Development and validation of a recombinant capsid protein-based ELISA for detection of antibody to porcine circovirus type 2

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Abstract

Porcine circovirus type 2 (PCV2) has been recently associated with a number of disease syndromes, especially postweaning multisystemic wasting disease (PMWS). Herein, an alternative indirect enzyme-linked immunosorbent assay (ELISA) for detection of PCV2 antibody was developed using nuclear localization signal-truncated capsid protein of PCV2 produced in Escherichia coli (CAP ELISA). This assay was validated by comparison with an indirect immunofluorescence assay (IIF) and a PCV2-based ELISA. The diagnostic sensitivity (DSN), specificity (DSP) and accuracy of the CAP ELISA were 95.3%, 93.9% and 95.1%, compared with IIF on 1080 field serum samples, and 93.3%, 84.2% and 91.1%, compared with the PCV2-based ELISA on 79 field sera, respectively. Cross-reactivity assay showed that this assay was PCV2-specific. Repeatability tests revealed that the coefficients of variation of positive sera within and between runs were less than 15%. This ELISA is simpler to produce and perform, time-saving and suitable for large scale surveys of PCV2 infection at low cost and the evaluation of the efficiency of various vaccines against PCV2.

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Keywords: Porcine circovirus type 2; Nuclear localization signal-truncated capsid protein; ELISA; Antibody detection

1. Introduction

Postweaning multisystemic wasting syndrome (PMWS), a multifactorial swine disease, characterized clinically by fever, progressive weight loss and respiratory and digestive disorders, was first identified in western Canada in 1996 (Clark, 1997). Thereafter, it has been reported worldwide (Allan and Ellis, 2000; Allan et al., 1999; Chae, 2004; Segales et al., 2005; Segales and Domingo, 2002). The main etiological agent of PMWS has been isolated and verified (Allan et al., 1998), and designated porcine circovirus type 2 (PCV2), which was different from non-pathogenic porcine circovirus type 1 (PCV1), a persistent non-cytopathic contaminant of the continuous PK-15 cell line (Tischer et al., 1982). Serological surveys indicated that both types of PCV were widespread in swine populations (Allan and Ellis, 2000; Segales and Domingo, 2002; Zhou et al., 2006), and there was significant cross-reactivity between PCV1 and PCV2 antigens (Magar et al., 2000).

The genomic organizations of both PCV1 and PCV2 are similar, containing two major open reading frames (ORFs): ORF1 and ORF2 (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). ORF1 of PCV1 and PCV2 encodes two replication-associated proteins (Rep and Rep') while ORF2 encodes a viral capsid protein (Cap) involved in
the yield of the 72-kDa MBP-His\textsubscript{8}-ORF2 fusion protein (IPMA) (Ellis et al., 1998), indirect immunofluorescence diagnostic method for monitoring the status of PCV2 infected herds (Allan and Ellis, 2000). To detect such carrier animals, it is essential to develop a simple and reliable diagnostic method for monitoring the status of PCV2 infection. The serological methods for detection of PCV2 infection, including immunoperoxidase monolayer assay (IPMA) (Ellis et al., 1998), indirect immunofluorescence assay (IIF) (Allan et al., 1998, 1999) and various enzyme-linked immunosorbent assays (ELISA) (Blanchard et al., 2003; Liu et al., 2004; Nawagitgul et al., 2002; Truong et al., 2001; Walker et al., 2000), have been developed by preparation of live virus in PK-15 cells or eukaryotic expression of capsid protein in insect cells or using synthetic B-133 peptide as a diagnostic antigen. In this study, we tried to use a prokaryotic-expressed recombinant protein as a diagnostic antigen in ELISA. Although the entire ORF2 gene of PCV2 had been expressed in the Escherichia coli (E. coli) “XL1-Blue MRF” strain (Liu et al., 2001b), the yield of the 72-kDa MBP-His\textsubscript{8}-ORF2 fusion protein was rather low and this antigen was not further used to develop an ELISA for PCV2. In our previous study, a nuclear localization signal-truncated capsid protein of PCV2 had been produced in E. coli as a soluble protein (Zhou et al., 2005). Herein, the recombinant capsid protein (rCAP) was used as antigen to develop an alternative ELISA (CAP ELISA) for specific and sensitive detection of PCV2 antibody.

