Development of an indirect ELISA detecting Swine acute diarrhea syndrome coronavirus IgG antibodies based on a recombinant spike protein

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Abstract

Swine acute diarrhea syndrome coronavirus (SADS-CoV) is a newly identified swine enteropathogenic coronavirus that causes watery diarrhea in neonatal piglets, leading to significant economic losses to the swine industry. Currently there are no suitable serological method to assess the infection of SADV-CoV and effectiveness of vaccines, making an urgent need to exploit effect enzyme-linked immunosorbent assay (ELISA) to compensate for this deficiency. In this study, an indirect ELISA (S-iELISA) based on recombinant spike (S) protein expressed in Baculovirus was developed and evaluated. The reaction conditions of S-iELISA were optimized and cut-off value determined as 0.3711 by analyzing OD450nm values of 40 SADS-CoV-negative sera confirmed by immunoinfluscent assay (IFA) and Western Blot. The coefficients of variation of 6 positive sera within and between runs of S-iELISA were both less than 10% and cross-reactivity assays demonstrated that S-iELISA was non-cross-reactive with other swine viruses' sera. Furthermore, the overall coincidence rate between IFA and S-iELISA was 97.3% based on testing 111 clinical serum samples. Virus neutralization test with 7 different OD450nm value sera showed that the OD450nm values tested by S-iELISA are positive correlated with virus neutralization. Finally, a total of 300 pig field serum samples were tested by S-iELISA and commercial kits of other swine enteroviruses showed that the IgG-positive for SADS-CoV, TGEV, PDCoV and PEDV were 81.7%, 54%, 65.3%, 6%, respectively. The results suggest this S-iELISA is specific, sensitive, repeatable and can be applied for vaccines evaluation and detection the SADS-CoV infection in swine industry.

Introduction

SADS-CoV, also name PEAV (Gong et al., 2017) and SeACoV (Y. Pan et al., 2017), as the newest strain of porcine coronaviruses (CoVs), was first detected in pig herds with diarrhea-outbreak in Guangdong, China in 2017 (P. Zhou et al., 2018). SADS-CoV belongs to the genus Alphacoronavirus of the family Coronaviridae (P. Zhou et al., 2018), which also includes *Transmissible gastroenteritis virus* (TGEV) (Doyle & Hutchings, 1946), *Porcine epidemic diarrhea virus* (PEDV) (Pensaert & de Bouck, 1978) and *Porcine deltacoronavirus* (PDCoV) (Woo et al., 2012). SADS-CoV genome is an enveloped, single-stranded, positive-sense RNA virus, which is approximately 27 kb in length, and arranged in the order of: 5' UTR-ORF1a/1b-S-NS3-E-M-N-NS7a-3' UTR, encoding 16 non-structural proteins and 4 structural proteins (Gong et al., 2017; Y. Pan et al., 2017; P. Zhou et al., 2018). SADS-CoV caused clinical symptoms similar to other porcine enteric pathogens, such as TGEV, PEDV and PDCoV, characterized by vomiting, diarrhea, dehydration, and a mortality rate as high as 90% in piglets (Xu et al., 2019; P. Zhou et al., 2018). Since SADS-CoV was first reported in southern China in 2017 (Gong et al., 2017), the retrospective investigation of 236 samples from 45 swine farms showed that the prevalence of PEAV in pigs was reported to be 43.53% in Guangdong, China in August 2016 to May 2017 (L. Zhou, Sun, et al., 2019). And yet from May 2017 to January 2019, there

were no new SADS cases reported in pig herds in Guangdong, but the re-emerging of SADS-CoV infection in pig herds in Guangdong on February 2019 (L. Zhou, Li, et al., 2019), indicating a continuing threat to pig farms. Apart from Guangdong, a SADS-CoV-like strain, CN/FJWT/2018, was recently discovered in Fujian, China (K. Li et al., 2018). Recently, it was reported that SADS-CoV could infect human cell lines (Edwards et al., 2020; Yang et al., 2019) and in view of the great harm of SARS-CoV2 (Hu, Guo, Zhou, & Shi, 2020), which same cross-species infection likely originate from bats as SADS-CoV (Hu et al., 2020; P. Zhou et al., 2018), reveal that the monitoring of epidemic situation of SADS-CoV in pig herds might have important public health significance.

