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Original article

Rapid screening of monoclonal antibodies against porcine circovirus type 2 using colloidal gold-based paper test

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Abstract

A proof of concept for using paper test as a suitable method in the production of monoclonal antibodies (MAbs) is reported. The paper test which detects antibodies against porcine circovirus type 2 (PCV2) using colloidal gold-labelled capsid protein as the antigen probe was applied exclusively in the screening of anti-PCV2 MAbs. It allowed the detection of 118 single cell clones within 30 min using naked eyes. MAbs with specific binding to authentic epitopes on the virus were selected using a blocking strategy in which the antibody was pre-incubated with PCV2 viral sample before applying to the test paper. Five hybridomas secreting MAbs against the capsid protein were obtained, with only three of them capable of binding to PCV2. The results were validated and confirmed using enzyme-linked immunosorbent assay and immunofluorescence assay. The paper test is simple, rapid, and independent on professional technicians and proves to be an excellent approach for the screening of MAbs against specific targets.

Key words: porcine circovirus type 2 (PCV2), screening of MAbs, paper test

Introduction

Monoclonal antibodies (MAbs) against complete and incomplete antigens have been produced and utilized in the development of assays for the detection of biomarkers, infectious diseases, hormones and antibiotics since cell fusion technique made it possible to merge immune spleen cells with immortal myeloma cells *in vitro* in 1975 (Kohler and Milstein 1975, Zhang et al. 2006, Warren et al. 2014, Kaushik et al. 2016). Selection of target-specific MAbs is generally achieved through the use of immunological methods such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), and immunoperoxidase monolayer assay (IPMA) (Johnson et al. 2011, Ma et al. 2016, Huang et al. 2019). Although these

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methods exhibit good specificity and sensitivity in aiding the screening of wanted MAbs, there are still some hindrances to them including incubation and washing steps, long operation time, and necessity for experienced professionals and expensive instruments. Immunochromatographic lateral flow strip test (ILFST), also called paper test, has been well-established for the qualitative and semi-quantitative detection of antigens, antibodies and haptens in many fields such as medicine, environmental science and food safety (Yang et al. 2018, Arsawiset and Teepoo 2020, Liu et al. 2020). It is a one-step test that is easier to perform, quicker and cost-effective and eliminates the need for trained personnel (Zhang et al. 2009, Huang et al. 2020). Colloidal gold nanoparticles as reporters are most widely used in paper test for colorimetric detection with naked eyes at the point of care.

To test if colloidal gold nanoparticle-based paper test would facilitate the rapid screening of specific antibodies in the production of MAbs, we prepared a paper test for the detection of antibodies against porcine circovirus type 2 (PCV2) using colloidal gold-labelled capsid protein as the antigen probe and evaluated its efficacy in the production of PCV2-specific MAbs. PCV2 is the causative agent of PCV2 systemic disease (PCV2-SD) that greatly affects the swine industry worldwide (Chae 2004, Nannucci et al. 2020). Capsid protein of PCV2, also the major structural protein containing several epitopes, is highly immunogenic and has been used as a good candidate for the development of subunit vaccines (Meyers et al. 2019, Liu et al. 2020). The production of anti-PCV2 MAbs is essential for the development of novel diagnostic devices that allow in-situ detection of swine viral diseases (Montagnese et al. 2019).

In this work, paper test for the specific detection of PCV2 antibodies was utilized in measuring serum antibody titers upon immunization, the presence of PCV2-specific antibody in cell culture supernatants after cell fusion and the antibody titer in ascites fluids. Experimental results demonstrated that the developed paper test greatly simplified and accelerated the screening of MAbs by providing instant results within 5 min. To our knowledge, this is the first report on the application of paper test in selection of MAbs.

