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Establishment and application of a solid-phase blocking ELISA method for detection of antibodies against classical swine fever virus

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ABSTRACT

Background: Classical swine fever (CSF) is a severe infectious disease of pigs that causes significant economic losses to the swine industry.
Objectives: This study developed a solid-phase blocking enzyme-linked immunosorbent assay (spbELISA) method for the specific detection of antibodies against the CSF virus (CSFV) in porcine serum samples.
Methods: A spbELISA method was developed based on the recombinant E2 expressed in *Escherichia coli*. The specificity of this established spbELISA method was evaluated using reference serum samples positive for antibodies against other common infectious diseases. The stability and sensitivity were evaluated using an accelerated thermostability test.
Results: The spbELISA successfully detected the antibody levels in swine vaccinated with the C-strain of CSFV. In addition, the detection ability of spbELISA for CSFV antibodies was compared with that of other commercial ELISA kits and validated using an indirect immunofluorescence assay. The results suggested that the spbELISA provides an alternative, stable, and rapid serological detection method suitable for the large-scale screening of CSFV serum antibodies.
Conclusions: The spbELISA has practical applications in assessing the vaccination status of large pig herds.

Keywords: Classical swine fever virus; E2 protein; blocking ELISA; antibody detection

INTRODUCTION

Classical swine fever (CSF) is caused by the CSF virus (CSFV). The disease is an OIE-listed, highly contagious, and often-fatal swine disease with a worldwide distribution. CSF can cause high morbidity and mortality in feral and domestic pigs, and it severely affects the swine industry and the international trade of live pigs and pig products [1]. The CSFV belongs to the genus *Pestivirus* of the family *Flaviviridae*, and it is genetically and serologically related to other pestiviruses, including the bovine viral diarrhea virus (BVDV)-1, BVDV-2, and border disease virus [2]. The CSFV is an enveloped virus with a single-stranded positive-sense RNA

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Conflict of Interest
The authors declare no conflicts of interest.

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genome, approximately 12.3 kb in length, encoding only a single open-reading frame [3]. The structural proteins include nucleocapsid protein C and three envelope glycoproteins, namely E^{rns}, E1, and E2. The viral glycoproteins, E^{rns} and E2, can induce the production of virus-neutralizing antibodies and trigger protective immunity in swine [4-6]. E2 consists of four relatively independent antigenic domains (A, B, C, and D) located in the amino acid residues 690–866 in the N-terminal of the E2 protein. A, B, and C are the main antigenic domains of E2 leading to the induction of neutralizing antibodies. The A antigenic domain contains three substructure domains: A1, A2, and A3 [7]. A synergistic interaction is present between A1 and B or C during the induction of neutralizing antibodies [8]. The membrane protein E2 is the most immunogenic protein of CSFV and the leading candidate protein for the design of genetic engineering vaccines and serum antibody detection methods [9].

Although the CSF has been controlled in developed countries by implementing a no-vaccination stamping-out policy or prophylactic vaccination strategy and eradicated in Australasia and North America, it is still epidemic in South and Central America, Eastern Europe, and Asia [10,11]. In China, CSF is effectively controlled by prophylactic vaccination using modified live vaccines, e.g., the C-strain. The timely and accurate diagnosis based on the detection of CSFV antibodies is essential for evaluating the effectiveness of vaccination and preventing and controlling CSF.

The tests commonly used for antibody detection include the virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA). VNT is the gold standard for CSFV antibody detection, given its sensitivity and specificity. Nevertheless, it is unsuitable for the mass analysis of samples in the field because of its cell-culture-dependent characteristics and labor-intensive and time-consuming operation. ELISA is characterized by high sensitivity, facile operation, standardization, and fast processing time. Therefore, this technique can be widely applied in high-throughput screening and analysis [12,13].

In this study, a recombinant E2 (rE2)-based solid-phase blocking ELISA (spbELISA) was developed. Its specificity was evaluated using reference positive sera by detecting the antibodies against other common swine infectious diseases. Its stability was determined using an accelerated thermostability test. The spbELISA was used to test the antibody levels of swine after immunization with the C-strain of CSFV. The ability of spbELISA to detect CSFV antibodies was compared with that of several commercial ELISA kits and validated using an indirect immunofluorescence assay (IFA).

