Development of a blocking ELISA for detection of serum neutralizing antibodies against porcine circovirus type 2

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A B S T R A C T

A monoclonal antibody (Mab)-based blocking ELISA was developed for the detection of serum neutralizing antibodies to porcine circovirus type 2 (PCV2). The Mab with neutralizing activity, which was produced by immunizing a recombinant capsid protein of PCV2 expressed in insect cells, was used as the detector antibody. The assay was evaluated in comparison with a serum neutralization assay, and its sensitivity and specificity were determined to be 98.8% and 88.5%, respectively. A significant positive correlation was found between results of the blocking ELISA and the serum neutralization assay (r = 0.9381). The assay was verified by testing experimental and commercial pig sera. A longitudinal antibody profile showed that serum neutralizing antibodies were detected 2 weeks after vaccination and that the detection rate reached 100% at 4 weeks. The serum neutralizing antibody profile showed a decrease from the age of 4 to 13 weeks, and seroconversion after 13 weeks in pigs from a commercial pig farm. Additionally, the positive detection rate in 703 sera collected from nine commercial pig farms was 73%. This report demonstrates that the assay is a simple, specific, sensitive and convenient method for epidemiological surveys and evaluations of serum neutralizing antibodies against PCV2.

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1. Introduction

Postweaning multisystemic wasting syndrome (PMWS) is caused by infection with porcine circovirus type 2 (PCV2) (Allan et al., 1999). PMWS occurs in pig herds that are usually healthy. It is associated with a low morbidity rate but a relatively high mortality rate in 5–12-week-old pigs. Clinically, the disease is characterized by progressive weight loss, respiratory and digestive disorders, and jaundice (Segalés and Domingo, 2002). In addition, PCV2 has also been associated with several other syndromes and diseases, such as porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, porcine respiratory disease complex, granulomatous enteritis, necrotizing lymphadenitis, and possibly exudative dermatitis (Chae, 2005). Recently, the diseases related to PCV2 have been referred to collectively as porcine circovirus disease (PCVD) (Segalés et al., 2005).

PCV2, a member of the genus Circovirus of the family Circoviridae, which is a non-enveloped, single-stranded, circular DNA virus with a genome of 1766–1768 nucleotides (Shang et al., 2009). PCV2 has two major open reading frames (ORFs). ORF1 is essential for viral DNA replication, whereas ORF2 encodes a major capsid protein (Mankertz et al., 1998; Nawagitgul et al., 2000). ORF1 proteins of the two types of PCV have been shown to be related antigenically, whereas ORF2 proteins contain type-specific epitopes (Mahé et al., 2000); this would suggest that PCV2 ORF2 might have a potential causative role in the PCV2-related disease. The conformational epitopes recognized by a monoclonal antibody (Mab) with neutralizing activity against PCV2 were detected in transfected PK-15 cells; residues 231–233 have been found to participate in the formation of conformational epitopes (Shang et al., 2009).

Current testing for antibodies to PCV2 in serum can be performed by an indirect immunofluorescence assay (IIF) (Walker et al., 2000; Racine et al., 2004), immunoperoxidase monolayer assay (IPMA) (Liu et al., 2004; Fort et al., 2007), indirect ELISA (Nawagitgul et al., 2002; Blanchard et al., 2003; Liu et al., 2004; Shang et al., 2008; Pérez-Martin et al., 2008), enzyme immunoassay (EIA) (Shkava et al., 2006), and competitive ELISA (Walker et al., 2000). These methods detect total antibodies, including serum neutralizing antibodies, but do not differentiate between neutralizing antibodies or non-neutralizing antibodies. The serum neutralization assay is the standard method for detection of neutralizing antibodies. However, it is a laborious procedure that takes 2–3 days to complete and requires a containment facility such as a biosafety cabinet. These aspects make the serum neutralization assay inappropriate for mass serological surveillance. In this study, five Mabs were produced and 1D2 Mab was shown to have neutralizing activity against PCV2. A blocking ELISA with 1D2 Mab as a detector antibody was
then developed and applied. This assay could not only be useful for detection of antibodies but also to determine the level of serum neutralizing antibodies against PCV2.