Materials and Methods

Reagents and materials

Dulbecco's Modification of Eagle's Medium (DMEM), RPMI-1640 media, hypoxanthine-aminopterin-thymidine (HAT), hypoxanthine-thymidine (HT), and fetal bovine serum (FBS) were purchased from ThermoFisher Scientific. Polyethylene glycol 4000 (PEG-4000), horseradish peroxidase (HRP)- or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG were bought from Solarbio Life Sciences. Porcine kidney epithelial cells PK-15, NS0 myeloma cell line, PCV2-positive swine sera, and PCV2-negative swine sera were kept in the Key Laboratory of Animal Immunology of the Chinese Ministry of Agriculture. BALB/c mice were purchased from the Experimental Animal Center of Zhengzhou University (Zhengzhou, China) and reared following Guidelines for the Care and Use of Laboratory Animals from the National Health Commission.

Preparation of colloidal gold-based paper test

The preparation of colloidal gold-based test paper was performed as previously described with minor modifications (Jin et al. 2012). In brief, purified capsid (Cap) protein of PCV2 was conjugated with colloidal gold nanoparticles with a mean diameter of 25 nm. Then the conjugate was dispensed onto the conjugate pad of the test paper. A master card consisting of the sample pad, the conjugate pad, the nitrocellulose (NC) membrane, and the absorbent pad was assembled and cut into 2.0 mm wide strips using a guillotine cutter. Staphylococcal protein A (SPA) and purified swine immunoglobulin G (IgG) against PCV2 were dispensed onto the NC membrane and used as test line (T) and control line (C), respectively.

Immunization of BALB/c mice

The animal experiment was approved by the Animal Ethics Committee of Henan Academy of Agricultural Sciences in accordance with China's legislation on animal welfare. For prime immunization, female BALB/c mice were immunized subcutaneously with purified PCV2 virus emulsified with Freund's complete adjuvant. Then, the mice were immunized two more times with PCV2 virus emulsified with Freund's incomplete adjuvant at intervals of two weeks. Serum samples were collected from the tail vein of mice and tested for the presence of PCV2-specific antibodies using paper test. Mice with the highest antibody titers were chosen to receive a final booster immunization by intraperitoneal injection of PCV2 virus four days before cell fusion. Antibody titer was defined as the reciprocal of the highest dilution giving a positive signal in paper test.

Screening of monoclonal antibodies

Spleen cells were harvested and fused with NS0 myeloma cells under the treatment of PEG-4000. Hybridoma cells were grown in HAT-containing RPMI-1640 media supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. Ten days

Table 1. Antibody titers in cell culture supernatants and ascites fluids tested using paper test.

Hydridomas	Cell culture supernatant	Ascites fluid
3C9	3.2×10^2	
6A5	2.5×10^2	4×10^5
8F9	6.4×10^2	3×10^5
9F4	3.2×10^2	4×10^5
15E4	6.4×10^2	

after cell fusion, supernatants of the cell culture were collected and tested for the presence of PCV2-specific antibodies using the paper test. Meanwhile, HT-containing RPMI-1640 media supplemented with 10% FBS were added into the wells to support cell growth. Positive hybridoma cells were transferred from 96 well plate into 24 well plate for expansion. Limiting dilution was performed to obtain single cell clones. Then, the supernatants were tested again using paper test. Expansion of positive hybridoma, limiting dilution, and the detection of PCV2-specific antibodies were repeated two more times to ensure the successful selection of hybridoma cell lines secreting MAbs against PCV2. The reactivity of these MAbs with PCV2 native virus was tested by incubating MAbs with the virus prior to paper test. Ascites fluid was produced by injecting the hybridoma cells into the abdomen of mice and collected for the purification of IgG by precipitation with ammonium sulfate.

Examination of monoclonal antibodies

The specificity of the MAbs was examined using enzyme-linked immunosorbent assay (ELISA). Recombinant E2 protein of classical swine fever virus (CSFV), GP5 protein of porcine reproductive and respiratory syndrome virus (PRRSV), gB protein of pseudorabies virus (PRV), VP1 protein of foot and mouth disease virus (FMDV) and S1 protein of porcine epidemic diarrhea virus (PEDV) were coated onto 96-well plates to determine the specificity of the MAbs in ELISA as previously described (Wang et al. 2016, Liu et al. 2020).

