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# Differentiation of Foot-and-Mouth Disease-Infected Pigs from Vaccinated Pigs Using Antibody-Detecting Sandwich ELISA

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ABSTRACT. The presence of serum antibodies for nonstructural proteins of the foot-and-mouth disease virus (FMDV) can differentiate FMDV-infected animals from vaccinated animals. In this study, a sandwich ELISA was developed for rapid detection of the foot-and-mouth disease (FMD) antibodies; it was based on an *Escherichia coli*-expressed, highly conserved region of the 3ABC nonstructural protein of the FMDV O/TW/99 strain and a monoclonal antibody derived from the expressed protein. The diagnostic sensitivity of the assay was 98.4%, and the diagnostic specificity was 100% for naïve and vaccinated pigs; the detection ability of the assay was comparable those of the PrioCHECK and UBI kits. There was 97.5, 93.4 and 66.6% agreement between the results obtained from our ELISA and those obtained from the PrioCHECK, UBI and CHEKIT kits, respectively. The kappa statistics were 0.95, 0.87 and 0.37, respectively. Moreover, antibodies for nonstructural proteins of the serotypes A, C, Asia 1, SAT 1, SAT 2 and SAT 3 were also detected in bovine sera. Furthermore, the absence of cross-reactions generated by different antibody titers against the swine vesicular disease virus and vesicular stomatitis virus (VSV) was also highlighted in this assay's specificity.
KEY WORDS: antibody detection, foot-and-mouth disease, non-structural protein, sandwich ELISA.

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Foot-and-mouth disease (FMD) is one of the most contagious diseases amongst artiodactyla (cloven-hoofed mammals) and thus can have economically devastating effects [27]. Foot-and-mouth disease virus (FMDV) belongs to the *Aphthovirus* genus in the family *Picornaviridae*. It is a non-enveloped virus with a positive-sense, single-stranded RNA genome that is 8.5 kb in length and is immediately translated into a polyprotein upon entry of the virus into a host cell. This polyprotein is posttranslationally cleaved to yield 12 mature proteins [30, 33]. The 12 proteins comprise, from 5' to 3', a set of four structural capsid proteins (1A, 1B, 1C and 1D, also known as VP4, VP2, VP3 and VP1, respectively) and eight nonstructural proteins (NSPs; Lpro, 2A, 2B, 2C, 3A, 3B, 3Cpro and 3Dpol) [31]. The structural proteins (SPs) 1A to 1D assemble to form the capsid of the virion. With the exception of 1A, they are the first line of contact between the host cell and the virus. The interaction between the biological systems occurs specically between the capsid protein VP1, the Arginine-Glycine-Aspartic acid (RGD)-dependent integrins and cell surface heparan sulfate (HS) glycosaminoglycan receptors [21]. The NSPs are involved in RNA replication, polyprotein processing, protease cleavage, inhibition of MHC class I expression, stimulation of VPg uridylylation, increasing membrane permeability and function of the early secretory pathway [16, 20, 28]. The high mutation rates [2, 14] and population bottlenecks that occur during the transmission of RNA viruses such as FMDV [15] account for the high genetic and antigenic variability of these viruses. This genetic variability is translated into the existence of seven immunologically diverse serotypes distributed around the world (southern African types [SAT] 1 to 3 and Asia 1, A, O and C), which have varied degrees of selective genetic variability [18, 22, 38]. Infection with one serotype does not confer protection against another. Globally, FMDV serotypes O and A are the most prevalent.

In 1997, an epidemic of FMD serotype O occurred in Taiwan and caused great economic losses (estimated at over US$ 1.6 billion) due to control measures and trade regulations [40]. The FMDV strain referred to as O/TW/1997 is a prototype of a porcinophilic strain that only infects swine [40]. The deletion at codons 93–102 within the 3A nonstructural protein region of the strain restricts viral replication in bovine epithelial cells *in vitro* [3].