2. Material and methods

2.1. Cells and viruses

*Spodoptera frugiperda* (SF21) cells (Invitrogen, Carlsbad, CA, USA) used for the expression of recombinant capsid protein of PCV2 were grown in Grace’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA-Strasse, Pasching, Austria) and 0.26% trypsin phosphate bean soup (Sigma–Aldrich, NY, USA) at 27 °C. PCV1-free PK-15 cells grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated FBS were used for virus propagation. SP2/0 cells cultured with RPMI 1640 medium containing 10% FBS were used for monoclonal antibody preparation. A high-titer seed recombinant baculovirus expressing recombinant capsid protein derived from a PCV2a strain was produced by Liu et al. (2004). Four different PK-15–adapted PCV2 strains were used in this study. Their origins and genotypes are shown in Table 1. The sequences from stains LG, CL, JF and AH have been deposited in GenBank under accession numbers HM038034, HM038033, HM038022 and HM038030, respectively. PCV2/LG strain (1.2 × 10^6 TCID50/ml) as virus seeds were inoculated simultaneously at a multiplicity of infection (MOI) of 10 into freshly digested PK-15 cell suspensions (2 × 10^5 cells/ml). Viruses were maintained in RPMI 1640 medium containing 2% FBS and 3 mM d-glucosamine (Sigma–Aldrich, NY, USA). After incubation at 37 °C for 120 h, the virus was collected by three freeze–thaw cycles of the cultured infected cells, and then centrifuged at 2500 × g for 10 min at 4 °C. The supernatants containing the viruses were aliquoted and stored at −80 °C.

2.2. Sera

PCV2-positive sera: a 35-day-old healthy piglet that had not been immunized and was negative (by PCR detection) for PCV1 and PCV2 nucleic acids was selected and inoculated intranasally with 1 ml of PCV2/LG strain cultures at 10^5 TCID50. Blood samples were taken weekly. When the antibody titer detected by IPMA was 1:51,200, the animal was killed, and its serum was isolated and designated PCV2-positive serum. Preparation methods of PCV2-positive serum were similar to those for PCV2-positive serum.

PCV2-negative serum: a healthy piglet negative for PCV1 and PCV2 by PCR detection. Pigs 1–3 were designated as the mock-immunized control group; pigs 4–11 were injected intramuscularly with PCV2-inactivated vaccine, at 1 ml/dose (1.0 × 10^5.5 TCID50). This immunization was repeated 3 weeks later. After 7 weeks, the vaccine-immunized group and the mock-immunized control group were challenged by intranasal and intramuscular inoculation of PCV2/YJ (PCV2b) cultures (1.0 × 10^5 TCID50/ml) at 1 ml/dose. Blood samples were taken weekly.

A set of 66 sera, which had not been vaccinated against PCV2, from a commercial pig farm in Heilongjiang province of China were also tested by the blocking ELISA. Blood samples were collected from different categories pigs including gilts (n = 6), sows (n = 6), boars (n = 6), 3-week-old pigs (n = 6), 4-week-old pigs (n = 6), 7-week-old pigs (n = 6), 10-week-old pigs (n = 6), 13-week-old pigs (n = 6), 16-week-old pigs (n = 6), 20-week-old pigs (n = 6), and 24-week-old pigs (n = 6).

A total of 703 sera from nine commercial pig farms in Heilongjiang, Jilin, and Liaoning provinces of China were also tested by the blocking ELISA.

Experimental animals used in the study were treated according to the American Physiological Society’s Guiding Principles in the Care and Use of Animals. Both experimental protocols were approved by the Institutional Animal Care and Use Committee.

2.3. Production, identification and horseradish peroxidase conjugation of monoclonal antibodies

Two 6-week-old female BALB/c mice (from the Laboratory Animal Center of Harbin Veterinary Research Institute, CAAS) were immunized with a recombinant capsid protein of PCV2 expressed by a baculovirus vector (Liu et al., 2004). A 0.5 ml mixture containing 50 μg recombinant capsid protein and 10% volume of ISA 15A VG (Seppic, Shanghai, China) was injected subcutaneously. This immunization was repeated 3 weeks later and after a further 2 weeks the mice were administered intraperitoneally 100 μg recombinant capsid protein without adjuvant. The mouse was subsequently euthanized 3 days later and spleen cells were fused with SP2/0 cells using standard procedures (Galfre and Milstein, 1981). The fused cells were cultured with RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing hypoxanthine–aminopurin–thymidine (Sigma–Aldrich, NY, USA) and supplemented with 20% FBS (PAA-Strasse, Pasching, Austria). Resulting hybridoma cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing hypoxanthine–thymidine (Sigma–Aldrich, NY, USA) and supplemented with 10% FBS (PAA-Strasse, Pasching, Austria). Hybridoma supernatants were removed and screened for the presence of PCV2-specific antibodies by an indirect ELISA (Liu et al., 2004). Selected hybridomas were then cloned three times by limiting dilution, expanded into 75-cm^2 tissue culture flasks, and frozen. Ascites containing Mab were prepared from the mice injected intraperitoneally with 0.5 ml sterile paraffin oil and the hybridomas (10^5 cells/mouse) suspended in RPMI 1640 medium; Mab titer was

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**Table 1** Origins of the PCV2 strains used in this study.