MATERIALS AND METHODS

Antigen, antibody, and horseradish peroxidase (HRP) conjugates

Preparation of the rE2 antigen

The details for preparing the rE2 antigen are described elsewhere [14]. Briefly, the E2 gene fragment coding the A–D antigenic regions of the C-strain was cloned into the pGEX-6p-1 vector and expressed in *Escherichia coli*. The E2 protein purified from *E. coli* was verified by Western-Blotting with using a monoclonal antibody against CSFV E2 which were kept in the lab (**Supplementary Fig. 1**). The soluble rE2 fraction was purified using glutathione–sepharose 4B resin affinity chromatography. This fraction was applied as an antigen in ELISA and used to induce hyperimmune serum production in rabbits.

Production of antisera and coupling antibodies of HRP-rE2 conjugates

A 0.15 mg/dose of purified rE2 immunogen was mixed vigorously with an equal volume of Freund's adjuvant in an isotonic salt solution and administered to each rabbit. Subsequent booster doses were administered at a rate of 0.1 mg/rabbit until the antibody titer reached the required level. The antisera were collected, and total immunoglobulins (Igs) were isolated with protein A. The specific Igs against rE2 were measured and labeled by covalent coupling to HRP using a two-step glutaraldehyde method to yield the HRP-rE2 conjugates [15].

Establishment of spbELISA

The optimal concentration of rE2 was determined using a checkerboard titration. After overnight incubation at room temperature (RT), the rE2 antigen was diluted with 50 mmol/L sodium carbonate buffer (pH 9.6) and coated on plates to the final volume of 100 μL per well. Each well of the antigen-coated plates was washed four times with 220–300 μL of phosphate-buffered saline (pH 7.2) containing 0.05% Tween-20 (PBST). Each well was blocked for 60 min at RT with 200 μL of PBST containing 10% (w/v) skimmed milk, 5% horse serum, and 5% trehalose. After washing, 100 μL of undiluted pig sera was added to each well, and the plates were incubated for 60 min at 37°C. The wells were washed four times with PBST. Subsequently, 100 μL of HRP–rE2 conjugates were added to each well, and the plates were incubated for 120 min at 37°C. The reaction plates were washed with PBST, and 100 μL of substrate solution (tetramethyl benzidine [TMB]; Sigma, USA) was added to each well. After 20 min incubation at RT in the dark, the reaction was quenched by adding 50 μL of 2 mol/L H_2SO_4. The absorbance was measured using a spectrophotometer at 450 nm. The blocking ratio of the serum sample was calculated using the following formula:

$$\text{Blocking ratio\%} = [(\text{NCOD}_{450}\text{ nm} - \text{SampleOD}_{450}\text{ nm})/\text{NCOD}_{450}\text{ nm}] \times 100\%$$

Validity criteria: 1. NCOD_{450} nm ≥ 0.5; 2. Blocking ratio of PC ≥ 40%

Interpretation of results: Antibody negative: blocking ratio% < 40%

Antibody positive: blocking ratio% ≥ 40%

Specificity of spbELISA

The specificity of spbELISA was tested by analyzing reference sera that were positive for porcine circovirus type 2 (PCV2), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), BVDV (from cattle), and BVDV (from pig) antibodies using the following described procedure. Each sample was tested in triplicate, and the blocking ratios were calculated.

Stability of spbELISA

An accelerated thermostability test was performed to test the stability of spbELISA. Four serum samples (two known CSFV antibody-negative sera and two known CSFV antibody-positive sera) were tested using two kits of the same manufacturing batch. All kit components, i.e., the CSFV antigen-coated plate, conjugate, negative control, positive control, TMB substrate, stop solution, wash concentrate (50×), polymer absorbent paper, and sealing sticker, were placed in an incubator at 37°C for one week. The four serum samples were tested daily eight times. The components of the other kits were stored at 4°C for one year and then used to test the four reference sera every three months for a total of four times.

Dynamics of CSFV antibodies in vaccinated pigs

Eight CSFV antibody-negative pigs were vaccinated with the attenuated lapinized CSFV C-strain. Three non-immunized CSFV-antibody-negative pigs were used as the negative

control group. The serum samples were collected from the jugular vein of each pig at 0, 7, 14, 21, 28, 35, and 50 days post-immunization (dpi). The serum samples were tested with spbELISA to analyze the dynamics of antibody production after vaccination.