2. Materials and methods

2.1. Viruses, cells and sera

The PCV2 strain HZ0201 was originally isolated from a superficial inguinal lymphnode of a pig with naturally occurring PMWS (Zhou et al., 2006). The PCV1 strain was isolated from a PCV1-contaminated PK-15 cell line. A PCV1-free PK-15 cell line was kept in our laboratory, and maintained in Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, GibcoBRL Life Technologies, Grand Island, NY).

Standard swine PCV2- and PCV1-positive sera were collected, respectively from colostrum-deprived (CD) piglets from 21 to 49 days after infection experimentally with PCV2 strain HZ0201 and PCV1 at a dose of $10^{5.6}$ TCID\textsubscript{50} by intra-nasal route. These sera were confirmed by IIF with a titer of 1:1600–12,800. Standard PCV2-negative sera were collected from those CD piglets before infection and 30–90-day-old specific-pathogen-free (SPF) pigs, which were shown to be free of PCV antibodies by IPMA. Positive sera against classic swine fever virus (CSFV), porcine parvovirus (PPV), pseudorabies virus (PrV) and porcine reproductive and respiratory syndrome virus (PRRSV) from SPF pigs were purchased from Chinese Institute of Veterinary Drug Control. One thousand and eighty field swine serum samples were selected randomly from 12 herds with or without PMWS in Zhejiang Province, China during 2001–2004 for evaluation of this assay.

2.2. IIF

IIF was used as the reference method to detect the presence of antibodies to PCV2. One thousand and eighty field swine serum samples were subjected to the IIF assay as described previously (Zhou et al., 2006). Briefly, the confluent monolayer of PK-15 cells infected with PCV2 or free of PCV were fixed in a mixture of equal volume of methanol and acetone for 30 min at −20 °C, and then the plate were thawed and washed with phosphate-buffered saline (PBS). After incubation for 1 h at 37 °C with 5% skimmed milk in PBS containing 0.05% Tween 20 (PBST), serum samples (50 μl) diluted either 1 in 20 or 1 in 100 in PBST were added to the plate and incubated for 1 h at 37 °C. Following three washes with PBST, fluorescein-conjugated anti-swine immunoglobulin G (Sigma) diluted 1 in 100 was added and incubated for 30 min at 37 °C. After washing, positive signal was observed using an inverted fluorescence research microscope IX71 (Olympus Optical Co. Ltd., Japan). Serum samples that gave a positive signal at a serum dilution of 1:20 or higher were called positive.

2.3. Antigen preparation

The rCAP protein was produced as described previously (Zhou et al., 2005). Briefly, recombinant E. coli strain BL21 (Amersham) containing nuclear localization signal-truncated capsid protein gene of PCV2 were grown in 2×YT medium (10 g yeast extract, 16 g tryptone, 5 g NaCl, 100 μg/ml ampicillin, pH 7.0) and induced by a final concentration of 0.1 mM isopropylthio-β-D-thiogalactoside (IPTG) for 5 h at 37 °C. The cells were pelleted and resuspended in PBS, and then lysed by sonication on ice. After centrifugation at 10,000 g for 10 min at 4 °C, the supernatant was loaded to GSTrap FF affinity column (Amersham) according to the manufacturer’s protocol. After washing twice with PBS, the rCAP protein was eluted with an elution buffer (50 mM Tris·HCl, 10 mM glutathione reduced, pH 8.0) and collected. The collected samples were identified by SDS-PAGE and Western blot assays as...
described previously (Sambrook and Russell, 2001). Protein concentration of the rCAP protein was determined by the Bradford assay with bovine serum albumin (BSA, Amresco, Inc., St. Louis, USA) as a standard, and was stored at −70 °C.