<table>
<thead>
<tr>
<th>Isolates name</th>
<th>Isolate region</th>
<th>Age (weeks)</th>
<th>Clinical history</th>
<th>Isolate tissue</th>
<th>Isolate time</th>
<th>Genotype</th>
<th>Genome length (nt)</th>
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<tr>
<td>LG</td>
<td>Jilin</td>
<td>12</td>
<td>PMWS</td>
<td>Inguinal lymph node</td>
<td>2008</td>
<td>PCV2a</td>
<td>1768</td>
</tr>
<tr>
<td>CL</td>
<td>Jilin</td>
<td>9</td>
<td>PMWS, respiratory signs</td>
<td>Lung</td>
<td>2007</td>
<td>PCV2a</td>
<td>1768</td>
</tr>
<tr>
<td>JF</td>
<td>Jilin</td>
<td>6</td>
<td>PMWS, respiratory signs</td>
<td>Inguinal lymph node</td>
<td>2008</td>
<td>PCV2b</td>
<td>1767</td>
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<tr>
<td>AH</td>
<td>Anhui</td>
<td>5</td>
<td>PMWS</td>
<td>Serum</td>
<td>2008</td>
<td>PCV2d</td>
<td>1766</td>
</tr>
</tbody>
</table>

Eleven pigs were used for the PCV2-inactivated vaccine immunization and challenge tests. These were 30-day-old healthy pigs that had not been immunized and were found to be negative for PCV1 and PCV2 by PCR detection. Pigs 1–3 were designated as the mock-immunized control group; pigs 4–11 were injected intramuscularly with PCV2-inactivated vaccine, at 1 ml/dose (1.0 × 10^5.5 TCID50). This immunization was repeated 3 weeks later. After 7 weeks, the vaccine-immunized group and the mock-immunized control group were challenged by intranasal and intramuscular inoculation of PCV2/YJ (PCV2b) cultures (1.0 × 10^5 TCID50/ml) at 1 ml/dose. Blood samples were taken weekly.

A set of 66 sera, which had not been vaccinated against PCV2, from a commercial pig farm in Heilongjiang province of China were also tested by the blocking ELISA. Blood samples were collected from different categories pigs including gilts (n = 6), sows (n = 6), boars (n = 6), 3-week-old pigs (n = 6), 4-week-old pigs (n = 6), 7-week-old pigs (n = 6), 10-week-old pigs (n = 6), 13-week-old pigs (n = 6), 16-week-old pigs (n = 6), 20-week-old pigs (n = 6), and 24-week-old pigs (n = 6).

A total of 703 sera from nine commercial pig farms in Heilongjiang, Jilin, and Liaoning provinces of China were also tested by the blocking ELISA.

Experimental animals used in the study were treated according to the American Physiological Society’s Guiding Principles in the Care and Use of Animals. Both experimental protocols were approved by the Institutional Animal Care and Use Committee.
determined by the indirect ELISA (Liu et al., 2004) and the ascites of 1D2 were used for purification and labeling.

The isotype of the produced Mabs was determined using the Mouse MonoAb-ID Kit (HRP) (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. This test identifies the IgG1, IgG2a, IgG2b, IgG3, IgA and IgM isotype classes and the κ and λ light chains using monospecific rabbit polyclonal antibodies (Pabs).

The reactivity of the Mabs to PCV2/LG was determined using a Western blot assay. PCV2/LG culture was subjected to three freeze/thaw cycles and was then centrifuged at 15,000 × g for 30 min at 4 °C. Subsequently, the supernatant was centrifuged at 180,000 × g for 4 h at 4 °C, and the pellet was lysed overnight at 4 °C. The ultracentrifuged PCV2/LG samples mixed with an equal volume of Laemmli buffer (2 ×) were boiled for 5 min and separated by standard SDS-PAGE and then transferred to a nitrocellulose membrane in transfer buffer (20 mM Tris–HCl, 192 mM glycine, 0.1% SDS, 20% methanol, pH 8.3) using a Trans-Blot SD Semi-dry Transfer Cell at 15 mA for 30 min. This membrane was blocked with PBS containing 2% skim milk overnight at 4 °C and then incubated with the Mabs and SP2/0 cells culture supernatant at 37 °C for 1 h. After three washes in PBS containing 0.05% Tween20 (PBS-T), membranes were incubated with a 1:4000 dilution of horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (H + L) (Invitrogen, Carlsbad, CA, USA) at 37 °C for 1 h. After three washes in PBS-T, the colorimetric reaction was developed using 3,3′-diaminobenzidine (DAB) substrate.

