 30°C for 6 h, bacteria were collected by centrifugation at 5,000 x g for 10 min. The CapΔ41 protein expressed by pET28a-ORF2Δ123, the Trx-CapΔ41 protein expressed by pET32a-ORF2Δ123, and the Trx protein expressed by pET-32a(+) were purified using the His-Bind Purification kit (Novagen, Madison, WI) according to the manufacturer's instructions. The purified proteins were analyzed by SDS-PAGE and Western blotting.

IIF. Confluent monolayers of uninfected or PCV2-infected PK-15 cells in 96-well plates (Costar, Corning, NY) were fixed in methanol-acetone (1:1) for 30 min at −20°C, and then the plate was washed 3 times with phosphate-buffered saline (PBS) (pH 7.2). After incubation with 5% skim milk-PBS containing 0.05% Tween 20 (PBST) for 1 h at 37°C, serum samples (50 μl) diluted either 1:20 or 1:100 in PBST were added to the plates and incubated for 1 h at 37°C. Following three washes with PBST, fluorescein-conjugated anti-swine immunoglobulin G (KPL, Gaithersburg, MD) diluted 1:200 was added and incubated for 30 min at 37°C. Fluorescence was observed using an inverted fluorescence microscope (BX51; Olympus, Tokyo, Japan). Serum samples showing positive fluorescence at a serum dilution of 1:20 or higher were considered positive.

PCV2 antibody detection with a commercial ELISA kit. Besides IIF, a commercial indirect ELISA kit (Jeno Biotech Inc., South Korea) based on recombinant Cap proteins immobilized on ELISA plates for detection of PCV2 antibody was used according to the manufacturer’s instructions as another reference method. Based on the determination criteria, the samples were positive if the S/P (sample OD450 - negative-control OD450/ positive-control OD450 - negative-control OD450) ratio was ≥0.4, negative if the S/P ratio was <0.3, and equivocal if the S/P ratio was between 0.3 and 0.4.

Preparation of horseradish peroxidase (HRP)-conjugated antigens. HRP-conjugated antigens (CapΔ41 and Trx-CapΔ41) were prepared according to the procedure described by Wilson and Nakane (43). The conjugate was purified by gel filtration chromatography on a Sephacore G200 column (Amersham Pharmacia Biotech). The purified HRP-conjugated antigens were mixed with an equal volume of glycerin and stored at −20°C.

Optimization of the working conditions of the double-antigen sandwich ELISA. The optimal concentrations of coating antigen and HRP-conjugated antigens and the ratio of HRP-conjugated antigens to the testing serum were determined by checkerboard serial-dilution analysis. The detailed optimizing process was as follows. CapΔ41 protein was serially diluted 2-fold from 8.0 μg/ml to 0.25 μg/ml in 0.05 M Na bicarbonate/carbonate buffer (pH 9.6). High-binding 96-well microtiter plates (Costar, Corning, NY) were coated with the protein solution at a volume of 100 μl per well at 4°C for 24 h. Thereafter, the wells were washed 3 times with PBST and blocked with 250 μl 5% dried skim milk in 0.01 mM PBST (pH 7.4) at 37°C for 2 h. Following 3 washes with PBST, the plates were dried at room temperature. HRP-conjugated CapΔ41 and HRP-conjugated Trx-CapΔ41 were serially diluted 2-fold from 1:1,000 to 1:8,000 in PBST. Each dilution was mixed with positive- and negative-control sera and with HRP-conjugated antigen and serum in ratios of 1:1, 4:1, 9:1, 19:1, 49:1, and 99:1. Each mixture was made in triplicate, and then 100-μl aliquots of the mixtures were added to the microtiter plate wells. After incubation for 60 min at 37°C followed by 5 rounds of washing with PBST, 50 μl/well of 3,3,5,5-tetramethylthylene diamine solution (SureBlue Reserve TMB Microwell Peroxidase Substrate; KPL) was added and the plates were incubated for 10 min at 37°C. The chromogenic reaction was stopped by the addition of 50 μl 2 M sulfuric acid, and the OD450 was recorded using a microplate reader (MK3; Thermo Labysystem, Helsinki, Finland). The conditions that gave the highest P/N (positive control OD450/negative control OD450) ratios and an OD450 value of positive serum close to 1.0 were considered to be optimal.