Existence of CSFV C-strain in vaccinated pigs

Anticoagulant blood samples were collected from the jugular vein of each individual pig at 0, 7, 14, 21, 28, 35, and 50 dpi. The peripheral blood lymphocytes were isolated from blood samples to extract the total RNA, which was used as a template for amplifying the viral E2 gene fragment through reverse transcriptase-polymerase chain reaction (RT-PCR). The RT-PCR results were observed on 1.0% agarose gel.

Detection of clinical samples

Two hundred and forty-six serum samples were collected from pigs from several pig farms in different regions of China. The samples were collected from pigs of different ages before and after vaccination against the CSFV. Two imported sera, i.e., BI and GIBCO, were purchased from Israel and New Zealand, respectively. In addition, three standard sera were obtained from the China Institute of Veterinary Drug Control. The antibody levels of these sera were measured using the proposed spbELISA method and three commercially available CSFV-Ab detection ELISA kits, including CSFV antibody test kit (IDEXX, USA), VDPro CSFV AB C-ELISA (MEDIAN, Korea), and PrioCHECK CSFV Ab 2.0 (Prionics, Switzerland) in strict accordance with the instructions for each kit.

IFA test of controversial sera

Some results obtained through spbELISA were inconsistent with those obtained using the three commercially available ELISA kits. IFA was conducted to validate the results of the controversial sera. The optimized experimental procedure is as follows. A porcine kidney cell line (PK-15) was maintained in a 24-well plate in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and essential antibiotics at 37°C under a humidified atmosphere containing 5% CO_2/95% air. When the PK-15 cells grew to approximately 75%–80% with a single layer, 200 μL of CSFV C-strain cell culture was added to each well. The samples were incubated in a cell culture box at 37°C for 1 h. The cell culture mixed with the C-strain was removed from the plate, and 500 μL of fresh cell culture maintenance medium (DMEM containing 2% fetal bovine serum and essential antibiotics) was added to each well. After 48 h of culturing, cell fixation was conducted by adding 200 μL of 4% polyformaldehyde to each well. The plates were incubated at 4°C for 25 min and washed three times with sterile PBST for 5 min each. For cell permeabilization, 200 μL of 1‰ Triton X-100 was added to each well. Each plate was incubated at RT for 20 min and washed with PBST. Subsequently, 200 μL of 1:10 diluted sera were added to each well. The plates were incubated overnight at 4°C and washed with PBST. After washing, 200 μL of 1:500 diluted fluorescein isothiocyanate conjugated-polyclonal goat anti-pig IgG (H+L) was added to each well. The plates were then incubated at 37°C for 2 h and washed with PBST. DAPI (200 μL) was added to each well. The plates were incubated at RT for 10 min and washed five times with PBST. The results were observed by fluorescence microscopy.

Ethics statement

In this study, all animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the study was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (permit number LVRIEC2018-008).

Table 1. Cross-reactivity with other reference sera positive for other diseases

Items	Positive sera				
	PCV2	PRV	PRRSV	BVDV (camel)	BVDV (pig)
OD_{450} nm	1.7132 ± 0.0497	1.6017 ± 0.0454	1.7645 ± 0.0771	1.5894 ± 0.0658	1.2800 ± 0.1700
Blocking rate (%)	8.73	14.67	6.00	15.32	18.67
Result	-	-	-	-	-

PCV2, porcine circovirus type 2; PRV, pseudorabies virus; PRRSV, porcine reproductive and respiratory syndrome virus; BVDV, bovine viral diarrhea virus.

RESULTS

Specificity of spbELISA

The specificity of spbELISA was tested using sera positive for PCV2, PRV, PRRSV, BVDV (from cattle), and BVDV (from pig) antibodies. No cross-reactions occurred between the sera positive for CSFV antibodies and those positive for antibodies against PCV2, PRV, PRRSV, and BVDV (**Table 1**).