In 1999, another FMD strain was discovered in subclinically infected cattle on Kinmen Island in Taiwan. This strain, O/TW/2/1999 (PanAsia strain), differed from O/TW/1997 in that it had a full-length 3A nonstructural protein that gave the virus the capability to infect cattle [19]. In response to the FMD outbreak, the Taiwanese government immediately took quarantine measures by serologically screening animals, humanely culling infected animals and

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disposing of the carcasses.

In addition, the government carried out precautionary screenings of farms suspected of FMDV infection to prevent the spread of FMDV.

In an attempt to eliminate FMD from Taiwan, systematic compulsory vaccination accompanied by serological surveys was implemented to ensure the decrease in residual viral activity. Since antibodies to FMDV NSPs are considered to be present only in infected animals, the antibodies can be used to discriminate infected from vaccinated animals. Moreover, since the NSPs are relatively conserved among different serotypes, the detection of anti-NSP antibodies has an additional advantage of serotypes independence [11, 12]. During the last decade, the use of immunoenzymatic tests based on the detection of antibodies to NSPs to assess viral circulation in susceptible populations has been extensively studied and has allowed for a "vaccination to live" policy, and this approach can be supported by testing vaccinated animals for the presence of antibodies to certain NSPs of FMDV, which are induced by infection with the virus, but not by vaccination with purified FMD vaccines [5, 29]. Many assay technologies, such as the latex bead agglutination test [37], enzyme-linked immunoelectrotransfer blotting [4], enzyme-linked immunosorbent assay [1, 5, 9, 11, 13, 19, 23, 25, 34–36], multiplexed Luminex assay [12] and chromatographic strip assay [8] have been employed, and several NSPs have been expressed or synthesized artificially for these assays.

The sensitivity results of previous chromatographic strip assays differed from those of commercially available kits testing the prokaryotic expression system. The performance of the strip assay was comparable to that of two commercial ELISA kits, Ceditest FMDV-NS and UBI FMDV NS EIA, and was better than that of CHEKIT FMD-3ABC po [8]. For this reason, we developed a recombinant protein monoclonal antibody sandwich ELISA for FMDV-NSP that was similar to the CHEKIT kit. We have modified this study design to improve the diagnostic sensitivity and diagnostic specificity, and to provide a safer method, which used recombinant protein instead of virus, for detecting anti-NSP antibodies. The aim of this study was to develop a sandwich ELISA to detect serum antibodies against the FMDV 3ABC core motif. In this study, a monoclonal antibody (mAb) against the 3ABC recombinant protein that was able to react to serotype-specific antiserum against seven serotypes of FMDV was produced. A sandwich ELISA was developed using the mAb to bind the 3ABC recombinant protein and indirectly capture anti-FMDV antibodies in the serum sample. The ELISA was shown to have higher sensitivity than a commercially available ELISA kit based on the same recombinant protein.

## MATERIALS AND METHODS

*Sera*: To investigate the sensitivity of the tests, two groups of 62 FMD-infected swine were evaluated according to their sera. Eight-week-old pigs were intradermally infected with 0.5 m*l* of a $10^5$ 50% tissue culture infective dose ($TCID_{50}$/500 $\mu l$) of the FMDV O/TW/97 strain at the heel bulb of the foot in level three biosafety facilities. Thirty infected sera were collected on the 28$^{th}$ day postinfection (dpi), and 32 infected sera (positive sera) were collected on the 14$^{th}$ dpi to determine the sensitivity. In addition, 255 sera from naïve pigs and 165 sera from vaccinated pigs were used to determine the specificity. The 255 swine sera included 96 sera collected from specific pathogen-free (SPF) pigs and 159 sera collected from commercial pigs in Taiwan before the 1997 outbreak. The 165 swine sera were collected from 165 noninfected pigs that had been vaccinated twice with the commercial FMD serotype O vaccine (Professor W. B. Chung, National Pingtung University of Science and Technology). The swine sera were used to evaluate the sandwich ELISA for possible serological cross-reactivity between FMDV and swine vesicular disease virus (SVDV) and vesicular stomatitis virus (VSV) to detect antibodies against other FMDV serotypes. In addition, six antisera against the SVDV UKG/27/72 strain (EU SVD reference serum batch 2002) and six bovine antisera against FMDV serotypes A, C, Asia 1, SAT 1, SAT 2 and SAT 3 were purchased from the Institute for Animal Health, Pirbright, United Kingdom. Three antisera against the New Jersey and Indiana strains of VSV were purchased from the National Veterinary Services Laboratories, United States of America. Fetal calf serum (FCS; HyClone) and normal bovine serum were used for negative controls. Only phosphate buffered saline (PBS; pH 7.4) buffer was added to the blank.