Since first detection in swine herds in 2017, multiple detection methods including real time reverse transcription loop-mediated isothermal amplification method (RT-LAMP) (H. Wang et al., 2018), SYBR green-based real-time PCR (Ma et al., 2019), multiplex*Taq* Man-probe-based real-time PCR (Z. Pan et al., 2020)), microfluidic-RT-LAMP chip (L. Zhou et al., 2020) are developed and widely used to detect SADS-CoV infection. However, these detection methods only target viral nucleic acid, serological assays for SADS-CoV to undertake epidemiological studies are not available to date. Enzyme-linked immunosorbent assay (ELISA), which mainly including indirect ELISA, double-antibody sandwich ELISA and blocking ELISA, has strong specificity and high sensitivity and is widely used in pathogens antibodies detection and vaccines evaluation (Knuchel, Ackermann, Muller, & Kihm, 1992). The basic principle of ELISA is the specific response between antigen and antibody. Therefore, for pathogen specific antibody detection, it is critical to select the appropriate detect antigen. S protein of CoVs belong to type I membrane fusion protein, locate on the envelope of the virus particle (Yu, Qiao, Guo, & Wang, 2020). It is known that the S protein, which can be divided into domain 1 (S1) and domain 2 (S2), plays an important role in virus attachment and entry, and induction of neutralizing antibodies*in vivo* (Wen et al., 2018). In addition, the S protein has higher antigenicity than any of the other CoVs' proteins, and anti-S antibodies persist longer than anti- nucleocapsid (N) antibodies *in vivo*(Knuchel et al., 1992). Therefore, Many studies have selected the whole or part of S protein as a coating antigen to develop indirect ELISA methods for the detection of TGEV (Sestak, Zhou, Shoup, & Saif, 1999), PEDV (Lin et al., 2018) and PDCoV (Lu et al., 2020) antibodies. Although SADS-CoV has the smallest S protein of only 1130 amino acids (Gong et al., 2017) compared to other reported CoVs, the structural features of SADS-CoV S protein are evolutionarily related to other CoVs genera (Guan et al., 2020), indicating that S protein of SADS-CoV can also be used as detect antigen.

SADS-CoV is an important enteropathogen in pigs, but currently no suitable serology assays to detect the specific antibodies for SADS-CoV. The objective of this study was to develop an anti-SADS-CoV IgG indirect ELISA based on recombinant S protein and utilize this ELISA to investigate the prevalence rates of SADS-CoV in the pig farms of China, which might provide a tool for advancing understanding of the epidemiology of SADS-CoV.

Materials and Methods

Virus and Cells

The SADS-CoV GDS04 strain was isolated from piglets with severe diarrhea in our laboratory (Xu et al., 2019), and the virus was propagated in DMEM supplemented with 10 μg/mL trypsin (Gibco, USA) and cultured under the conditions described below.

Human embryo kidney (HEK) 293 T (ATCC CRL-1573) and Vero cells were obtained from ATCC (ATCC number: CCL-81) (USA). IPI-FX cells (X. Wang et al., 2019) were kindly provided by Professor Shaobo Xiao (Huazhong Agricultural University, China). All cells cultured in Dulbecco's modified eagle medium (DMEM) (Hyclone, USA), supplemented with 10% fetal bovineserum (FBS) (BOVOGEN, Australia). Human embryo kidney (HEK) 293 F and insect Sf9 cells were obtained from Wen' s Foodstuffs Group Co., Ltd (Guangdong, China), grown in Celkey CD HEK293 202 medium (IOSCIENCES, China) and SF-FSM serum-free medium (SuZhou World-medium Biotechnology Co. Ltd, China), respectively. All mediums supplemented with 100 U/mL penicillin, and 100 U/mL streptomycin. All cells were incubated in 37 with 5% CO_2 incubator.