Stability of spbELISA

All kit components were placed in a freezer at 4°C and used to test the four reference sera five times every three months. The results are shown in **Supplementary Table 1**. In the accelerated thermostability test, all kit components were incubated at 37°C and used to test the four reference sera tested daily a total of nine times. The results are presented in **Supplementary Table 2**. In the two experimental groups, the OD_{450} nm values of the positive control sera and the two known positive sera did not change significantly. On the other hand, the OD_{450} nm values of the negative control sera and the two known negative sera decreased significantly with time during the experiment. The blocking ratio was calculated based on the OD_{450} nm value each time the sera were tested. The blocking ratio of the positive control and the two known positive sera decreased with time but was always higher than the cut-off blocking ratio of 40%. By contrast, the blocking ratio of the two known negative-sera was consistently less than 40% throughout the experiment. These findings suggest that the change in the OD_{450} nm values of these samples did not affect the result. In addition, the OD_{450} nm value of the negative control was always greater than 0.5, and the blocking ratio of the positive control was always higher than 40%, satisfying the validity criteria of spbELISA (**Figs. 1** and **2**). Overall, the results of the stability experiments are reliable.

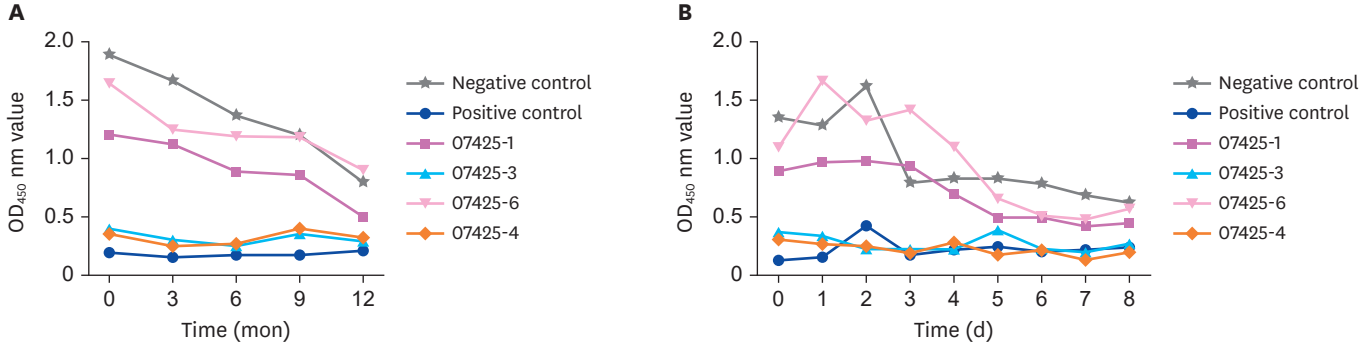

Fig. 1. Change in the OD_{450} nm value (A) and the blocking ratio (B) of the control sera and four reference serum samples in the 4°C group. All kit components were placed in a freezer at 4°C and used to test sera every three months. 07425-1 and 07425-6 are CSFV-antibody-negative sera. 07425-3 and 07425-4 are CSFV-antibody-positive sera. The black line shown in (B) represents the cut-off value of 40% of the blocking ratio.
OD, optical density; CSFV, classical swine fever virus.

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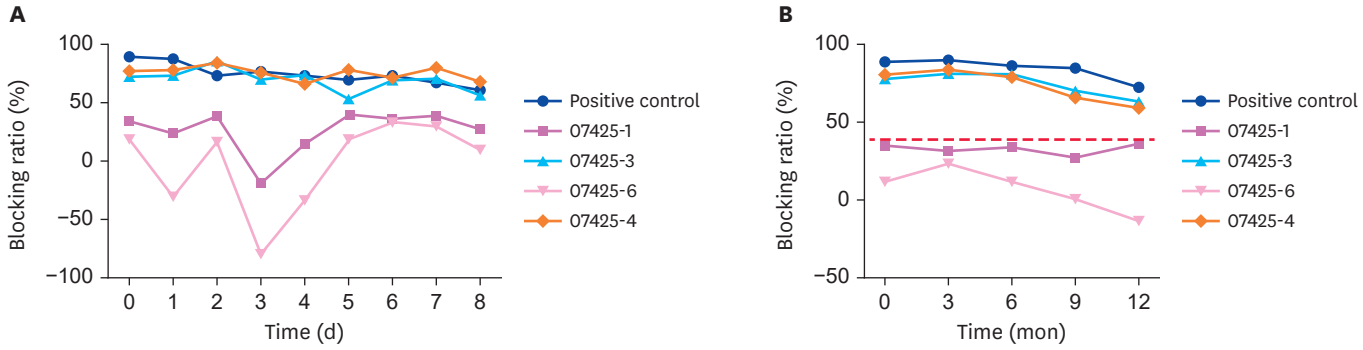

Fig. 2. Change in the OD_{450} nm value (A) and the blocking ratio (B) of the control sera and four serum samples in the 37°C group. All kit components were incubated at 37°C and used to test sera daily. 07425-1 and 07425-6 are CSFV-antibody-negative sera. 07425-3 and 07425-4 are CSFV-antibody-positive sera. The red dotted line shown in (B) represents the cut-off value of 40% of the blocking ratio.
OD, optical density; CSFV, classical swine fever virus.