To compare the sensitivity and specificity of the sandwich ELISA with those of three commercial ELISA kits, a serum panel containing 320 swine sera from 32 SPF pigs was purchased from Animal Technology Institute Taiwan and experimentally infected with the FMDV O/TW/97 strain. The sera in the panel were sampled at 0, 2, 4, 6, 8, 10, 14, 21, 28 and 34 dpi.

Convalescent swine sera from pigs experimentally infected with the FMDV O/TW/97 strain or the O/TW/99 strain, with a VNT titer of 1:256 and 1:512, respectively, were used as the positive control sera for the sandwich ELISA. An SPF swine serum was tested using western blotting and was used as the negative control for the sandwich ELISA.

*Virus neutralization test (VNT)*: The sera collected from experimentally infected pigs were tested using the VNT as previously described [39]. Antibody titers were expressed as the reciprocal of the final dilution of serum in the serum/virus mixture that neutralized an estimated 100 $TCID_{50}$ of virus at the 50% end-point.

*Expression, purification and identification of the FMDV 3ABC polypeptide*: Production of the FMDV 3ABC polypeptide was described previously by Chen *et al*. (2009) [8]. Briefly, *E. coli* that was transformed with the pTH 162-B plasmid carrying a partial 3ABC gene was cultivated. Expression of the 3ABC polypeptide was induced by the isopropylthiogalactoside. Next, the transformed *E. coli*

cells were harvested and lysed, and the soluble 3ABC polypeptide within the lysate was purified using affinity chromatography. The specificity of the purified polypeptide was confirmed using western blotting.

*Mouse immunization and production of a mAb against 3ABC*: One hundred milligrams of the FMDV 3ABC recombinant protein was subcutaneously injected into 3 five-week-old BALB/c mice with Genesisbio (GB) pristine adjuvant four times at four-week intervals. Three days before cell fusion, the mice were boosted with the same amount of the protein diluted in phosphate-buffered saline by subcutaneous injection. Spleen cells obtained from the immunized mice were then fused with Sp2/0 myeloma cells in 1 m*l* of polyethylene glycol 1500 (Roche, Mannheim, Germany). After a two-week incubation in Hypoxanthine-Aminopterin-Thymidine (HAT) selection medium, the supernatant from the fused cells was screened using western blotting and indirect ELISAs [23] based on the recombinant protein of O/TW/1999. Fused cells, or hybridomas, with a positive/negative (P/N) ratio greater than 2 in the indirect ELISA were cloned by limiting dilution. The positive hybridoma (CmA 40) and 0.5 m*l* of GB pristine adjuvant was mixed and injected into a BALB/c mouse. The mAb was harvested from the ascites of the mouse, and the isotype of the mAb was determined using a mouse monoclonal isotyping kit (IsoQuick™ strips, Envirologix, Portland, ME, USA) according to the manufacturer's instructions.