Construction of Plasmids and recombinant baculovirus generation

The region encoding the S protein of SADS-CoV strain GDS04 (GenBank no. MG742313.1) was analyzed by using Uniprot software (https://www.uniprot.org/uniprot/A0A3G1II32). The domain containing amino acids (aa) 19-1068 of S protein, which remove signal peptides, transmembrane regions and cytoplasmic regions, was 3' terminally fused with a cysteine protease cleavage aequence (TEV, encoding ENLYFQG) and flexible peptide (FP, encoding GGSGG), followed by Fc domain of human IgG1 (GenBank no. MH975516.1), synthesized by Suzhou GENEWIZ Biotechnology Co. Ltd (China), and then cloned into the*Nhe* I-*Xho* I restriction sites of the eukaryotic expression vector pCMV (Wen' s Foodstuffs Group Co., Ltd, China) to yield the recombinant plasmid pCMV-S-Fc (Fig 1).

The plasmid pCMV-S-Fc were then chemically transformed into competent DH10BacTM *Escherichia coli* (*E. coli*) cells (Invitrogen, USA), and the clones were confirmed with sequencing analysis by Suzhou GENEWIZ Biotechnology Co. Ltd (China). Recombinant *baculovirus*generation was performed as previously described with some modifications (Yin et al., 2016). Briefly, the obtained recombinant bacmids were transfected into Sf9 cells using Cellfection® II Reagent (Gibco, USA) according to the manufacturer's instruction, and incubated for 7 days. The target recombinant *baculoviruses* were then harvested from the supernatant.

Expression and purification of the recombinant S protein

For recombinant proteins expression, HEK 293F cells were cultured at an orbital shaker incubator in two 1 L flasks containing 500 mL culture volume until the cell density reached 2 x 10^6cells/mL, and the cell survival rate was above 95%, then infected with recombinant or wild-type *baculoviruses* (rB or wB) at a multiplicity of infection (MOI) of 20 and incubated at 37 . After 8 h, sodium butyrate (Shanghai Macklin Biochemical Co., Ltd, China) was added to the final concentration of 10 mM, and incubated for 3 days at 37 in 5% CO_2. Cells were then collected, lysed in 1% Triton X-100, and then centrifuged at 12000 x *g* for 30 min at 4 . The supernatants were prepared and examined with Western Blot as described below.

The resulting precipitates were resuspended in 1 x phosphate buffer saline (PBS) (pH = 7.4), ultrasonicated, and then centrifuged at 12000 x*g* for 10 min at 4 . The supernatants were collected and purified using protein G REsin (GenScript, Nanjing, China) according to the manufacturer's instructions. The purified proteins were analyzed using Coomassie Blue stained SDS-PAGE electrophoretogram, then quantified by NanoDrop spectrophotometry (IMPLEN, Germany) and supplemented with 1% protease inhibitor cocktail C (Beijing Yataihengxin Company, China) and frozen at -80 degC until use.

Western Blot Analysis

The 293F cell lysates infected recombinant or wild-type*baculoviruses* (rB or wB) were resuspended with 120 μL 1 × SDS-PAGE loading buffer and boiled for 10 min before fractionated by electrophoresison 8% SDS-PAGE gels, and the resolved proteins were transferred onto PVDF membranes (Millipore, USA). After blocking with 5% skim milk, the membranes were probed with HRP-conjugated goat anti-Human Fc polyclonal antibody (1:2000; DingGuoShengWu, China) or primary antibodies [SADS-CoV-positive pig serum (1:200; Wen's Foodstuffs Group Co., Ltd, China), or SADS-CoV-negative specific pathogen free (SPF) pig serum (1:200; Wen's Foodstuffs Group Co., Ltd, China)], followed by incubation with HRP-conjugated goat anti-pig IgG (1:2000; Solarbio, China) secondary antibody. The blots were visualized using the ECL reagent according to the manufacturer's instructions (GE Healthcare, UK).