To produce the detector antibody, the ascites of 1D2 was purified using protein A Sepharose CL-4B (GE Healthcare, Uppsala, Sweden) and labeled using a peroxidase labeling kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions.

2.4. Immunoperoxidase monolayer assay (IPMA)

IPMA was used to detect the reactivity of Mabs to PCV2. IPMA was performed as described previously by Liu et al. (2004). Briefly, 96-well plates containing PCV2/LG-, PCV1- and mock-infected cells were fixed in 33% acetone-PBS for 20 min at room temperature and dried (stored at −20 °C). Supernatants from five Mabs and SP2/0 cells, PCV1 positive sera, and PCV2 positive sera were added to PCV2-PCV1- and mock-infected cells, respectively, and then incubated at 37 °C for 1 h. After the unbound antibodies were washed three times with PBS, a 1:3,000 dilution of HRP-labeled goat anti-mouse IgG (H + L) (Invitrogen, Carlsbad, CA, USA) at 37 °C for 1 h. After three washes in PBS-T, the colorimetric reaction was stopped by adding 50 μl of 1% NaO. Optical density (OD) was measured at 405 nm. The blocking ELISA conditions for each step were investigated by varying conditions for each step while maintaining conditions for all other steps constant, except for the steps of colorimetric reaction and reaction stopping. The blocking ELISA tests were performed in triplicate. The OD value was converted to a percent inhibition (PI) value using the formula: PI (%) = 100 × [1 − (test serum OD405 nm/negative reference serum OD405 nm)]. The cut-off value between positive and negative sera was calculated from the mean percentage inhibition of 100 PCV2 IPMA-negative sera plus 2 or 3 standard deviations (SD) of the mean. This calculation gives 95% or 99% confidence that all negative values will fall within the defined range.

The known reference positive sera against PCV1, PPV, PRV, TGEV, PEDV, CSFV, and PRRSV were detected by the blocking ELISA. Interassay variations for the blocking ELISA were evaluated by testing five sera samples in triplicate.

2.7. Serum neutralization assay

Analytical sensitivity and specificity of the blocking ELISA was compared with the serum neutralization assay by testing 132 serum samples from the PCV2-inactivated vaccine immunization and challenge tests. A sensitive neutralization assay adapted from the method of Meerts et al. (2006) was used. Briefly, after inactivation at 56 °C for 30 min, pig sera were diluted 1:50. PCV2/LG strain cultures were diluted to 2 × 103 TCID50/ml as an indicator virus; 200 μl samples of indicator virus were mixed with the diluted sera at equal volume, and incubated at 37 °C for 2 h. After incubation, this mixture was added to semi-confluent monolayers of PCV-negative PK15 cells in 4 wells of a 96-well plate. After 1 h incubation at 37 °C, cell cultures were washed twice in RPMI 1640 and fresh medium was added. Cell cultures were fixed 36 h later. IPMA with PCV2-positive serum was performed as described by Liu et al. (2004). The number of infected cells per well was determined by light microscopy. The procedure was performed for the standard positive and negative serum control groups, virus control group, and the mock-infected cell control group. The neutralizing activity of a serum was expressed as percentage reduction in the number of infected cells compared with negative serum control. A serum was considered positive when the percentage reduction was greater than 50%. The sensitivity and specificity of the blocking ELISA were calculated by the following formulae: sensitivity = true...
positives × 100/(true positives + false negatives), specificity = true negatives × 100/(true negatives + false positives).

Twenty-four pig serum samples with different levels of neutralizing antibodies from the PCV2-inactivated vaccine immunization and challenge tests were analyzed by the serum neutralization assay, IPMA and the blocking ELISA. Each serum was diluted serially two fold from 1:50 to 1:51,200, and then analyzed by the serum neutralization assay, IPMA and the blocking ELISA, respectively. The serum neutralizing antibodies titers were calculated as the reciprocal of the last dilution in which percentage reduction was greater than 50%. The IPMA and the blocking ELISA titers were calculated as the reciprocal of the last dilution that was positive. The correlation coefficient of the serum neutralization assay and the blocking ELISA, and that of the serum neutralization assay and IPMA was calculated, respectively.