2.4. ELISA procedure

High binding 96-well microtiter plates (Canada JET Biochemicals Int’l., Inc. Ontario, Canada) were coated with 100 μl rCAP protein in 0.05 M bicarbonate/carbonate buffer (pH 9.6) overnight at 4 °C after incubation for 1 h at 37 °C. Following three washes in PBS containing 0.05% Tween 20 (PBST), the plates were blocked with 5% skimmed milk for 3 h at 37 °C, and incubated with 100 μl serum samples diluted in PBST containing 0.1% BSA at 37 °C, each sample in duplicate. Following five washes with PBST, the plates were further incubated with 100 μl peroxidase-conjugated goat anti-swine immunoglobulins (Kirkegaard & Perry Laboratories, KPL, USA) in PBST at 37 °C. Then, the plates were washed again, and the colorimetric reaction was developed using 100 μl chromogenic substrate (0.1 mg/ml tetramethylbenzidine (TMB, Sigma), 100 mM acetate buffer, pH 5.6, 1 mM urea hydrogen peroxide) for 10 min at 37 °C. Color development was stopped with 2 M H2SO4, and optical density in PBST at 37 °C. The plates were further incubated with BSA at 37 °C/C176 and the colorimetric reaction was developed using 100 l (TMB, Sigma), 100 mM acetate buffer, pH 5.6, 1 mM urea hydrogen peroxide. After the antigen and antisera dilutions were checked, the conditions that gave highest OD450 ratio after the antigen and antisera dilutions were checked, the conditions that gave highest OD450 ratio was recorded using universal Microplate Reader ELx800 (Bio-Tek Instruments, Inc., Winoski, VT, USA).

2.5. Optimization of ELISA working conditions

On the basis of the procedure described above, the optimal antigen concentration and sera dilution were determined through standard checkerboard titration procedures (Crowther, 2000). Briefly, the rCAP protein was immobilized onto 96-well microtiter plates in serial twofold dilutions from 4.96 μg/ml to 0.31 μg/ml. Correspondingly, standard swine PCV2-positive serum and negative control serum were also diluted in serial twofold dilutions from 1:100 to 1:1600 for optimization. To determine the optimal conjugate dilution in the CAP ELISA, after the antigen and antisera dilutions were checked, the HRP-labeled goat anti-swine IgG (KPL) were added onto the plate at the dilutions of 1:500, 1:1000, 1:2000, 1:4000 and 1:8000. The conditions that gave highest OD450 ratio between positive and negative serum (P/N value) and the OD450 value of positive serum close to 1.0 were scored as optimal working conditions.

After the conditions mentioned above were fixed, the coating buffer was optimized from 0.1 M NaOH solution at pH 13 (NaOH), 0.05 M carbonate–bicarbonate buffer at pH 9.6 (CB), 0.05 M Tris–HCl buffer at pH 8.5 (TB), 0.05 M phosphate-buffered saline at pH 7.4 (PBS), distilled water at pH 6.5 (H2O) and 0.1 M HCl solution at pH 3.0 (HCl). Subsequently, to test optimal exposure time for serum samples and the conjugate, the reaction was stopped by washing after exposure for 8 min, 15 min, 30 min, 45 min, 60 min and 90 min, respectively.

2.6. Reproducibility experiments

Evaluation of assay reproducibility within and between runs was performed as proposed by Jacobson (1998). Sixteen field serum samples (10 IIF positive samples, 6 IIF negative samples) were selected for the reproducibility experiments. 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. Mean S/P ratio, standard deviation (SD) and coefficient of variation (CV) were calculated. The S/P value was calculated by the following formula: S/P = (OD450 of sample – OD450 of negative control)/(OD450 of standard positive control – OD450 of negative control).

2.7. Validation of CAP ELISA

To set a negative–positive cutoff value for this assay, 1080 field serum samples were also tested by the CAP ELISA in duplicate. The S/P ratios of 1080 field sera obtained from the CAP ELISA were compared with the IIF results. A receiver-operating characteristic analysis (ROC) was performed, and a cutoff point was determined so that the diagnostic sensitivity (DSN) and specificity (DSP) were maximized while the sum of false negative and false positive results were minimized.

IIF was used as the reference method to detect the presence of antibodies to PCV2. The DSN and DSP and accuracy of the CAP ELISA 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 indicated true-positive, false-negative, true-negative and false-positive, respectively.