SADS-CoV S protein indirect ELISA (S-iELISA) development

Conventional indirect ELISA detection serum antibodies as described previously with some modifications (Lin et al., 2018). ELISA plates with 96 wells (Costar, USA) were coated with 100 μL/well of purified recombinant S-Fc protein in 50 mM bicarbonate buffer (pH = 9.6) and incubated overnight at 4 °C. After three washs with PBST (1 × PBS containing 0.05% Tween-20), the plates were blocked with 5% NON-Fat Powdered Milk (Shanghai Sangon Company, China) in 1 × PBST for 2 h at 37 °C. After plates were washed three times, 100 μL/well porcine serum samples diluted in 1 × PBST was added and incubated for 1 h at 37 °C. After the same washing step, plates were reacted with 100 μL diluted secondary antibody (HRP-goat anti-pig IgG, Solarbio, China) for 30 min at 37 °C for the purpose of detecting IgG against SADS-CoV in

serum samples. Plates were then washed three times and the peroxidase reaction was visualized using 100 μL 3,3',5,5'-tetramethylbenzidine (TMB) (KPL, USA) as the substrate for 15 min at 37 °C in complete darkness and stopped by adding 50 μL of 2 M H_2SO_4 to each well. Finally, the OD_{450nm} values was measured and recorded immediately using Spectra-Max M2 (Molecular Devices, USA). Positive, negative and blank (1 × PBST) samples were tested in duplicate and included on each plate.

The optimal concentration of coating antigen and second antibody dilution for the S-iELISA were determined using a checkerboard titration as described previously with some modifications (Li et al., 2015). Briefly, the concentration of S-Fc protein was coated on 96-well microtiter plates gradually reduced from 0.25 μg/mL to 8 μg/mL. The standard pig SADS-CoV-positive serum and negative serum were diluted in 1:100. After the antigen and antiserum were added, HRP-labeled secondary antibody was added correspondingly to the plate with dilutions from 1:1000 to 1:8000 to determine the optimal conjugate dilution. When the conditions that gave the highest OD_{450nm} ratio between the positive and negative serum (P/N value) and an OD_{450nm} value for positive serum close to 1.0 with the OD_{450nm} value of negative serum [?] 0.2 were consider optimal.

After the conditions mentioned above were determined, the serum were diluted in serial twofold dilutions from 1:100 to 1:3200. Then, the blocking buffers with 1% BSA, 5% skimmed milk, 5% fetal bovine serum, 10% goat serum were used and blocked for 1, 2, 3 and 4 h at 37°C. The incubation time of serum sample was optimized with 0.5, 1, 1.5, 2 h. The HRP-goat anti-pig IgG was optimized with 15, 30, 45, 60 min. The reactions were stopped and optimized by assessing 10, 15, 20, and 25 min, respectively.

Determination of the cut-off value of S-iELISA

The cut-off value of S-iELISA was determinated as described previously with some modifications (Li et al., 2015). Forty SADS-CoV-negative serum samples were tested using the S-iELISA as described above to determine the cut-off value. Each sample was tested three times, and the mean OD_{450nm} value of SADS-CoV-negative serum samples plus three standard deviations (SDs) was used to calculated the cut-off value. The OD_{450nm} value of serum samples showing greater than or equal to this cut-off value were considered SADS-CoV-seropositive. This calculation gives 99% confidence that all negative values will fall within the defined range.

Determination of repeatability of S-iELISA

The reproducibility within and between runs of S-iELISA was evaluated, as described previously with some modifications (Li et al., 2015; Lin et al., 2018). Six control serum samples (5 IFA-positive samples and 1 IFA-negative samples) were selected for reproducibility experiments. For intra-assay reproducibility, each serum sample was tested in three replicates on the same plate within the same occasion. For inter-assay reproducibility, each serum sample was tested in three replicates on different plates of different occasions. The results were presented as the coefficient of variation (CV), which is the ratio of the standard deviation (SD) to the mean OD_{450nm} value of each group of samples (S). A CV value criterion of 10% was used to meet the repeatability requirement of the test.