3. Results

3.1. Production and characterization of monoclonal antibodies

Thirty-five hybridomas that produced Mabs against the recombinant capsid protein of PCV2 were frozen. Cloning by limiting dilution resulted in five stable hybridomas. These Mabs were designated 1D2, 2E8, 3A10, 5F2, and 6F10. The isotypes of Mabs were identified using the Mouse MonoAb-ID Kit (HRP). It was shown that the isotypes of 1D2, 2E8, 5F2, and 6F10 were IgG1; also, the light chain of 2E8, 3A10, 5F2, and 6F10 was k type, and that of 1D2 was \( \kappa \) type. Antibody titers of culture supernatants of the five hybridomas and the ascites prepared with them were measured by the indirect ELISA. Antibody titers in the culture supernatants of 1D2, 2E8, 3A10, 5F2, and 6F10 Mabs were 1:1280, 1:1280, 1:640 and 1:1280, respectively, and those in the ascites were 1:2,048,000, 1:512,000, 1:1,024,000, 1:512,000, and 1:1,024,000, respectively. High antibody titers of 1D2 were suitable for further blocking ELISA testing.

The reactivity of the five Mabs to purified PCV2 particles by ultracentrifugation was determined by Western blot analysis. Mabs 2E8, 3A10, 5F2, and 6F10 gave a strong and specific reaction with the 28-kDa capsid protein of PCV2 (Fig. 1, lanes 2, 3, 4, and 5). However, the 1D2 Mab did not give a positive reaction (Fig. 1, lane 1). It was presumed that 1D2 might be directed against the conformational epitope of the capsid protein. No reaction was observed with the SP2/0 cell culture supernatant control (Fig. 1, lane 6).

Neutralizing activity assays of 1D2 Mab for four PCV2 strains and one PCV1 strain by the sensitive neutralization assay. The neutralizing activity of a hybridoma supernatant was expressed as a percentage reduction in the number of infected cells compared with medium.

Fig. 2. Neutralizing activity assays of 1D2 Mab for four PCV2 strains and one PCV1 strain by the sensitive neutralization assay. The neutralizing activity of a hybridoma supernatant was expressed as a percentage reduction in the number of infected cells compared with medium.

The blocking ELISA was established using a PCV2-positive serum, PCV2/LG strain and the neutralizing Mab (1D2) was standardized by checker board titrations. The optimal dilution of the coated PCV2-positive serum was 1:1000. The optimal titer of virus culture was 1.0 × 10⁵ TCID50/ml. The tested pig serum was diluted to 1:100. A working concentration of the enzyme-labeled Mab (1D2) was determined to be a 1:4000 dilution. Coloration was developed by 0.21 mg/ml of 2,2-azino-di [3-ethylbenzthiazoline sulfonic acid] in 0.1 M citrate (pH 4.2) containing 0.003% hydrogen peroxide, and terminated with 1% NaF; the value of OD405 nm of each well was read using a microplate reader.

To determine the cut-off level of PI for the blocking ELISA, 100 known negative pig sera (as determined by IPMA) were examined by the blocking ELISA. The average PI (\( \bar{X} \)) of 100 pig sera samples by the blocking ELISA was 14.19% ± 10.47% (mean ± SD); \( \bar{X} ± SD = 35.13\% \pm 45.6\% \) was considered positive. When PI ≤ 10\% and PI >45.6\%, the serum was considered negative.

3.2. Establishment of the blocking ELISA

Based on the cut-off level of the blocking ELISA, the results of comparative experiments using 132 sera from the PCV2-inactivated vaccine immunization and challenge tests are shown in Table 2. Using the serum neutralization assay, 80 samples were positive and 52 negative, whereas with the blocking ELISA, 85 were positive and 47 were negative. One sample was negative by the
blocking ELISA but positive with the serum neutralization assay, and six samples were positive with the blocking ELISA but negative with the serum neutralization assay. Using the serum neutralization assay as a reference standard, the sensitivity of the blocking ELISA was 98.8% (79/80) and specificity was 88.5% (46/52), indicating that the blocking ELISA approach is associated with high sensitivity and specificity.

Twenty-four pig sera from the PCV2-inactivated vaccine immunization and challenge tests were detected by the serum neutralization assay, IPMA and the blocking ELISA, and antibodies titers were calculated. The results are shown in Table 3. The sensitivity of antibody detected by the blocking ELISA was higher than that detected by the serum neutralization assay, which was about fourfold that of the serum neutralization assay. Furthermore, there was a higher correlation coefficient between the serum neutralization assay and the blocking ELISA (0.9381) than between the serum neutralization assay and IPMA (0.8593), indicating that the antibodies titers detected by the blocking ELISA were more similar to the results of the serum neutralization assay than the results of IPMA. It can be concluded that the antibody titer detected by the blocking ELISA may reflect directly the level of the neutralizing antibody and the sensitivity of the blocking ELISA was higher than that of the serum neutralization assay.