To detect the specificity of the CAP ELISA, positive sera against PCV1 (IIF titer 1:2560), CSFV, PPV, PrV and PRRSV were tested according to the CAP ELISA procedure. Each sample was tested in triplicate, and the S/P ratios were calculated.

For further validation of this assay, 79 serum samples, also tested by IIF, were selected and detected by the CAP ELISA and the modified PCV2-based ELISA (Nawagitgul et al., 2002). For the PCV2-based ELISA, positive and negative antigens were prepared, respectively from PCV2-infected and mock-infected PK-15 cells. The plates were coated with a concentration of 10 μg/ml positive and negative antigens diluted in a carbonate–bicarbonate coating buffer, pH 9.6, and incubated at 4 °C for 36–40 h. Serum samples were diluted 1:100 in the PCV2-based ELISA. The DSN, DSP and accuracy of the CAP ELISA compared with the PCV2-based ELISA for the 79 serum samples were calculated using the above-mentioned formulae. The discrepant samples were further confirmed by IIF.
3. Results

3.1. Results of field serum samples by IIF

The results of field serum samples tested by IIF are summarized in Table 1. Out of 1080 field sera, 933 samples were positive and 147 samples were negative for PCV antibodies. Therein the seroprevalence of PCV2 was 97.4% for sows and gilts, 94.8% for 90–180-day-old pigs, 71.8% for 60–90-day-old pigs, 66.7% for 30–60-day-old pigs and 0 for 0–30-day-old pigs, respectively, and the seroprevalence of PCV2 in swine populations increased from 70.9% to 90.3% in Zhejiang Province during 2001–2004 (Table 1).

3.2. Purification of rCAP antigen

Recombinant capsid protein was expressed as a soluble form and purified by an affinity chromatography. As shown in Fig. 1, most rCAP proteins were eluted from GSTrap FF affinity column with some degraded bands including GST. However, only the 48 kD-rCAP protein could react strongly with swine anti-PCV2 serum in Western blot analysis, whereas the degraded product did not react with anti-PCV2 serum. In addition, the reactivity of rCAP protein with swine anti-PCV1 serum was not observed by Western blot assay (data not shown). All these data confirmed that this antigen could be used to detect specific antibodies against PCV2.

3.3. Optimization of CAP ELISA procedure

In the checkboard ELISAs, the optimal antigen concentration and serum sample dilution were set at 1.24 μg/ml and 1:400 (Fig. 2a and b), based on the standard that the OD450 value of positive serum was close to 1.0, the OD450 ratio between positive and negative serum (P/N value) was highest and the background is lower. Using the same standard, the optimal dilution of the conjugate was defined as 1:2000 (Fig. 2c). After the above-mentioned conditions were checked, the coating buffer was optimized. It was found that 0.05 M Tris–HCl buffer at pH 8.5 was the best coating buffer, giving the highest P/N value, compared with other coating buffers (Fig. 2d). For the optimal exposure time of serum samples or conjugate, it was shown that incubation for 30 min was sufficient and time-saving in the CAP ELISA, as the P/N value was relatively stable following exposure for 30 min (Fig. 2e and f).

3.4. Reproducibility of CAP ELISA

The reproducibility of the test was determined by comparing S/P ratios of each field serum sample. The intra-assay CV of 10 positive serum samples tested ranged from 1.62% to 12.18%, whereas the interassay CV of these positive serum samples ranged between 4.66% and 14.93%. In addition, the CAP ELISA also showed good repeatability for six negative field sera (data not shown). These data showed that this assay was repeatable and yielded a low and acceptable variation.