Serum cross-reactivity of S-iELISA to other swine pathogens

To determine the cross-reactivity, porcine positive sera [All porcine positive sera were kindly provided by Professor Lang Gong (South China Agricultural University, China)] against SADS-CoV, PEDV, PDCoV, TGEV,*Porcine circovirus type 2* (PCV2), Classical swine fever virus (CSFV), *Pseudorabies virus* (PRV), *Foot-and-mouth disease virus* (FMDV), *Porcine reproductive and respiratory syndrome virus* (PRRSV), and *African swine fever virus* (ASFV) were tested using the S-iELISA. Each sample was tested three times, and the mean OD_{450nm} value was calculated to determine whether the sample was positive or negative as above described.

Immunofluorescence assay (IFA)

To evaluate the practicality of the S-iELISA, a total of 111 pig serum samples were evaluate by as above described S-iELISA and IFA, respectively. IFA was conducted as described previously with some modifica-

tions (X. Wang et al., 2019; Xu et al., 2019). Briefly, IPI-FX cells (1×10^5) were seeded on 24-well plates and cultured overnight, then infected with SADS-CoV at a multiplicity of infection (MOI) of 0.01. At 24 hours after inoculation, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then permeabilized with 0.2% Triton X-100 for 15 min on the ice. The cells were then blocked with 1% bovine serum albumin (BSA) at 37 for 2 h, and incubated with pig sera (1:200; Shenzhen agriculturalproduct Quality and Safety Inspection and Testing Center, China) at 37 for 1 h, followed by Cy3-labelled rabbit anti-pig secondary antibody (1:1000; Solarbio, China) at 37 for 50 min. The cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) [Beyotime Biotechnology (Nanjing, China)] for 5 min at room temperature. After the cells were washed with 1 x PBST, the stained cells were observed with a fluorescence microscope (LEICA DMi8, Germany).

The results of S-iELISA and IFA were compared and the results of IFA were used as the standard for positive and negative to determine the sensitivity and specificity of the S-iELISA as described previously (Lin et al., 2018). Briefly, sensitivity was defined as the ratio of positive tests from the S-iELISA to the positive tests from the reference IFA. Specificity was defined as the ratio of negative tests from the S-iELISA to the negative tests from the reference IFA.

Virus neutralization assays

The neutralizing antibody titers of SADS-CoV in sera were examined according to described previously with some modifications (Li et al., 2015; Wen et al., 2018). Briefly, seven serum samples with different OD_{450nm} values (0.1054, 0.6131, 0.8040, 1.0042, 1.1795, 1.3908, 1.7055) were heated at 56 for 30 min and were serial 10-fold diluted in serum-free DMEM medium before mixing with an equal volume of 200 $TCID_{50}$ SADS-CoV strain GDS04 and incubated for 1 h at 37 . A positive control and a negative control were included on each plate. The mixture was added to washed (three times with sterile 1 x PBS) Vero cell monolayers grown in micro-titerplates (Nunc 96-well tissue culture plate, Corning, USA) (100 μL/well) and incubated for 1 h at 37 . Cells were then washed again and incubated in the maintenance medium at 37 in 5% CO_2. After 5 days, CPE was observed using an inverted microscope and the neutralizing concentration was defined as the lowest concentration of antibodies in the serum that prevented the occurrence of cytopathic effect (CPE).

Detection of SADS-CoV, PEDV, TGEV and PDCoV IgG antibodies in field serum samples by ELISA

From July 1 to September 30, 2020, a total of 300 serum samples were collected from commercial growing pigs on different farms with reported diarrhea outbreaks from eleven provinces in the China (Shanxi, Yunnan, Guangdong, Jiangxi, Henan, Hubei, Hebei, Hunan, Qinghai, Anhui, Shanxi), and stored at -20 until further tested. All pig serum samples were tested for presence of anti-SADS-CoV IgG antibodies using the S-iELISA as above described, and each sample was tested three times. In addition, all pig serum samples also were tested for presence of anti-PEDV (Shenzhen Lvshiyuan Biotechnology Co., Ltd, China), TGEV (Shenzhen Lvshiyuan Biotechnology Co., Ltd, China) and PDCoV (Jianglaibio, China) IgG antibodies using the commercial kits.