For the optimized conditions, positive-control and negative-control sera were applied in each test, and the antibody levels of the samples were indicated by S/P ratios calculated according to the following formula: S/P = (sample OD450 - negative-control OD450)/(positive-control OD450 - negative-control OD450). In addition, the detection abilities of HRP-conjugated CapΔ41 and HRP-conjugated Trx-CapΔ41 were compared on 92 of the 334 serum samples to determine which was the more suitable HRP-conjugated antigen for the double-antigen sandwich ELISA.

Confirmation of negative-positive cutoff. Sixty swine serum samples sequentially collected from 12 PCV-free pigs that tested negative for PCV2 antibody by both IIF and commercial indirect ELISA were tested using the double-antigen sandwich ELISA. The mean S/P ratio (x) and standard deviation (SD) were calculated, and the cutoff value was determined to be x + 3 SD.

Reproducibility of the double-antigen sandwich ELISA. Evaluation of the assay reproducibility within and between runs was performed as proposed by Jacobson (13). Twelve IIF-positive and 12 IIF-negative field serum samples were selected. For intra-assay (within-plate) reproducibility, three replicates of each serum sample were assigned in the same plate. For interassay (between-run) reproducibility, three replicates of each sample were run in different plates. The mean S/P ratio, SD, and coefficient of variation (CV) were calculated.

Performance evaluation of the double-antigen sandwich ELISA. Both IIF and the commercial kit were used as reference methods to evaluate the performance of the double-antigen sandwich ELISA. The diagnostic sensitivity (DSN), specificity (DSP), and accuracy were calculated using the following formulae: DSN = TP/(TP + FN) × 100, DSP = TN/(TN + FP) × 100, and accuracy = (TP + TN)/total number of serum samples tested × 100, where TP, FN, TN, and FP represent true positive, false negative, true negative, and false positive, respectively (13). The kappa statistic was used to measure the strength of agreement between the results of the double-antigen sandwich ELISA and the reference methods. A kappa statistic of >0.75 represents excellent agreement, 0.40 to 0.75 good to fair agreement, and <0.40 poor agreement (31).

For further validation of the assay, the mean PCV2 antibody S/P ratios in pigs of different categories from farms A, B, C, and D were calculated,
and the overall changes of PCV2 antibody levels in pigs of different age groups were analyzed.

RESULTS

Expression of the recombinant protein. As determined by agarose gel electrophoresis, the PCR product was about 600 bp, corresponding to the expected size of the cap gene without the nuclear localization signal sequence. Both recombinant expression plasmids pET28a-ORF2Δ123 and pET32a-ORF2Δ123 were successfully constructed and expressed in soluble as well as in inclusion body forms. The approximate molecular masses of the CapΔ41 and Trx-CapΔ41 proteins were 26 kDa and 40 kDa, respectively, which are the corresponding theoretical molecular masses of the fusion proteins (Fig. 1A). The soluble forms of these proteins were used in the subsequent development of the ELISA. Western blot results indicated that the CapΔ41 and Trx-CapΔ41 proteins, but not Trx, were recognized by PCV2-specific polyclonal antibody (Fig. 1B).

Results of IIF testing for PCV2 antibody on serum samples from different pig farms. Test results for 60 serum samples sequentially collected from the 12 PCV-free pigs were all negative for PCV2 antibodies. Of 334 field sera, 277 samples were positive and 57 samples were negative for PCV2 antibodies. Most of the piglets and sows from farms A, B, C, and D were PCV2 antibody positive. PCV2 antibody-negative samples were mainly from nursery pigs (Table 1).

Working conditions of the double-antigen sandwich ELISA. The optimal working conditions were 100 ng/well of the coating antigen combined with 1:4,000-diluted HRP-Trx-CapΔ41-conjugated antigen and 1:10 dilution of serum sample, which gave an OD450 value of about 1.0 for the positive-control serum and the coated antigen and 1:4,000-diluted HRP-Trx-CapΔ41-conjugated antigen for the double-antigen sandwich ELISA. The optimal working conditions were 100 ng/well of the coating antigen and 1:10 dilution of serum sample, which gave an OD450 value of around 1.0 for the positive-control serum and the coated antigen and 1:4,000-diluted HRP-CapΔ41-conjugated antigen for the double-antigen sandwich ELISA. The sensitivity of the double-antigen sandwich ELISA was not as high as that of the HRP-Trx-CapΔ41-based ELISA, particularly with positive serum samples tested shortly after seroconversion (some data are shown in Table 2). Therefore, HRP-Trx-CapΔ41 was chosen as the HRP-conjugated antigen for the double-antigen sandwich ELISA.