3.5. Validation of CAP ELISA

Based on ROC analysis of the CAP ELISA, the S/P ratios of serum samples varied from a minimum of -0.135 to a maximum of 0.258 for negative sera and from a minimum of 0.044 to a maximum of 3.25 for positive sera. A cutoff of 0.16 was found to give an optimal result with a DSP of 93.9% and a DSN of 95.3% (Fig. 3). Thus, samples with S/P ratios less than or equal to 0.16 were considered negative, those with ratios more than 0.16

Table 1

<table>
<thead>
<tr>
<th>Year</th>
<th>PCV antibodies in different age of serum samples*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0–30 days</td>
<td>30–60 days</td>
</tr>
<tr>
<td>2001</td>
<td>0/10</td>
<td>5/7</td>
</tr>
<tr>
<td>2002</td>
<td>0/6</td>
<td>9/17</td>
</tr>
<tr>
<td>2003</td>
<td>0/5</td>
<td>32/51</td>
</tr>
<tr>
<td>2004</td>
<td>–</td>
<td>42/57</td>
</tr>
<tr>
<td>Total</td>
<td>0/21</td>
<td>88/132</td>
</tr>
</tbody>
</table>

* Results are presented as number of IIF positive sera/total sera tested.

–, no serum tested.
Fig. 2. Optimization of ELISA working conditions. (a) and (b) Optimization of rCAP protein and serum sample dilutions. (c) Optimization of dilution of conjugates (HRP-labeled goat anti-swine IgG). (d) Optimization of coating buffers: 0.05 M Tris–HCl buffer (TB, pH 8.5), 0.1 M NaOH solution (NaOH, pH 13), 0.05 M carbonate–bicarbonate buffer (CB, pH 9.6), 0.05 M phosphate-buffered saline (PBS, pH 7.4), distilled water (H₂O, pH 6.5) and 0.1 M HCl solution (HCl, pH 3.0); (e) and (f) Optimization of exposure time of sera and the conjugate.

Fig. 3. Frequency plots of number of IIF positive (n = 933) and negative (n = 147) samples of field sera and natural log of S/P ratios obtained from the CAP ELISA demonstrating two overlapping populations. The number in parentheses is the S/P ratio. The negative–positive cutoff was defined as 0.16 with an OD₄₅₀ value of positive serum control close to 1.0 for the CAP ELISA, at which the DSN and DSP of the assay was greater than 90%.
were considered positive. In addition, an OD_{450} value close to 1.0 for positive serum control was also required to achieve the specified negative–positive cutoff. Based on this cutoff, out of 1080 serum samples, 898 and 182 serum samples were classified as positive and negative, respectively (Table 2).

After the cutoff was determined, the specificity of this assay was evaluated by testing the reactivity of antibodies against other porcine viruses with the CAP antigen. The S/P ratios of standard positive sera against PCV1, CSFV, PPV, PrV and PRRSV are shown in Table 3. The S/P ratios of all antisera were significantly less than the cutoff value. These data revealed that there was no cross-reactivity between PCV2 rCAP antigen and antibodies against other porcine viruses, proving that the rCAP antigen was specific for antibody to PCV2.

Compared with the PCV2-based ELISA, 59 of 79 samples were detected as PCV2 antibody positive and 20 samples as negative by the CAP ELISA at a cutoff of 0.16, while 60 of 79 samples were judged as PCV2 antibody positive and 19 samples as negative by the PCV2-based ELISA. There were 56 samples positive and 16 samples negative judged by both methods (Table 4). The DSN, DSP and accuracy of the CAP ELISA were 93.3%, 84.2% and 91.1%, respectively. After further confirmation of the discrepant samples by IIF, the four samples positive in the PCV2-based ELISA were all IIF negative while the three samples negative in the PCV2-based ELISA were two negative and one positive.

### Table 2
Comparison of the CAP ELISA with IIF for 1080 field serum samples

<table>
<thead>
<tr>
<th>CAP ELISA</th>
<th>IIF results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>889</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
</tr>
<tr>
<td>Total</td>
<td>933</td>
</tr>
</tbody>
</table>

* The negative–positive cutoff was 0.16. The DSP, DSN and accuracy of the CAP ELISA was 93.9%, 95.3% and 95.1%, respectively.