*Sandwich ELISA*: A sandwich ELISA for the O serotype was established to detect serum antibodies against 3ABC NSP. The optimal dilution of the purified 3ABC recombinant protein was determined using chessboard titrations of mAb and antigen. The working dilution gave an absorbance on the upper part of the linear region of the titration curve (between 1.5 and 2 optical density units) [6]. First, 96-well microtiter plates (Nunc Maxisorp, Rochester, NY, U.S.A.) were coated with the saturating dilution of mAb diluted in 0.06 M carbonate/bicarbonate buffer, pH 9.6, coating buffer (KPL, Gaithersburg, MD, U.S.A.) and incubated overnight at 4°C or for 1 hr at 37°C. The 3ABC recombinant protein in dilution buffer (PBS, pH 7.4) was added, and the plate was incubated at 37°C for 1 hr. Test pig serum (100 $\mu l$) diluted 1:100 in blocking buffer [1% (w/v) casein; Hammersten grade] in 100 mM sodium phosphate (150 mM NaCl, pH 7.4, containing Kathon® antimicrobial agent) was added to each well and incubated at 37°C for 1 hr. Horseradish peroxidase-labeled goat anti-swine IgG (100 $\mu l$, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) diluted 1:7000 in blocking buffer was added to each well, and the plate was incubated at 37°C for 1 hr**.** The substrate solution (3,3',5,5'-tetramethylbenzidine, TMB; Thermo, Rockford, IL, U.S.A.) was added, and the plate was incubated at room temperature for 15 min. To each well, 50 $\mu l$ of 1.0 M sulfuric acid stop solution (KPL, Gaithersburg, MD, U.S.A.) was added to stop the color development. Optical density at 450 nm ($OD_{450}$) was measured using a spectrophotometer. Each incubation step lasted 1 hr at 37°C and was followed by washing six times with PBS (pH 7.4). Results were expressed as a test to control (T/C) index.

*Commercially available ELISA kits for the detection of antibodies to FMDV NSPs*: Three commercially available ELISA kits were used in our study for comparison with our sandwich ELISA with regard to antibody detection ability. PrioCHECK FMDV-NS (Prionics Lelystad B.V., Lelystad, The Netherlands) is a blocking ELISA based on the baculovirus-expressed 3ABC polypeptide [35, 36]. UBI FMD NS EIA (Swine; United Biochemical Inc., Hauppauge, NY, U.S.A.) is an indirect ELISA based on a synthetic 3B peptide [34]. CHEKIT FMD-3ABC po (IDEXX Laboratories, Inc., Westbrook, ME, U.S.A.) is an indirect ELISA based on the *E. coli*-expressed 3ABC polyprotein [13]. The assays were conducted according to the manufacturers' instructions. Agreement between ELISA kits was evaluated using kappa statistics.

## RESULTS

*Isotype of the mAb*: The isotype of the produced mAb was identified as $IgG_1$, and its light chain was a kappa chain.

*Stability of the control sera in the sandwich ELISA*: In preliminary studies of analytical specificity, the optimal dilutions of reagents were determined using checkerboard titrations, employing known strong positive, weak positive and negative sera. The optimal dilution of test sera was first established. A dilution of 1/100 was selected as the dilution that best discriminated positive from negative sera while minimizing false positive or false negative results. The limit dilutions of strong and weak positive control sera were 1/800 and 1/200, respectively (Fig. 1). The stability of positive and negative control sera was evaluated with sandwich ELISA in each of the eighteen 96-well plates. The statistics were calculated, and the results showed a mean ± SD of 0.90 ± 0.09 for the positive control and 0.09 ± 0.02 for the negative control in the 18 plates.

*Determination of the cut-off value in sandwich ELISA*:

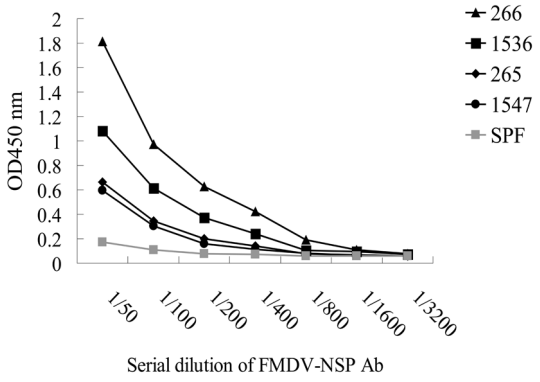


Fig. 1. Analytical specificity of the sandwich ELISA for detecting the anti-3ABC antibody. Swine sera No. 266, 1536, 265 and 1547 were FMD-positive sera, and the SPF serum was the serum obtained from five specific pathogen-free pigs. The blank value was 0.05 of the $OD_{450}$.