The reactivity of the MAbs with PCV2 native virions was validated using immunofluorescence assay (IFA). PK-15 cells grown in DMEM media in 96-well plates were infected with PCV2 for 24 h before treatment with pre-chilled ethanol at room temperature for 15 min. Then the plates were sequentially incubated with 5% skimmed milk, MAbs, and HRP-conjugated goat anti-mouse IgG at 37°C for 1 h. PCV2-positive swine sera and PCV2-negative swine sera were used as the control to validate the assay and probed with HRP-conjugated goat anti-swine IgG. During each step, the plates were washed with phosphate-buffered saline containing 0.05% tween-20 (PBST). Finally, the cells were observed under a fluorescence microscope and images were taken at 50× magnification using the integrated software of the Leica system.

Results

Principle of the developed paper test

The principle of the developed paper test was based on a sandwich format in which MAbs against PCV2 could be sandwiched by colloidal gold nanoparticle-labelled Cap protein (the antigen probe) and SPA protein at the test line. When PCV2-specific MAbs are present in the sample, they will bind to the antigen probe and be captured at the test line, forming a red band due to the accumulation of gold nanoparticles. In the absence of these antibodies, the antigen probe will not be captured at the test line and thus no red band could be observed. Irrespective of the presence of target antibodies, swine IgG against PCV2 in the control line will combine with the antigen probe to ensure the validity of the test.

Selection of positive hybridoma against PCV2

Previously it has been shown that the sensitivity of the developed paper test is comparable to that of ELISA (Jin et al. 2012). Therefore, paper test was exclusively used to determine serum antibody titers upon vaccination, the presence of antibodies in cell culture supernatants, and the antibody titer in ascites fluids. Spleen cells from the mouse giving a serum antibody titer of more than 1.28×10^4 were harvested for fusion with NS0 myeloma cells. Ten days after cell fusion, 875 wells with stable cell clones were obtained among 1440 wells distributed in 15 cell culture plates. Among them, 118 were confirmed to be single cell clones under light microscopy and tested using paper test. Fourteen positive single cell clones were detected and then subcloned to ensure their monoclonality. Five hybridomas, named 3C9, 6A5, 8F9, 9F4 and 15E4, gave the best reaction in paper test as shown in Table 1 and were selected for further characterization. To select MAbs capable of reacting with PCV2 native virus, paper test based on a blocking format was used in which MAbs 6A5, 8F9, 9F4, 3C9, and 15E4 were pre-incubated with PCV2 viral sample before applying to the test paper.

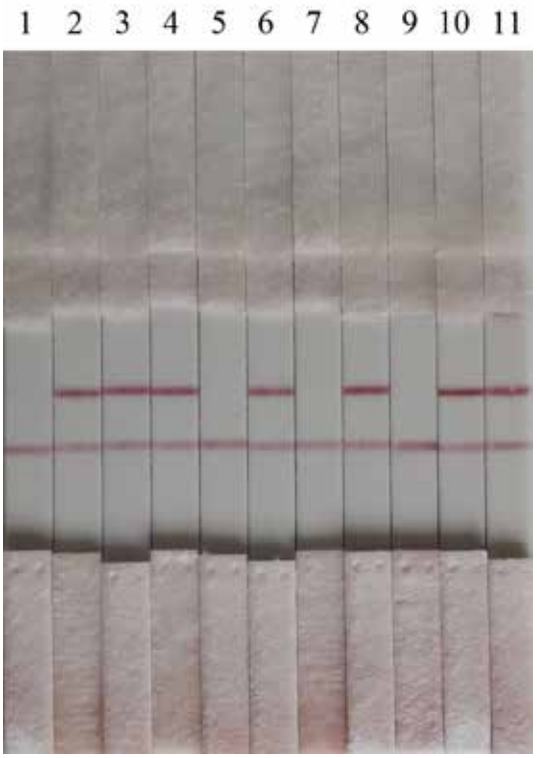

Fig. 1. Selection of MAbs reacting with authentic epitopes on PCV2 virus using a blocking strategy in paper test. MAbs were preincubated with normal saline or PCV2 viral sample and then tested. 1. Normal saline (NS); 2. NS-diluted 3C9; 3. PCV2-diluted 3C9; 4. NS-diluted 6A5; 5. PCV2-diluted 6A5; 6. NS-diluted 8F9; 7. PCV2-diluted 8F9; 8. NS-diluted 9F4; 9. PCV2-diluted 9F4; 10. NS-diluted 15E4; 11. PCV2-diluted 15E4.