The cross-reactivity seen by the blocking ELISA with several positive reference sera of porcine virus (PCV1, PPV, PRV, TGEV, PEDV, CSFV and PRRSV) showed that, with the exception of PCV2-positive sera (which were positive), all the sera were negative. This demonstrates that the blocking ELISA can detect specifically PCV2 neutralizing antibodies and there was no serum cross-reaction with other viruses. The blocking ELISA reaction plates coated in the same medium and different batches were used to detect five pig sera. The intra-batch variation coefficients ranged from 1.47% to 3.89% and the inter-batch variation coefficients ranged from 2.57% to 7.90%, indicating that the results obtained by the blocking ELISA were easily reproducible.

### 3.4. The dynamics of serum neutralizing antibodies in experimental pigs

Based on the cut-off level of the blocking ELISA, the results of testing experimental pig sera by this method are shown in Fig. 3. Antibodies in 25% (2/8) of the vaccination group were positive at 2 weeks after immunization, in 75% (6/8) at 3 weeks after immunization, and 100% (8/8) at 4 weeks after immunization, without an antibody reaction in the control group. Two weeks after virus challenge (9 weeks after immunization), the antibodies in the control group were positive; the antibody levels in the vaccination group were maintained at a high level from 4 weeks until the challenge tests.

### 3.5. Detection of serum neutralizing antibodies in commercial farms

Sixty-six sera from pigs of different ages from a commercial farm in Heilongjiang province were detected by the blocking ELISA; the PI mean values per category were calculated and are shown in Fig. 4. Based on the cut-off level of the blocking ELISA, the antibody profile found in this commercial farm exhibited a decrease in antibody level from 4- to 13-week-old pigs and seroconversion after 13 weeks. High levels of antibody against PCV2 were detected in 20- and 24-week-old pigs (gildts, sows and boars). In addition, the PI level against PCV2 of 3-week-old pigs was as high as that in sows.

A total of 703 pig sera from nine commercial pig farms in Heilongjiang, Jilin, and Liaoning provinces of China were detected by the blocking ELISA (Table 4). Positive sera were detected in all three regions, with a positive detection rate of 22.7% to 100%, and a total

### Table 2

Coincidence of the blocking ELISA and the serum neutralization assay for 132 sera from the PCV2-inactivated vaccine immunization and challenge tests.

<table>
<thead>
<tr>
<th>Detection methods</th>
<th>The serum neutralization assay</th>
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<tbody>
<tr>
<td></td>
<td>Testing positive numbers</td>
<td>Testing negative numbers</td>
</tr>
<tr>
<td>The blocking ELISA</td>
<td>79</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>46</td>
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<tr>
<td></td>
<td>80</td>
<td>52</td>
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### Table 3

Detection of antibody titers to PCV2 by the serum neutralization assay, IPMA and the blocking ELISA.

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Antibody titer</th>
<th>Serum no.</th>
<th>Antibody titer</th>
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<tbody>
<tr>
<td></td>
<td>SNA</td>
<td>IPMA</td>
<td>B-ELISA</td>
</tr>
<tr>
<td>1</td>
<td>1:50</td>
<td>1:100</td>
<td>1:200</td>
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<tr>
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</table>

SNA: the serum neutralization assay. B-ELISA: the blocking ELISA.
positive detection rate of 73%. These results show that PCV2 was highly prevalent in pig farms in China.

4. Discussion

PCV2 is now recognized as the causal agent of PMWS, an economically harmful wasting disease of young pigs. Although most herds and pigs are PCV2 seropositive, only a few suffer PMWS outbreaks or have individual PMWS cases. The pathogenesis of PCV2 has attracted much attention. PCV2 infection can cause directly cell damage to the immune system, resulting in immune suppression (Darwich et al., 2004). It seems that secondary or opportunistic infections are common in pig infected with PCV2 (Segalés et al., 2004). Not only is PCV2 detected in PMWS-affected pigs, but PRRSV, CSFV, PRV, PPV, and some bacteria have also been detected (Ellis et al., 2004). A high level of viremia and a high titer of PCV2 in lymphoid tissues have been found in pigs with PMWS (Segalés et al., 2005). The persistence of the virus in the body and its high replication rate indicates that the pig’s immune system cannot clear effectively the virus and infected cells. Meerts et al. (2005) showed a correlation between the lack of neutralizing antibodies and an increase in viral replication, and the same investigations later demonstrated an association between a poor neutralizing antibodies response and the development of PMWS (Meerts et al., 2006). Fort et al. (2007) reported that PMWS-affected pigs had lower neutralizing antibodies titers, if any, than healthy animals; neutralizing antibodies titers were also correlated inversely with the PCV2 load in the serum. Neutralizing antibodies thus have an important role in the removal of PCV2 in the circulation and the recovery from disease, suggesting that the humoral immune response may have an important role in PCV2-related diseases.