Table 1. Distribution of the T/C ratio profile tested using sandwich ELISA

| T/C interval ($OD_{450}$nm) | Naïve | | Vaccinated[a)] | Experimentally noninfected (0 dpi)[a)] | Experimentally infected (dpi)[b,c)] |
|---|---|---|---|---|---|
| | Pre-outbreak[a)] | SPF[a)] | | | |
| 0–0.05 | 1 | 7 | 100 | 0 | 0 |
| 0.06–0.11 | 108 | 80 | 40 | 29 | 0 |
| 0.12–0.17 | 46 | 9 | 14 | 3 | 0 |
| 0.18–0.23 | 4 | 0 | 11 | 0 | 1 |
| >0.23 | 0 | 0 | 0 | 0 | 61 |
| Total | 159 | 96 | 165 | 32 | 62 |

a) Cut off ≤$OD_{450}$ 0.23 as negative reaction. b) Cut off >$OD_{450}$ 0.23 as positive reaction. c) From 30 infected sera and 32 infected sera panels at 28 and 14 dpi, respectively.

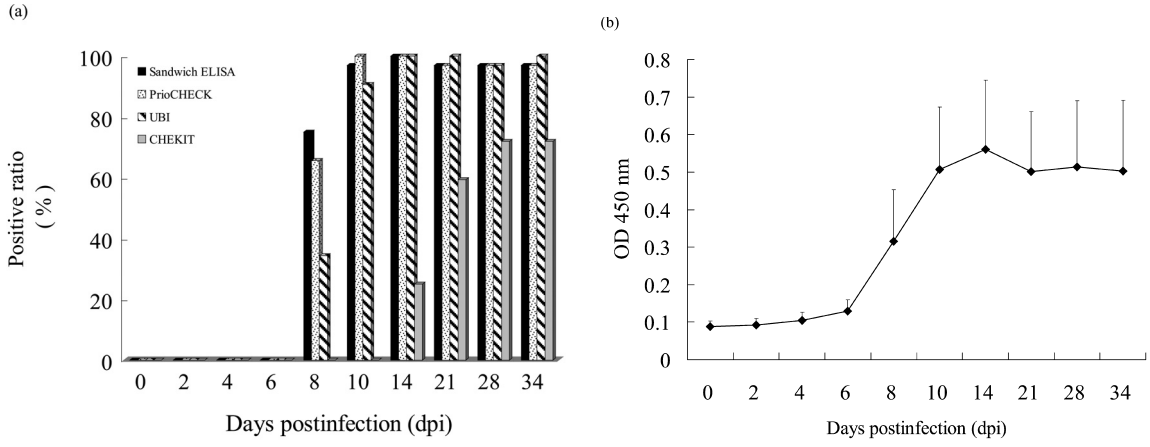


Fig. 2. (a) Results of 32 positive pigs tested using the sandwich ELISA and the commercially available ELISA kits PrioCHECK FMDV-NS, UBI FMDV NS EIA and CHEKIT FMD-3ABC po. The standardized $OD_{450}$ values measured with the positive ratio (positive pigs/total pigs, %) using the developed assay are shown. (b) The solid line indicates the standardized values measured by the developed assay with the $OD_{450}$ values. The error bar indicates the standard error of the mean at each day postinfection.

Results were normalized and expressed by dividing the $OD_{450}$ value of the test sera by the mean $OD_{450}$ value of the positive control sera. A total of 287 negative swine sera (255 from naïve pigs and 32 from zero dpi sera in the serum panel) and 62 experimentally-infected pig sera were used to determine the cut-off values for the sandwich ELISA. Sera collected from 96 SPF pigs were used to validate the specificity of the sandwich ELISA. Most of the test results had a value less than 0.15 at $OD_{450}$ nm, and only 83.3% were higher at $OD_{450}$ 0.06–0.11. In addition, the mean $OD_{450}$ nm value of the duplicate testing sera from 165 vaccinated pigs was less than 0.23. The cut-off value was therefore determined to be $OD_{450}$ 0.23. Samples were considered negative when $OD_{450}$ was less than or equal to 0.23 and positive when $OD_{450}$ was greater than 0.23 (Table 1).