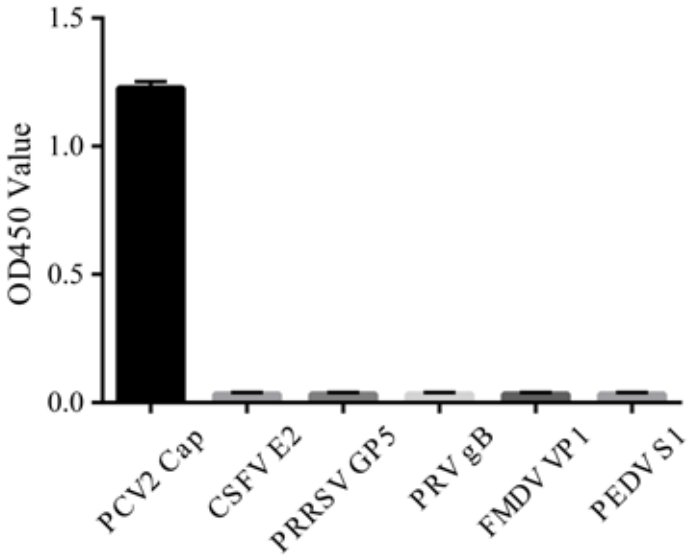

Fig. 2. Cross-reactivity of MAb with different swine pathogens in ELISA.

As shown in Fig. 1, MAbs diluted in normal saline all gave positive reactions in paper test; however, when using PCV2 viral sample as the diluent, only MAbs 3C9 and 15E4 exhibited positive reactions while MAbs 6A5, 8F9, and 9F4 displayed no reaction. The blockage of reaction between MAbs and the gold-labelled antigen probe demonstrated that MAbs 6A5, 8F9, and 9F4 were able to recognize native virions. Ascites fluids were produced only for these three MAbs and the further characterization of MAbs 3C9 and 15E4 was considered unnecessary and stopped. Antibody titers in ascites fluids from the three hybridoma cell lines were determined using paper test and summarized in Table 1. Hence, by using viral samples as diluent in the screen-