Methods for detection of serum antibodies against PCV2 include IIF, IPMA, indirect ELISA, and competitive ELISA, of which IIF and IPMA are used most commonly. Although IIF and IPMA have many advantages, the interpretation of the results is somewhat subjective and it is difficult to achieve high-throughput and automated detection, which limits their application. In contrast, ELISA is rapid, the interpretation of results is objective, and throughput is high. The indirect ELISA approach based on recombinant protein had a high specificity, but its sensitivity was lower than that of IPMA (Liu et al., 2004). In addition, although these methods detect total antibodies, including serum neutralizing antibodies, they do not differentiate between neutralizing antibodies or non-neutralizing antibodies. The 1D2 Mab in this study had PCV2-neutralizing activity and did not cross-react with PCV1, indicating that it was feasible for use as a detector antibody. The blocking ELISA established using 1D2 Mab could detect directly serum neutralizing antibodies against PCV2, and has advantages of large-scale production and meets the requirements of automated detection. With high specificity and sensitivity similar to those of IPMA, this assay provided an effective method for epidemiological surveys and evaluation of vaccination with the PCV2 vaccine.

All five Mabs were positive by an indirect-ELISA based on recombinant capsid protein but only 1D2 was able to detect the PCV2 antigen by IPMA. The indirect-ELISA could be used to detect antibodies against inside, outside, linear and structural epitopes because the recombinant capsid protein used in the ELISA was refolded partly. PCV2-IPMA could be used to detect those antibodies against epitopes outside the virus. Therefore, the 1D2 Mab may be specific to the epitope that is outside the PCV2, whereas the other four Mabs may be specific to those inside PCV2. Mabs 2E8, 3A10, 5F2, and 6F10 gave a strong and specific reaction with the 28-kDa capsid protein of PCV2. However, the 1D2 Mab did not give a positive reaction. The SDS-PAGE used in this study was reducing, which denatures the proteins by reducing disulfide linkages, thus overcoming some tertiary protein folding and disrupting quaternary protein structure. Therefore, the 1D2 Mab might be specific to the structural epitope of PCV2.

The 1D2 Mab used as a diagnostic detector in this study was generated by immunizing a recombinant capsid protein of PCV2 that is known to be important immunologically. Strains of PCV2 share 91–100% and 89–100% identity at the nucleotide and protein levels of the capsid protein, respectively (Larochelle et al., 2002). Several studies have suggested that genetic differences in PCV2 are associated with the geographical region from which the isolates originated (Fenaux et al., 2000; Hamel et al., 2000; Mankertz et al., 2000; Kim & Lyoo, 2002; Wen et al., 2005). Therefore, it was necessary to determine if the 1D2 Mab can bind to different genotypes of PCV2. In this regard, 1D2 Mab was analyzed using four different strains of PCV2: LG strain (PCV2a), CL strain (PCV2a), JF strain (PCV2b), and AH strain (PCV2d). More extensive studies with other strains of PCV2 are needed to ensure that 1D2 Mab can bind to a broad spectrum of PCV2. In this study, PCV2a/LG strain was neutralized almost completely by 1D2 Mab. For this reason, the LG strain was selected as the antigen in the blocking ELISA.

Previously, a competitive ELISA was developed for the detection of serum antibodies to PCV2 (Walker et al., 2000). This competitive ELISA used a cell culture isolate of PCV2 as antigen and a PCV2-specific Mab as competing reagent. This competitive ELISA was shown to be highly sensitive, specific and suitable for screening large numbers of sera. In contrast to this competitive ELISA, the blocking ELISA developed in the present study was a sandwich ELISA in which PCV2-positive serum was coated to capture PCV2 particles; therefore, it was not necessary to purify PCV2 to remove other proteins derived from PK-15 cells. Purification of PCV2 is laborious and time-consuming. Therefore, the blocking ELISA plates were coated easily and with a high specificity. The PCV2 capsid protein expressed by recombinant baculovirus was also tested as a coating antigen, but resulted in low specificity and sensitivity (data
bodies titer (Fort et al., 2007). Pigs with high levels of serum PCV2 may invade easily the body and replicate when maternal piglets from being infected with PCV2. With increasing age, the positive; this was related to higher serum neutralizing antibodies in pigs. A serologic study was performed in a commercial pig farm showing a decrease in serum neutralizing antibodies of 3-week-old piglets were positive using the IPMA, which made the procedure more simple and rapid.