*Kinetics of the antibody response to 3ABC*: A positive serum panel of sequentially sampled swine sera was tested using the VNT and commercial kits. Anti-3ABC antibodies in 32 experimentally infected pigs were first detected at 8 dpi. All the pigs tested positive at 10 dpi. From 10 dpi, the positive percentage stayed over 90% throughout the period of the animal experiment, as schematically depicted in Fig. 2a. All experimentally infected pigs had virus neutralization antibody titers ranging from 1:16 to 1:1,024 after 4 dpi, confirming infection.

*Comparison of the sandwich ELISA and the commercially available ELISA kits*: The PrioCHECK kit and UBI kit gave similar results, but the CHEKIT kit did not. With the CHEKIT kit, antibodies were initially detected at 14 dpi, and the positive percentage stayed under 80% throughout the rest of the experiment (Fig. 2a). Results obtained from the sandwich ELISA were compared with those from the PrioCHECK, UBI and CHEKIT kits. The kappa statistics given by the comparison were 0.95 (ELISA/PrioCHECK), 0.87 (ELISA/UBI) and 0.37 (ELISA/CHECK). There was 97.5, 93.4 and 66.6% agreement between the results obtained from our ELISA and those obtained from the PrioCHECK, UBI and CHEKIT kits, respectively. The diagnostic sensitivity of the assay was 98.4%, and the diagnostic specificity was 100% for naïve and vaccinated pigs. The results obtained from our assay were similar to those of the PrioCHECK and UBI kits; however, when testing vacci-

Table 2. Sensitivity and specificity of the sandwich ELISA and the commercially available ELISA kits in swine

| Tests | Sensitivity | Specificity | | |
|---|---|---|---|---|
| | Infected[a)] | SPF | Pre-outbreak | Vaccinated |
| Sandwich ELISA | 98.40% (61/62) | 100% (96/96) | 100% (159/159) | 100% (165/165) |
| PrioCHECK kit | 98.40% (61/62) | 100% (96/96) | 100% (158/158) | 100% (167/167) |
| UBI kit | 98.40% (61/62) | 100% (96/96) | 100% (158/158) | 85.30% (93/109) |
| CHEKIT kit | 35.50% (22/62) | 100% (96/96) | 100% (158/158) | 100% (167/167) |

a) The sensitivity was calculated at 14 DPI from 32 infected sera panels and 30 infection pig sera.

nated pigs, the specificity of our assay was better than that of the UBI kit (85.3%), and when testing sera from infected pigs, the sensitivity of our assay was better than that of the CHEKIT kit (35.5%; Table 2).

*Identification of recombinant protein to other FMDV serotypes using bovine antiserum*: All the bovine antisera, composed of antisera against serotypes A, C, Asia 1, SAT 1, SAT 2 and SAT 3 FMDV, gave positive results with the optic density at 450 nm greater than 0.23 (Fig. 3). Four out of six antisera (serotype C, Asia 1, SAT 1 and SAT 3) had an $OD_{450}$ value above 0.5.

*Specificity evaluated using antisera against SVDV and VSV*: As determined using sandwich ELISA, the six antisera against the UKG/27/72 strain of the SVDV and the three antisera against the New Jersey and Indiana strains of the VSV were negative without nonspecific cross-reactions between FMDV and SVDV.

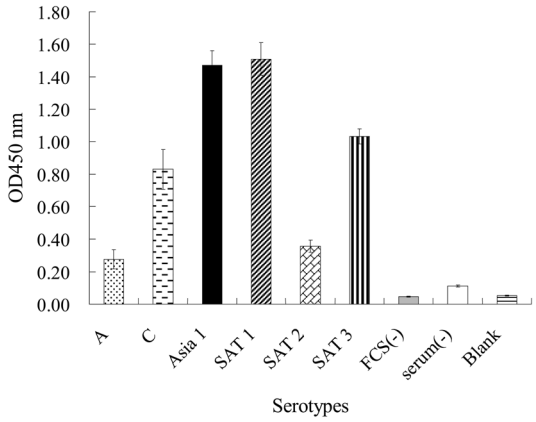


Fig. 3. Detection of the NSP antibody in bovine sera using the sandwich ELISA. The tested sera were obtained from cattle infected with the FMDV of serotypes A, C, Asia 1, SAT 1, SAT 2 and SAT 3. Fetal calf serum (FCS) and normal bovine serum were used for negative controls. Only PBS (pH 7.4) buffer was added to the blank.