Table 2. Dynamics of CSFV antibodies in pigs vaccinated with the CSFV C-strain

dpi	Blocking ratio of serum samples at different time points (%)										
	942*	507*	903*	553*	570*	946*	926*	958*	908†	955†	593†
0	−28.98	22.90	−26.79	10.35	12.78	−142.56	18.35	24.77	13.25	−9.32	21.4
7	−26.93	66.28	58.98	39.59	68.77	−134.6	73.73	71.26	8.54	−9.88	15.02
14	42.58	74.3	57.41	80.29	69.81	28.53	78.21	75.4	10.87	3.11	9.96
21	74.38	82.06	70.85	77.56	81.19	72.15	82.66	66.86	10.2	1.94	36.79
28	78.28	80.5	63.08	82.37	48.89	81.64	81.01	74.25	11.59	2.76	14.90
35	78.43	80.97	57.50	80.44	75.06	82.18	79.35	78.23	13.08	8.57	13.57
50	65.36	76.44	67.74	73.44	71.18	55.39	74.48	69.06	8.24	4.66	26.76

Antibodies were detected through solid-phase blocking enzyme-linked immunosorbent assay.
CSFV, classical swine fever virus; dpi, days post-immunization.
*Vaccinated pigs: 942, 507, 903, 553, 570, 946, 926, 958; †Unvaccinated control pigs: 908, 955, 593.

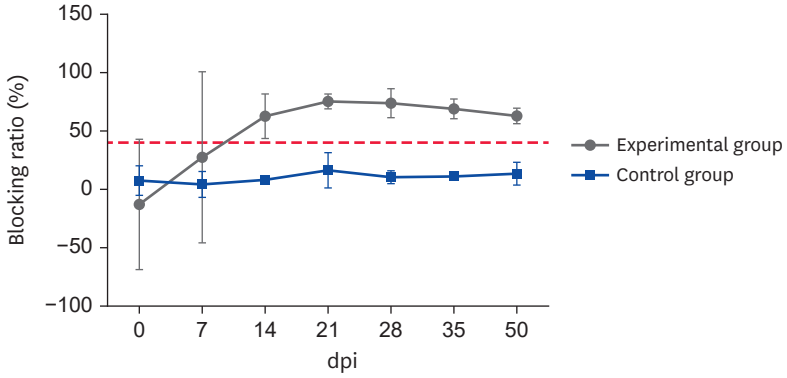

Fig. 3. Antibody dynamics in pigs vaccinated with classical swine fever virus C-strain. Serum samples were collected at 0, 7, 14, 21, 28, 35, and 50 dpi and subjected to solid-phase blocking enzyme-linked immunosorbent assay. Data were shown as mean ± SD. The red dotted line shown in the figure represents the cut-off value of 40% of the blocking ratio.
dpi, days post-immunization.

Dynamics of the CSFV antibodies in vaccinated pigs

All serum samples collected pre-vaccination were negative. spbELISA could detect CSFV antibodies on seven dpi (**Table 2**). The antibody levels increased initially, peaking at 21 dpi, and then decreased (**Fig. 3**).

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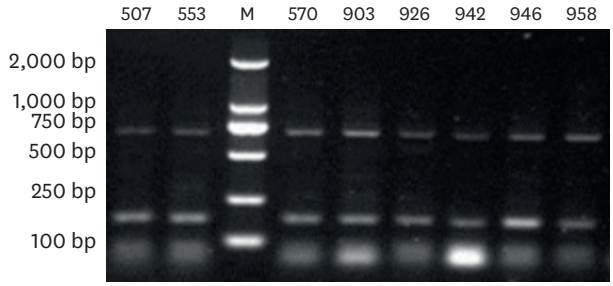

Fig. 4. Detection of the classical swine fever virus-E2 gene in peripheral blood lymphocytes. The target gene band, which is approximately 700 bp in length, was detected in 8 samples.
M, DL 2, 000 DNA marker.