Results

Expression, purification and identification of SADS-CoV S protein

To express the S protein of SADS-CoV *in vitro* , eukaryotic expression of the synthesized *S* gene was done using the vector pCMV with an Fc tag (Fig 1), then the cell lysates infected by rB or wB was detected by Western Blot with anti-huamn Fc PcAb and porcine anti-SADS-CoV positive or negative serum, respectively. As shown in Fig 2A, a specific band could be detected by Western Blot, indicating that S-Fc protein have been expressed and have good antigenicity. Since the S-Fc protein was confirmed successfully expressed in HEK 293F cells, the S-Fc protein was purified with protein G Resin. As shown in Fig 2B, more 95% purity protein was examined by SDS-PAGE, indicating that purified S-Fc protein can be used to develop ELISA.

Optimization of S-iELISA

To develop an indirect ELISA for detecting SADS-CoV IgG antibodies, a checkerboard titration was per-

formed to determine the optimal work conditions of S-iELISA based on the standards that the OD_{450nm} value of positive serum was close to 1.0, the P/N value was highest, and the negative serum was low. As shown in Fig 3, the optimal dilution of coated antigen S-Fc protein and second antibody dilution were set at 4 μg/well and 1:8000. Furthermore, the optimal serum sample dilution was defined as 1:200. After the above-mentioned conditions were determined, it was found that 5% skimmed milk in 1 × PBST was the best blocking solution and the best blocking time for 2 h at 37°C were sufficient and time-saving in the S-iELISA. In addition, the optimal reaction times for serum samples and second antibody were 2 h and 45 min, respectively. Finally, the optimal stopping time was determined as 20 min.

Cut-off value, repeatability and specificity of S-iELISA

To determine the cut-off value of the S-iELISA, 40 SADS-CoV-seronegative samples, verified by both IFA and Western Blot assays, were tested by this ELISA method. As shown in Fig 4A, the mean OD_{450nm} value of these negative serum samples was 0.2870, and the SD of these samples was 0.0326. Therefore, the cut-off value of S-iELISA was calculated to be 0.3711, indicating that the sample OD_{450nm} value[?]0.3711 was considered to be SADS-CoV-seropositive and vice versa. In the repeat ability experiment, six SADS-CoV-positive serum samples and one SADS-CoV-negative serum sample were used to determine the intra- and inter-assay CV of the S-iELISA, which was 0.49%-2.09% and 2.41%-5.20%, respectively (Fig 4B). To determine the specificity of S-iELISA, porcine positive sera against common swine viruses were examined. As shown in Fig 4C, the average OD_{450nm} values of positive serum samples for PEDV, TGEV, PDCoV, FMDV, ASFV, CSFV, PRV, PRRSV and PCV-2 were 0.2507, 0.3018, 0.282, 0.2732, 0.1881, 0.2140, 0.2659, 0.2205, 0.2686, respectively. These values are all less than 0.3711, indicating that these serum samples were PEDV-seronegative and non-cross-reactive with this S-iELISA.

Validation of S-iELISA

To analysis the specificity and sensitivity of S-iELISA, a total of 111 pig serum samples were tested by IFA (Fig 5A) and S-iELISA, respectively. As shown in Fig 5B, the IFA detected 92 SADS-CoV-positive samples, of which 90 tested SADS-CoV-positive by S-iELISA. On the other hand, of the remaining 19 samples that tested SADS-CoV-seronegative by IFA, 18 of them were tested SADS-CoV-negative by S-iELISA. Hence, the sensitivity of S-iELISA was 97.8% among SADS-CoV-seropositive individuals, and the specificity was 94.7% among SADS-CoV-seronegative individuals using IFA as standard evaluation method. These results suggest that the overall coincidence rate of the S-iELISA to IFA was 97.3%.