The blocking ELISA in this study was evaluated in comparison with the serum neutralization assay, and its sensitivity and specificity were determined to be 98.8% and 88.5%, respectively. The sensitivity was higher than that of the serum neutralization assay, whereas the specificity was lower than that of the serum neutralization assay. Furthermore, a significant positive correlation (0.9381) was found between results of the blocking ELISA and the serum neutralization assay. The sensitivity of antibody detected by the blocking ELISA was higher than that detected by the serum neutralization assay, which was about fourfold that of the serum neutralization assay. However, a lower positive correlation (0.8593) was found between results of IPMA and the serum neutralization assay. The serum neutralization assay was specific for the detection of serum neutralizing antibodies against PCV2, whereas IPMA was specific for the detection of total antibodies against PCV2. Thus, the blocking ELISA was verified to have the capability to detect exclusively PCV2 neutralizing antibodies. The serum neutralization test is the standard method for detecting serum neutralizing antibodies, but this method is complex, laborious, and time-consuming. The blocking ELISA in this study was simple, convenient, had a high-throughput, and was easily standardized. More importantly, it can be concluded that the antibody titer detected by the blocking ELISA may reflect directly the level of the neutralizing antibody. In the future, this comparison should be made with sera derived from other genotypes of PCV2 to validate more thoroughly the blocking ELISA method.

The use of the blocking ELISA for the detection of porcine serum antibody showed that pigs inoculated with inactivated vaccine produced serum neutralizing antibodies earlier and that these antibodies persisted longer. This assay could be utilized to monitor the effects of the PCV2 vaccine. A serologic study was performed in a commercial pig farm using the blocking ELISA. The longitudinal antibody profile found in this commercial pig farm showed a decrease in serum neutralizing antibodies from 4- to 13-week-old pigs and seroconversion after 13 weeks. This period coincides with the decline in the level of antibodies and often with the onset of PMWS cases. The serum neutralizing antibodies of 3-week-old piglets were positive; this was related to higher serum neutralizing antibodies levels in sows. The maternal antibodies could have prevented piglets from being infected with PCV2. With increasing age, the level of maternal antibodies decreased gradually. According to the serological epidemiology survey and the incidence of PMWS, PCV2 may invade easily the body and replicate when maternal antibodies decline. The decrease in viremia most often occurred simultaneously with an increase in the serum neutralizing antibody titer (Fort et al., 2007). Pigs with high levels of serum neutralizing antibodies after infection with PCV2 can resist PCV2 replication and gradually clear the virus in the blood, inducing a higher level of serum neutralizing antibodies. In pigs with low or absent serum neutralizing antibodies, PCV2 replicates significantly and damages the immune system, resulting in secondary infection or synergistic infection, and together with other factors, ultimately develops into PMWS. Although the clinical PCV2-positive rate is high, few cases progress eventually into PMWS. The potential pathogenesis of PMWS through changes in antibodies remains unclear and its pathogenic mechanism requires further investigation.

The results of sera detected from nine commercial pig farms showed that PCV2 is highly prevalent in the pig population in China. Although PCV2 serology has no diagnostic value, this bioindicator can be used to determine when the infection occurs in the herd through analysis of longitudinal or transversal studies. Analysis of the serological profile may increase our knowledge of viral circulation and may be useful for the implementation of vaccination strategies and effective control measures according to the characteristics of an individual herd.

In summary, this study demonstrated that a Mab (with neutralizing activity)-based blocking ELISA could be used as an alternative to the serum neutralization assay for measuring the level of serum neutralizing antibodies to PCV2. One Mab has been produced and shown to have the ability to neutralize four PCV2 strains. The analytical sensitivity and specificity of the Mab, when used as a detector antibody in a blocking ELISA, appeared high for detecting antibodies in pig sera compared with the serum neutralization assay. A significant positive correlation was found between the results obtained with the blocking ELISA and the serum neutralization assay. Preliminary application suggested that this assay could offer the prospect of a convenient and standardized serological test suitable for PCV2 surveillance in pigs and monitoring seroconversion in vaccinated pigs.

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References