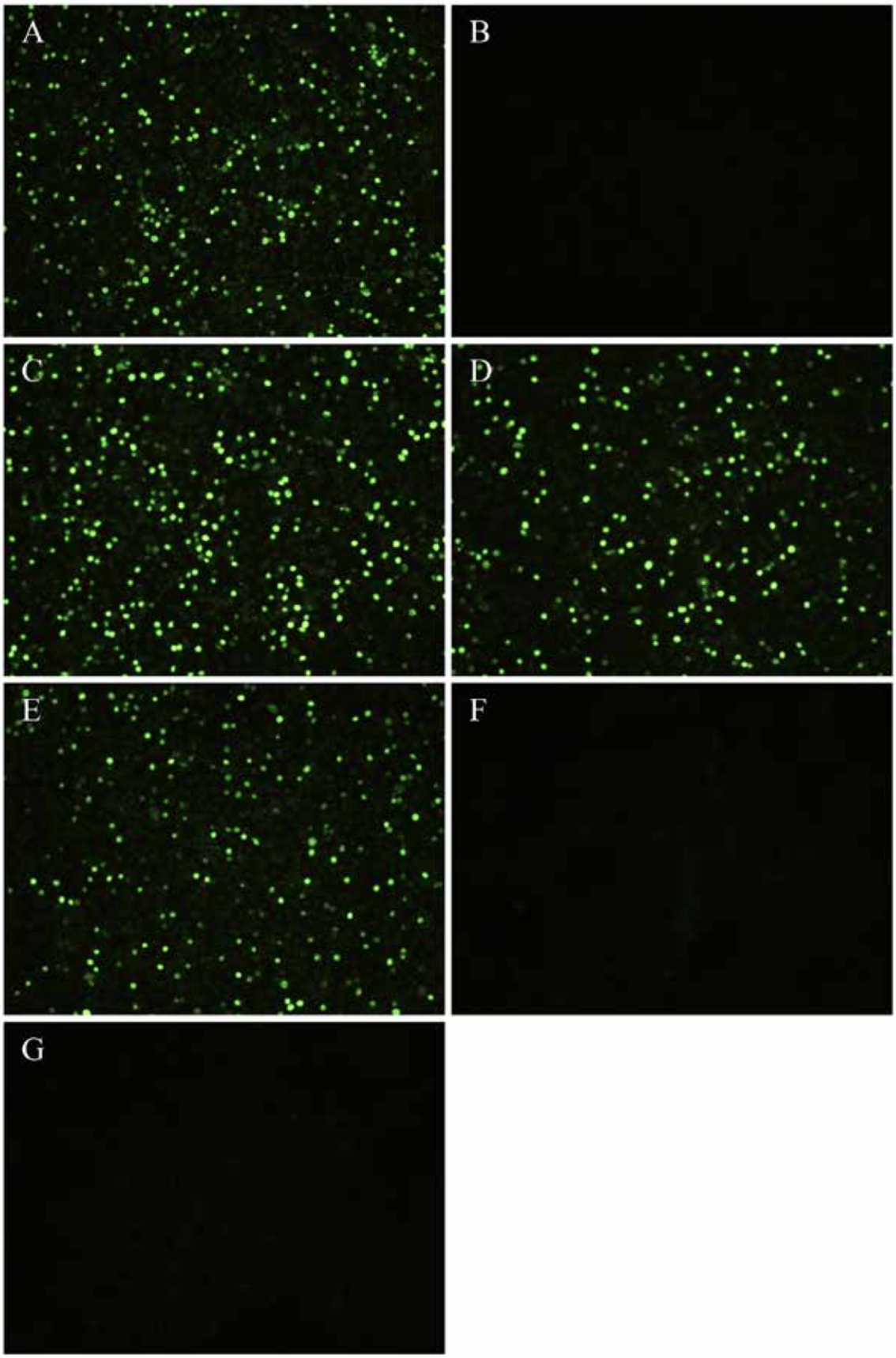

Fig. 3. Reactivity of MAbs with PCV2-infected PK-15 cells in IFA. (A) PCV2-positive swine serum, (B) PCV2-negative swine serum, (C) MAb 6A5, (D) MAb 8F9, (E) MAb 9F4, (F) MAb 3C9, (G) MAb 15E4. x50

ing step, the blocking strategy assures the selection of MAbs able to bind authentic epitopes on the virion.

Validations of monoclonal antibodies to PCV2

The specificity of the MAbs was examined by testing their cross-reactivities with recombinant E2 protein of CSFV, GP5 protein of PRRSV, gB protein of PRV, VP1 protein of FMDV and S1 protein of PEDV using ELISA. It was shown that all the 5 MAbs only gave positive signal in Cap protein-based ELISA and showed no cross-reactivity with proteins of other important swine pathogens (Fig. 2).

The reactivity of these MAbs with PCV2 native virus was determined by testing their reactions with PCV2-infected PK-15 cells using IFA. As shown in Fig. 3, PCV2-positive swine serum gave an obvious signal while PCV2-negative swine serum showed no reaction in IFA, which guaranteed the effectiveness of the assay. MAbs 6A5, 8F9 and 9F4 could apparently bind to PCV2-infected cells, while MAbs 3C9 and 15E4 displayed no signal. This meant these two MAbs only react with Cap protein but show no reaction with PCV2 native virus and confirmed the results observed in the screening step.