Reproducibility of double-antigen sandwich ELISA. The reproducibility of the ELISA was determined by comparing the S/P ratios of each serum sample. The intra-assay CV of these samples ranged from 2.06% to 8.27%, with a median value of 5.16%, whereas the interassay CV of these samples ranged between 3.32% and 12.73%, with a median value of 7.95%. In addition, the ELISA also showed good repeatability for 12 negative samples taken shortly after seroconversion (some data are shown in Table 2). Therefore, HRP-Trx-CapΔ41 was chosen as the HRP-conjugated antigen for the double-antigen sandwich ELISA.

TABLE 1 Detection of antibodies to PCV by IIF in swine serum samples from 4 farms

<table>
<thead>
<tr>
<th>Farm</th>
<th>No. of PCV2-specific serum samples from age group (wk)</th>
<th>Sows</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0 2 4 5 6 8 10 11 12</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>12 11 9 8 6 9 10 12 12</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>8 8 7 5 8 9 12 12</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>12 12 12 12 12 12 12 12 12</td>
<td>12</td>
</tr>
</tbody>
</table>

a – no serum tested. The results are presented as the numbers of IIF-positive sera. In each category, 12, 10, and 12 serum samples, respectively, taken from farms A, B, C, and D was tested. Most 0- to 5-week-old piglets and sows were serum positive for PCV2 antibody. In 6- to 12-week-old piglets, the PCV2-positive rates were lower than in 0- to 5-week-old animals, and most samples showed only low fluorescence.

TABLE 2 Comparison of HRP-Trx-CapΔ41 and HRP-CapΔ41-based ELISAs

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>HRP-Trx-CapΔ41</th>
<th>HRP-CapΔ41</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2-1</td>
<td>1.337</td>
<td>1.124</td>
</tr>
<tr>
<td>A2-2</td>
<td>0.997</td>
<td>0.961</td>
</tr>
<tr>
<td>B4-1</td>
<td>0.624</td>
<td>0.685</td>
</tr>
<tr>
<td>B4-2</td>
<td>0.732</td>
<td>0.648</td>
</tr>
<tr>
<td>A14-1</td>
<td>0.902</td>
<td>0.775</td>
</tr>
<tr>
<td>A14-2</td>
<td>0.557</td>
<td>0.489</td>
</tr>
<tr>
<td>B14-1</td>
<td>1.083</td>
<td>0.921</td>
</tr>
<tr>
<td>B14-2</td>
<td>1.194</td>
<td>1.040</td>
</tr>
</tbody>
</table>

a Eight serum samples of the 92 sera testing positive for PCV2 antibody by HRP-Trx-CapΔ41- and HRP-CapΔ41-based ELISA.

b Eight serum samples taken shortly after seroconversion of the 92 sera testing positive for PCV2 antibody by HRP-Trx-CapΔ41-based ELISA but negative by HRP-CapΔ41-based ELISA.
field sera (data not shown). These data indicated that the double-antigen sandwich ELISA was repeatable with low variation.

**Confirmation of negative-positive cutoff:** The mean S/P ratios (x) and SD of the 60 negative sera were calculated as 0.088 and 0.059, respectively. The negative-positive cutoff S/P value of approximately 0.27 was obtained based on the formula x + 3 SD.

**Performance evaluation of the double-antigen sandwich ELISA:** The sensitivity and specificity of the double-antigen sandwich ELISA were evaluated in comparison to those of IIF and the commercial ELISA kit. Based on the results of the 394 serum samples tested by the three methods (Table 3), the DSN and DSP of the ELISA were calculated as 90.61% and 94.02%, respectively, compared with IIF and 94.38% and 95.28% compared with the commercial kit. The positive rates of the 334 field serum samples tested by IIF, the commercial kit, and double-antigen sandwich ELISA were 82.93, 79.94, and 77.25%, respectively. The agreement between the double-antigen sandwich ELISA and IIF was excellent, with a kappa value of 0.88, and that between the double-antigen sandwich ELISA and the commercial indirect ELISA kit was also excellent, with a kappa value of 0.88.

Serum samples from the 12 PCV-free pigs tested negative for PCV2 antibodies by both double-antigen sandwich ELISA and the commercial indirect ELISA kit over the entire 5-week test period (Fig. 2). Seroprofiles based on the mean PCV2 antibody titers over the time covering the whole growth stages of pigs from farms A, B, C, and D are shown in Fig. 3. Similar results were obtained with both the commercial indirect ELISA and the double-antigen sandwich ELISA. All four farms showed seroconversion with similar serological profiles. Initially, the newborns had varying levels of maternal antibody, which declined gradually with the growth of piglets. On farms A, B, C, and D, titers were lowest at 10, 8, 11, and 12 weeks of age, respectively, with all increasing to adult levels over the next 4 weeks.