## DISCUSSION

Vaccination is one of the main methods that can be implemented to control FMD, and it is currently the primary control strategy used in Taiwan. In countries and areas where FMD vaccination is implemented, the serological differential diagnosis of FMD can be difficult because vaccinated animals cannot be distinguished from infected animals simply by the VNT, since neutralizing antibodies are elicited by vaccination, natural infection or both. Several countries that vaccinate against FMD now use antibodies against NSP of FMDV as replaced serological markers for identifying FMDV-infected animals [9, 26]. Developing novel serological methods or improving the currently available ones, especially ELISA, is a crucial step toward the eradication of FMD [1, 13, 25, 35]. In this study, a 3ABC NSP-based sandwich ELISA was developed to test for the serum antibody against 3ABC.

In our study, the cut-off value was determined to be 0.23 of the $OD_{450}$ value based on testing sera from naïve, vaccinated and experimentally infected pigs (Table 1). One of the four naïve sera collected prior to the first outbreak showed an $OD_{450}$ value of 0.18, and the other three showed an $OD_{450}$ value of 0.21. All four sera were determined to be negative by our sandwich ELISA and the other ELISA kits (Table 1). All the experimentally infected pigs at 8 dpi still had strong positive reactions and remained positive up to 34 dpi (Fig. 2a and 2b). These results were consistent with a previous report in which strong NSP antibody responses were observed by 30 dpi in experimentally infected pigs tested by both 3B and 3ABC ELISA kits [7]. Sera sampled from one pig at 21, 28 and 34 dpi had $OD_{450}$ values of 0.17, 0.12 and 0.15, respectively, using the sandwich ELISA. The three sera were negative according to the PrioCHECK kit, CHEKIT kit and our in-house chromatographic strip [8]. In contrast, they tested positive using the UBI kit. However, the three sera had neutralizing antibody titers of 1:256, 1:256 and 1:64, respectively, implying that the pig was truly infected. The difference in results might be due to the antibody type used in the test procedures, such as type-specic polyclonal versus FMD-specic monoclonal antibodies or the difference in the specicity of reactions [32]. The inconsistency might also be attributed to the conformational differ-

ences among the polypeptides produced in different expression systems [9].

Comparison of the performance for the detection of 320 experimentally infected pig sera between the sandwich ELISA and the PrioCHECK, UBI and CHEKIT kits demonstrated kappa statistics of 0.95, 0.87 and 0.37, respectively, demonstrating a high level of agreement between the results obtained from the sandwich ELISA and those from the PrioCHECK and UBI kits. Our assay could be carried out within a single day or 4–5 hr, which is shorter than the time required for the PrioCHECK kit, which needs an overnight incubation of samples. In addition, when testing vaccinated pigs, the specificity of our assay was better than that of the UBI kit (85.3%; Table 2). The results showed that the CHEKIT kit had a high specificity of 100%, which is similar to the specificity reported elsewhere [24].

Our study revealed a difference in the diagnostic sensitivity among the developed sandwich ELISA, CHEKIT kit and 3ABC-based indirect ELISA. The sensitivity of the sandwich ELISA was higher than that of the CHEKIT kit. We believe the higher sensitivity of our assay may result from the strong and specific binding of the FMDV-NSP recombinant protein to the monoclonal antibody used.