Existence of the C-strain in vaccinated pigs

Viral nucleic acids could only be detected in the peripheral blood lymphocytes on 7 and 14 dpi (**Fig. 4**). The target gene band, approximately 700 bp in length, was sequenced and analyzed. The results showed that the gene is the hog cholera lapinized virus E2 gene of the CSFV vaccine strain. This gene was not detected at other time points, suggesting that the CSFV vaccine will not persist in pigs. Instead, it will exist in the peripheral blood lymphocytes for approximately 14 days.

Comparison of the detection ability of spbELISA and commercial CSFV Ab kits using the clinical serum samples

spbELISA yielded a positive rate of 74.68% when used to detect the antibody levels of the 158 serum samples, whereas three commercial kits yielded positive rates of 52.53% (IDEXX), 73.42% (VDPro), and 73.42% (PrioCHECK) (**Table 3**). The positive rate of spbELISA was highest among all the methods tested and was slightly higher than that of VDPro and PrioCHECK. The IDEXX ELISA kit showed the lowest positive rate among all the methods tested. spbELISA showed a positive rate of 69.32% when used to test 88 other serum samples, which was higher than the positive rate of the IDEXX ELISA kit (57.95%) (**Table 4**). spbELISA and the three commercial kits yielded negative results for the two imported sera and consistent results for the three reference sera.

IFA test of the controversial serum samples

The spbELISA and IDEXX ELISA kit yielded inconsistent results for 117 serum samples. These results included 81 IDEXX ELISA−/spbELISA+ sera and 36 IDEXX ELISA+/spbELISA− sera. These samples were analyzed further using IFA (**Fig. 5**, **Supplementary Fig. 2**). As shown in **Table 5**, IFA indicated that 54 of the 81 IDEXX ELISA−/spbELISA+ samples and 10 of the 36 IDEXX ELISA+/spbELISA− were positive. These results suggested that spbELISA provides slightly higher positive detection rates than the other methods.

Table 3. Comparison of the detection results of spbELISA with those of three commercial kits for 158 field pig serum samples

Items	IDEXX		VDPro		PrioCHECK		Total
	+	−	+	−	+	−	
spbELISA							
+	64	54	91	27	92	26	118
−	19	21	25	15	24	16	40
Total	83	75	116	42	116	42	158

The IDEXX ELISA kit yielded suspicious results for eight samples. These results were considered negative by statistical analysis.
Positive rate of the spbELISA: (118/158) × 100 = 74.68%.
Positive rate of the IDEXX: (83/158) × 100 = 52.53%.
Positive rate of the VDPro: (116/158) × 100 = 73.42%.
Positive rate of the PrioCHECK: (116/158) × 100 = 73.42%.
spbELISA, solid-phase blocking enzyme-linked immunosorbent assay.

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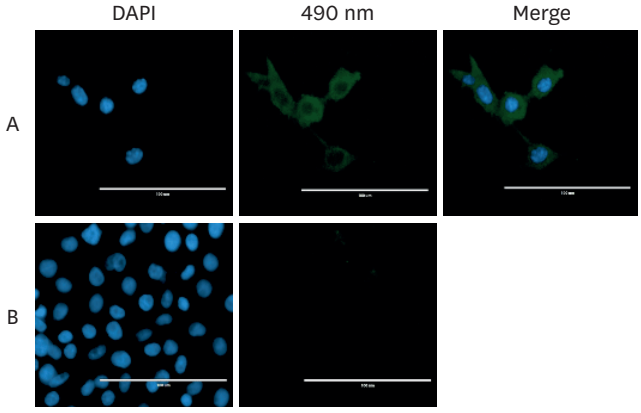

Fig. 5. IFA detection results. PK-15 cells were harvested at 48 h post-immunization and processed for IFA. All nuclei were stained with DAPI. The specific immunostaining of a sample indicated the presence of classical swine fever virus antibodies in the serum sample (magnification: 40×).
IFA, immunofluorescence assay; A, the positive sample; B, control.

Table 4. Comparison of the detection results of spbELISA with those of the IDEXX ELISA kit for 88 field pig serum samples

Items	IDEXX ELISA kit		
	+	–	Total
spbELISA			
+	34	27	61
–	17	10	27
Total	51	37	88

The IDEXX ELISA kit yielded suspicious results for one sample. This result was considered negative in statistical analysis.
Positive rate of the spbELISA: (61/88) × 100 = 69.32%.
Positive rate of the IDEXX: (51/88) × 100 = 57.95%.
spbELISA, solid-phase blocking enzyme-linked immunosorbent assay.