**DISCUSSION**

Several indirect ELISAs using the recombinant Cap protein expressed in insect cells or *E. coli* as a coating antigen have been described (4, 20, 25, 28, 34, 44). These studies indicated that the recombinant PCV2 Cap protein expressed in either prokaryotes or eukaryotes showed good immunoreactivity and that these proteins were effective diagnostic antigens for establishment of an ELISA method for PCV2 epidemiological investigation and clinical diagnosis. However, the prokaryotic expression system is easier to use in terms of cost and time saving. In the present study, therefore, prokaryotic expression was chosen to produce the recombinant PCV2 Cap protein to develop the double-antigen sandwich ELISA.

Cap protein encoded by the ORF2 gene contains a nuclear localization signal (NLS) of 41 amino acids at its N terminus (22), which seriously affects the heterologous expression of the Cap protein in *E. coli* (39). Fortunately, it has been reported that this NLS has poor reactivity with PCV2-positive swine sera and that deletion of NLS has little effect on the immunoreactivity of the Cap protein (18, 44). Therefore, the 41-amino-acid signal peptide sequence at the 5’ end of the ORF2 gene was deleted, and the resulting ORF2 gene was inserted into the pET28-a(+) and pET32-a(+) expression vectors to construct recombinant plasmids pET28a-ORF2Δ123 and pET32a-ORF2Δ123. Instead of using the inclusion body form of the recombinant Cap protein, which required renaturation before developing the ELISA, we used the soluble form of the protein to establish the double-antigen sandwich ELISA because of its good immunoreactivity without a need for renaturation. While establishing the double-antigen sandwich ELISA using either HRP-CapΔ41 or HRP-Trx-CapΔ41 as the conjugated antigen, we found that inconsistent test results were observed with some positive serum samples, mainly from 10- to 12-week-old pigs shortly after seroconversion. Antibodies in these samples were recognized by HRP-Trx-CapΔ41, but not by HRP-CapΔ41. This is probably due to the modification of some epitopes on the CapΔ41 protein during HRP conjugation, resulting in loss of specific binding activity to serum PCV2 antibody. In this study, the conjugation method was based on the principle that HRP conjugated to a protein by the reaction between the aldehyde group (formed by oxidation of the hydroxyl group) of HRP and a primary amine on a protein and lysine side chain ε-amines on proteins are primary amine groups which are usually present in abundant quantities for modification or conjugation reactions (11). In recombinant Trx-CapΔ41, there are 21 lysine residues, with 8 in CapΔ41 and 13 in Trx. Consequently, the proportion of HRP conjugated to Trx will likely be greater than that conjugated to CapΔ41. Conjugation of HRP to Trx may therefore have spared the important CapΔ41 epitopes in Trx.

**FIG 2** Mean PCV2-specific serum antibody titers of 12 PCV-free pigs over a 5-week period. Standard deviations are indicated by the error bars. The S/P cutoffs of the commercial indirect ELISA kit and the double-antigen sandwich ELISA were 0.4 and 0.27, respectively.

**TABLE 3** Comparison of double-antigen sandwich ELISA with IIF and commercial indirect ELISA kit

<table>
<thead>
<tr>
<th>Double-antigen sandwich ELISA result</th>
<th>No. of serum samples</th>
<th>Commercial kit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IIF</td>
<td>Commercial kit</td>
</tr>
<tr>
<td>Positive</td>
<td>251</td>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>26</td>
<td>Negative</td>
</tr>
<tr>
<td>Total</td>
<td>277</td>
<td>Total</td>
</tr>
<tr>
<td>S/P ratio cutoff</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Total</td>
<td>394</td>
<td>Total</td>
</tr>
<tr>
<td>Positive</td>
<td>258</td>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>127</td>
<td>Negative</td>
</tr>
<tr>
<td>Total</td>
<td>394</td>
<td>Total</td>
</tr>
</tbody>
</table>

a S/P ratio cutoffs: positive, ≥0.27; negative, <0.27.

b For better statistical analysis, the S/P ratio cutoffs of the commercial kit were adjusted as follows: positive, ≥0.35; negative, <0.35 (the original S/P ratio cutoffs were positive, ≥0.40; equivocal, ≥0.30 and <0.40; negative, <0.30).