The analytical sensitivity was established by the detection of NSP antibody. The chromatographic test strip and sandwich ELISA could detect the positive sera that were diluted $10^{-5}$ and $10^{-2}$ fold, respectively. The analytical sensitivities of the two tests were 10,000 and 10 times higher than that of the commercial PrioCHECK ELISA (diluted $10^{-1}$ fold), respectively. This revealed that the chromatographic test strip could rapidly accomplish the detection of NSP antibodies and was more suitable than the ELISAs when applied to clinical diagnosis. However, the ELISAs could detect a larger number of samples at a time in a laboratory when used by skilled personnel. Furthermore, the analytical sensitivity of the sandwich ELISA was slightly more sensitive than the commercial PrioCHECK ELISA. Consequently, the sandwich ELISA could be used for the detection of NSP antibodies in vaccinated animals that have been infected by FMD virus and maybe become carriers. Because the protection provided by the vaccine-induced antibodies might limit the replication of FMDV and result in presentation of a very low level of NSP antibodies in vaccinated animals, the detection of NSP antibodies needs a higher sensitivity test than is available currently [10]. The pig has not been considered a carrier animal of FMDV. The levels of NSP antibody detected by the UBI and PrioCHECK kits in infected pigs were gradually decreasing and may become negative after 60 to 180 dpi [7]. However, 15 infected sera collected from pigs that received one dose of FMD vaccine at 12 weeks old and were infected with $10^5$ $TCID_{50}$ O/TW/97 FMDV at 31 weeks old. All of the 15 vaccinated pigs showed typical vesicle lesions on the feet and snout at 4 days postchallenge. All 15 sera were NSP antibody positive, as detected by the chromatographic strip. However, 11 out of the 15 sera were positive by the sandwich ELISA. In contrast, only 9 of the 15 sera were positive by the PrioCHECK ELISA. The results indicated that the former two methods were more sensitive than the latter one. However, these methods should be applied to the detection of NSP antibody for subclinical infected animals in the future.

This paper described a sandwich ELISA for the detection of serum antibodies to the 3ABC NSP of the FMDV serotype O. To evaluate the potential ability of the test to detect anti-NSP antibodies elicited by FMDVs of other serotypes, bovine sera from cattle infected with serotypes other than serotype O were also tested using our method. The results suggested that our sandwich ELISA was effective in detecting the NSP antibodies against other FMD serotypes in swine (Fig. 3).

For biosafety consideration, the 3ABC NSP of FMDV was used as an immunogen instead of the inactivated virus in the production of mAb. The results showed that our sandwich ELISA using the 3ABC and anti-3ABC antibody derived from the FMDV O/TW/99 strain could detect antibodies induced by both the O/TW/99 and O/TW/97 strains in swine antisera (data not shown).

In this study, we found that using the expressed proteins to immunize BALB/c mice was a safer strategy to prepare mAb, since this lowered the risk of live virus escaping from the laboratory [13, 17]. To enhance safety and efficacy, we designed our sandwich ELISA using expressed protein instead of live FMDV as a coated antigen. Therefore, this assay may be safely performed in laboratories with basic biosafety requirements.

In addition, the purpose of the assay was to detect anti-NSP antibodies so as to identify previously infected animals in populations. As discovered through our experiments, the sandwich ELISA demonstrated satisfactory sensitivity and specificity, both higher than 95%. The method was able to distinguish antibodies produced by naturally infected animals from those produced by vaccinated animals. Furthermore, with antisera against SVDV and VSV, the assay proved that it could specifically recognize antibodies against FMDV, but not cross-react with antibodies against SVDV and VSV, indicating that it can be used in areas where the three diseases might be present.

Employment of mAb in our sandwich ELISA improved the specificity. Our study revealed that the performance of sandwich ELISA was unsatisfactory, either in terms of low specificity when testing vaccinated pigs by the UBI kit or in terms of low sensitivity when testing infected pigs by the CHEKIT kit (Table 2). To minimize these drawbacks, a 3ABC-specific mAb was introduced to capture the coated antigen. Introduction resulted in a satisfactory specificity without losing diagnostic sensitivity.

To sum up, the sandwich ELISA described in the present article had the advantages of convenience, stability, high sensitivity, high specificity, safety, and high throughput. With a reliable immunoassay system, we hope to be able to diagnose vesicular diseases in swine more effectively, thereby further improving veterinary disease control and the culling of infected animals in the early stages.

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