Discussion

The selection of MAbs against any pathogen or protein molecule relies on the specific method used. Such a method should at least be specific and sensitive. Currently, immunological methods such as ELISA, IFA and IPMA are generally preferred. However, these methods are limited to be operated by laboratory technicians and are time-consuming. The ideal choice in the selection of MAbs or the detection of pathogens is to have faster, efficient and portable devices and systems that allow on-field detection of analytes by non-specialized personnel (Griol et al. 2019). Paper test, which integrates all the reagents needed into the test paper, is a representative of such a system. It eliminates the use of trained personnel and expensive equipment and allows users to see the results with naked eyes within 5 min in point of care settings (Zhang et al. 2009). It has been applied in the specific and sensitive detection of antigens, antibodies, and haptens as other immunological methods (Li et al. 2017, Na et al. 2020, Li et al. 2021).

In this study, we integrated the convenience and rapidness of paper test into the screening step of MAb production and demonstrated its use in the preparation of MAbs against PCV2. Using colloidal gold-labelled capsid protein as the antigen probe, paper test was applied exclusively in the screening of anti-PCV2 MAbs. It greatly enhanced the efficiency of monoclonal antibody selection by enabling the detection of 118 single cell clones within 30 min using naked eyes. This is superior to other immunological method such as ELISA which takes at least 30 min for a single round of incubation. Besides, the result is easy to be judged with naked eyes and the same read-out could be achieved by two different readers. In the screening step, although the cells were distributed in 15 cell culture plates upon cell fusion, only wells containing singe cell clones chosen with the aid of light microscopy were screened for the presence of PCV2-specific antibodies. Such an operation also accelerated the selection of positive hybridomas secreting anti-PCV2 MAbs and was performed out of the consideration that only a handful of epitopes are available on the Cap protein of PCV2 (Shang et al. 2009). It avoids the characterization of wells containing several cell clones and saves time and cost, albeit risking the loss of MAbs with high-affinity.

In practice, virus-infected cells or purified virus are preferred to select MAbs capable of binding with authentic epitopes on the virus. However, the harvest of large quantity of purified virus or even the culture of virus is hard to be achieved in resource-limiting settings. Recombinant proteins, on the other hand, are relatively inexpensive to obtain, especially when expressed in *Escherichia coli*. Hence, recombinant Cap protein of PCV2 was used in this study as a substitute of the virus itself. Among the 118 single cell clones, 14 wells were detected to be positive for anti-PCV2 antibodies. During expansion of these cell clones, 5 hybridomas showing vigorous growth gave the best reaction in paper test and were selected for further characterization. Yet, the reactivity of these MAbs with recombinant protein cannot fully guarantee their reactivity with native virions. In a blocking format in which MAbs were pre-incubated with PCV2 viral sample before applying to the test paper, only 3 MAbs were blocked while the other 2 MAbs exhibited positive reactions. The blockage of reaction between the MAbs and the gold-labelled antigen probe demonstrated that they were able to recognize native virions. The results were validated and confirmed using immunofluorescence assay. Therefore, the blocking strategy in paper test assured the selection of MAbs reacting with authentic epitopes on native viruses.

In summary, MAbs against PCV2 were produced using paper test as the exclusive screening method in this work. It facilitated the rapid detection of antibodies in serum samples, cell culture supernatants and ascites fluids. Besides, by using a blocking strategy in which PCV2 viral sample was used as the diluent, MAbs that react with PCV2 native virus were selected in the screening step using paper test, avoiding the fur-

ther characterization of MAbs only showing positive reaction with recombinant Cap protein but not PCV2 virus. These results were confirmed by both ELISA and IFA which examined the specificity and reactivity of these MAbs with Cap protein and PCV2, respectively. Hence, paper test has excellent performance in screening wanted antibodies and can be used as a potential method in the production of target-specific monoclonal antibodies. To our knowledge, this is the first report on the application of paper test in selection of monoclonal antibodies.

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