---

September 2012 Volume 19 Number 9 cvi.asm.org

A Novel ELISA To Detect PCV2 Antibodies

---
CapΔ41 conjugates, resulting in higher detection sensitivity in the double-antigen sandwich ELISA.

IIF is a traditional and classical method for immunodetection, especially for those viruses in which cross-reactivity is not a problem. However, there is cross-reactivity between the Rep proteins of PCV1 and PCV2, so the cross-reaction of IIF on PCV1-infected PK15 cells and on PCV2-infected PK15 cells exists (23). This may explain why the positive rate obtained by IIF was higher than that obtained by the ELISAs with the Cap protein as a coating antigen and may explain why such ELISAs have higher specificity than IIF for PCV2 antibody detection. Despite previous studies describing the high prevalence of PCV1 in swine herds, recent investigations have shown that the prevalence of PCV1 is low in PCV field cases, with PCV2 being the prevailing strain (32). This may explain why the incidence of PCV2 as measured by IIF was in good agreement but slightly higher than with the ELISAs.

Compared with IIF, the commercial indirect ELISA is the main reference method concerned, because there are more common characteristics between the indirect and double-antigen sandwich ELISA. The concordance of the two methods is excellent, but the double-antigen sandwich ELISA may be superior and more suitable for detection of antibodies to PCV2. In recent years, the double-antigen sandwich assay has been used for detection of antibodies to several viruses and toxin, including human hepatitis B virus (19), hepatitis C virus (10), hepatitis E virus (12), influenza A virus (41), rabies virus (45), and verocytotoxin (26), and some clinical trials have shown that the assay is more sensitive and specific than the indirect assay (19,26). In the indirect ELISA system, some of the nonspecific IgG antibodies in the test serum with strong binding ability may be directly adsorbed to either the solid-phase carrier or the surface of the coated antigen, and these nonspecifically bound antibodies, which are not removed in subsequent washing steps, bind to the conjugated secondary antibodies, resulting in a high background or false-positive reactions (10, 26).

Instead of using conjugated secondary antibodies in the indirect ELISA, conjugated antigens were used in double-antigen sandwich ELISA. Therefore, in the double-antigen sandwich ELISA system, serum specific IgG antibody binds to the antigen on the solid phase by one of the two binding sites and captures the conjugated antigen in the liquid phase with the other. Even if the nonspecific IgG molecules attached to the solid phase, they would not capture the conjugated antigen. Thus, double-antigen ELISA generally exhibits low backgrounds. This was demonstrated in the present work, in which the OD450 values of most of the negative sera were below 0.1 by the double-antigen ELISA but between 0.1 and 0.3 in the commercial indirect ELISA (data not shown). Improving the specificity of an indirect ELISA generally requires preparation of high dilutions of test sera, which is inconvenient and prone to dilution error or might decrease sensitivity, whereas the double-antigen sandwich ELISA can be performed without

FIG 3  Mean PCV2-specific serum antibody titers of pigs in different categories from farms A (A), B (B), C (C), and D (D). Standard deviations are indicated by the error bars. The S/P cutoffs of the commercial indirect ELISA kit and the double-antigen sandwich ELISA were 0.4 and 0.27, respectively.
sample dilution (10), which increases sensitivity and simplifies the procedure.

Serological analysis performed on pigs at farms A and B, with severe PMWS; at farm C, with less severe PMWS; and at farm D, with mild PMWS, provided a further check on the validity of the double-antigen sandwich ELISA for detection of antibodies against PCV2. Seroconversion was found to occur earlier at farms A and B than at farms C and D, indicating a relationship between the time of seroconversion and the severity of the disease, i.e., seroconversion occurred earlier in pigs that developed more severe PMWS. This observation is similar to a previous report that seroconversion on PMWS-affected farms appeared earlier than on non-PMWS-affected farms (4). The double-antigen sandwich ELISA indicates similar serological profiles for the 4 farms, which were consistent with the profiles obtained by the reference commercial indirect ELISA and also in agreement with results of previous reports (5, 14, 25).

In conclusion, this is the first report of a sensitive and specific double-antigen sandwich ELISA for detection of PCV2 antibody. Compared with other ELISA methods, this technique is rapid and simple and should prove to be a useful tool for detection of PCV2 antibodies in pigs.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (grant no. 30972167).

REFERENCES