Table 5. Summary of the 117 inconsistent sera detection results by IFA

Items	81 IDEXX ELISA–/spbELISA+ samples	36 IDEXX ELISA+/spbELISA– samples
Specific fluorescence		
+	54	10
–	27	26

IFA, immunofluorescence assay; spbELISA, solid-phase blocking enzyme-linked immunosorbent assay; +, samples showed specific fluorescence in IFA; –, samples showed no specific fluorescence in IFA.

DISCUSSION

Numerous diagnostic methods for CSF are available and include viral isolation and identification, immunofluorescence tests, and serological and molecular biological detection. Thus far, ELISA is the most widely used method for detecting CSFV antibodies in serum owing to its simple operation and high specificity, sensitivity, and repeatability. Another important advantage of ELISA is that it can detect large sample quantities simultaneously. Many rE2 or Erns protein-based ELISA methods have been established for detecting CSFV antibodies; these methods include indirect ELISA [16-18], blocking ELISA [19-21], competitive ELISA [22], multiple ELISAs [23], and double-antigen ELISA [24]. These ELISA methods offer advantages in sensitivity, specificity, and clinical applications. Several of these methods have been applied to CSF prevention in many countries.

The recombinant protein E2 shows great antigenicity. In the present study, an E2-based spbELISA was developed to detect CSFV antibodies. spbELISA does not require serum

sample dilution, thereby saving time, facilitating operation, and reducing the likelihood of error. Thus, spbELISA provides results closer to the real antibody levels of the samples. The antibodies induced by CSFV may cross-react with those induced by other pestiviruses [25]. The present study found that spbELISA prevents serological cross-reactions between CSFV and BVDV. In addition, spbELISA yielded negative results when used to detect reference sera positive for antibodies against other swine viruses (PCV2, PRV, and PRRSV), highlighting the excellent specificity of this method. In the accelerated thermostability test, the OD_{450}nm of the negative control and the blocking ratio of the positive control always satisfied the validity criteria. At the same time, the final determination results of the serum samples in the 4°C and 37°C groups remained unchanged during all stability experiments. Therefore, the stability and reliability of the kit can be guaranteed for a year when stored at 4°C.

Moreover, the kit has a reasonable shelf life when stored under 2°C–8°C for one year. Investigating the dynamics of CSFV antibodies in pigs after vaccination showed that the antibody levels peaked at 21 dpi. This result is consistent with those of other relevant studies [26,27]. In this study, the viral nucleic acid in the peripheral blood lymphocytes could only be detected at 7 and 14 dpi, suggesting that the CSFV-attenuated vaccine persisted in the vaccinated pigs for only 14 days. spbELISA and the three commercial kits showed different positive rates when used to test the clinical serum samples. The possible reason is that these kits had different coating antigens or testing principles. spbELISA showed the highest positive rate among all kits. The spbELISA and IDEXX ELISA kit yielded inconsistent results for 117 serum samples. These samples were analyzed further by IFA, indicating that 54 of the 81 IDEXX ELISA−/spbELISA+ samples and 10 of the 36 IDEXX ELISA+/spbELISA− were positive. These results showed that spbELISA is more sensitive than the IDEXX ELISA kit.

In conclusions, the spbELISA approach established in this study is sensitive and specific for the CSFV antibodies. This method provides an alternative, inexpensive, and rapid serological detection method suitable for the large-scale screening of CSFV antibodies. This method has potential applications in assessing the vaccination status of large pig herds.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Results of the OD_{450} nm value and the blocking ratio of the control sera and four serum samples detected by the solid-phase blocking enzyme-linked immunosorbent assay in the 4°C group

Click here to view

Supplementary Table 2

OD_{450} nm values and the blocking ratios of the control sera and four serum samples detected by the solid-phase blocking enzyme-linked immunosorbent assay in 37°C group

Click here to view

Supplementary Fig. 1

Western blot analysis of the recombinant E2 protein.

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Supplementary Fig. 2

IFA detection results of controversial samples. All nuclei were stained with DAPI. The specific immunostaining of a sample indicated the presence of classical swine fever virus antibodies in the serum sample (magnification: 40×).

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