### Table 3
Specificity of the CAP ELISA to antisera against other swine viruses

<table>
<thead>
<tr>
<th>Antisera</th>
<th>OD_{450} value (mean ± SEM)</th>
<th>S/P ratio (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSFV</td>
<td>0.176 ± 0.008</td>
<td>0.0293 ± 0.0051</td>
</tr>
<tr>
<td>PRV</td>
<td>0.204 ± 0.004</td>
<td>0.0484 ± 0.0029</td>
</tr>
<tr>
<td>PRRSV</td>
<td>0.186 ± 0.005</td>
<td>0.0363 ± 0.0036</td>
</tr>
<tr>
<td>PCV1</td>
<td>0.192 ± 0.005</td>
<td>0.0403 ± 0.0031</td>
</tr>
<tr>
<td>PPV</td>
<td>0.168 ± 0.007</td>
<td>0.0242 ± 0.0047</td>
</tr>
<tr>
<td>PCV2</td>
<td>1.753 ± 0.035</td>
<td>1.0896 ± 0.0236</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.137 ± 0.003</td>
<td>0.0038 ± 0.0016</td>
</tr>
</tbody>
</table>

* Samples positive for PCV2 have S/P ratio higher than 0.16.

### 4. Discussion

The dramatic increase in reported cases of PMWS in pig farms became a potential threat to global swine industry. As PCV2 is associated with PMWS, detecting PCV2 in pig herds is essential to help control of PMWS. Therefore, PCV2-specific diagnostic tools are necessary to elucidate the course of infection. To date, most serological studies used viral particles of PCV2 as an antigen for the detection of PCV2 infection. PCV2 antibodies are currently detected by IIF or IPMA assays (Allan et al., 1999; Ellis et al., 1998; Magar et al., 2000; Raye et al., 2005; Vicente et al., 2004; Zhou et al., 2006). Thereafter, a competitive ELISA (c-ELISA) and a modified PCV2-based ELISA for specific detection of PCV2 antibodies were developed using PCV2 viral particles (Nawagitgul et al., 2002; Walker et al., 2000). Recently, the ORF2-based ELISA assays were also developed using recombinant capsid protein expressed in baculovirus (Blanchard et al., 2003; Liu et al., 2004; Nawagitgul et al., 2002). However, using antigen from PCV2-infected cells lacked PCV2 specificity for serodiagnosis of PCV2 infection, owing to antigenic cross-reactivity between Rep proteins of PCV1 and PCV2 (Magar et al., 2000), and it was difficult to prepare a purified ELISA antigen from PCV2-infected cells and baculovirus-expressed Cap protein of PCV2 (Blanchard et al., 2003; Nawagitgul et al., 2002). In the present study, an alternative indirect ELISA for detection of antibody against PCV2 was successfully developed using a soluble and purified rCAP protein expressed in E. coli as an antigen, which avoided using the mock antigen as a negative control as described previously (Blanchard et al., 2003; Nawagitgul et al., 2002). Thus this ELISA is simpler to produce and perform, time-saving and more suitable for large scale surveys of PCV2 infection.

The comparison of the CAP ELISA with IIF displayed that the CAP ELISA seemed to be slightly less sensitive than IIF, since a considerable number of the IIF positive sera were classified as negative by the CAP ELISA (Table 2). However, it was believed that antibodies to Rep protein of PCV1 in serum samples might raise sensitivity and non-specificity of the IIF assay for antigenic cross-reactivity between Rep proteins of PCV1 and PCV2 (Magar et al., 2000). The similar conclusion was also drawn from the comparison of the CAP ELISA with the PCV2-based ELISA (Table 4). But the confirmatory
test by IIF showed that the four discrepant samples positive in PCV2-based ELISA sera were negative, which was consistent with the result of the CAP ELISA, indicating that the PCV2 antigen used in the PCV2-based ELISA might cause false positive for bad purity of the antigen and the CAP ELISA was more specific for using a purified specific antigen. Also, the high seroprevalence of PCV2 detected by the CAP ELISA and IIF revealed that PCV2 infection in swine population was widespread in Zhejiang Province, China. The growing trend of seroprevalence of PCV2 implied in this study might highlight the importance of monitoring PCV2 infection in swine population. And the CAP ELISA established here might be helpful for that.

The CAP ELISA established in the present study was sensitive and specific for PCV2 antibodies detection, simple to produce and perform and time-saving, compared with existing serological methods for PCV2 infection. These data in this study would facilitate development of a reliable tool or kit for large scale detection of PCV2 antibodies at low cost, and the evaluation of the efficiency of various vaccines against PCV2.

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