Neutralization tests

Considering the S protein of CoVs can induce neutralizing antibodies *in vivo* (Wen et al., 2018), we attempted to analyze the relationship between OD_{450nm} values tested by S-iELISA and neutralization titers. As shown in Fig 6, 6 ELISA-positive sera had different level of virus neutralization activity and the higher OD_{450nm} value and the higher neutralization antibody, indicating that OD_{450nm} values of pig serum might positively correlated with virus neutralization activity.

Detection of SADS-CoV, PDCoV, TGEV and PEDV in field samples

To determine the prevalence of swine enteropathogenic CoVs in pig farms, a total of 300 serum samples were collected from commercial growing pigs on different farms with reported diarrhea outbreaks from eleven provinces in China from July 1 to September 30, 2020, and all serum samples were tested for presence of anti-SADS-CoV, PDCoV, TGEV and PEDV IgG antibodies using the ELISA kits. As shown in Fig 7, 81.7% (245/300) of the pig serum samples were positive for anti-SADS-CoV IgG antibodies and the IgG positive for TGEV, PDCoV and PEDV were 54% (162/300), 65.3% (196/300), 6% (18/300), respectively. In addition, of 300 pig serum samples examined, 4 (1.3%) were positive for both SADS-CoV and PEDV, 59 (19.7%) were positive for both SADS-CoV and PDCoV, 53 (17.7%) were positive for both SADS-CoV and TGEV. Of note, 11 (3.7%) were positive for SADS-CoV, PEDV, PDCoV and TGEV, indicating that swine enteropathogenic CoVs coinfections exist in pig farms.

Discussion

Since SADS-CoV was first reported in pigs in early February 2017 in Guangdong, China (P. Zhou et al., 2018), this novel swine enteric CoV has been widely detected in areas of southern China, including Guangdong and Fujian (K. Li et al., 2018; L. Zhou, Li, et al., 2019), results in significant economic losses to the pig industry. Vaccines are essential for the prevention and control of porcine enteric CoVs. Serological methods are helpful to evaluate the immune effect of vaccines and monitor the prevalence of viruses. Currently there are no suitable serological method to assess the infection of SADV-CoV and effectiveness of vaccines. In the present study, an indirect ELISA method using a recombinant S protein from SADS-CoV was developed and utilized this ELISA to investigate the prevalence rates of SADS-CoV in the pig farms of China.

The basic principle of ELISA is based on the specific reaction between antigen and antibody. Therefore, the key to establish a specific ELISA method for detecting virus antibodies is to select the appropriate coating antigen. S protein of CoVs has higher antigenicity and persist longer *in vivo* (Knuchel et al., 1992). In addition, the S protein from SADS-CoV has low homology (22.4%-23.9%) with the S proteins from TGEV, PEDV and PDCoV, indicating that there may be no cross-reaction of antibodies against SADS-CoV S protein. These informations prompted us to select the expression and purification of S protein from SADS-CoV as the coating antigen. Prokaryotic and eukaryotic are two common protein express systems (Verma, Boleti, & George, 1998). The prokaryotic expression mainly depends on the expression system of*Escherichia coli* (*E. coli*), which is characterized by high expression of exogenous proteins but no capable of glycosylating proteins (Verma et al., 1998). On the contrary, the eukaryotic expression systems like *baculovirus* to express exogenous proteins can retain the functional activity of viral proteins (Verma et al., 1998). Our previous study found that the PEDV S protein expressed by *E. coli*immunized mice could not induce the body to produce neutralizing antibodies (data not shown), while the expression of *Infectious bronchitis virus* (IBV) S1 protein by *baculovirus* could provide a protective immune response in chickens (Yin et al., 2016), indicating that loss of glycosylation of S protein expressed by *E. coli* might affect its immunogenicity. We also confirmed the presence of glycosylation of S protein from SADS-CoV by using *O* -Glycosidase &α2-3,6,8,9 Neuraminidase (data not shown). Therefore, the S protein from SADS-CoV was expressed by *baculovirus* -HEK 293F cells expression system in this study, and finally obtained the S protein with high purity and good immunogenicity that met the coating antigen requirements of establishing ELISA (Fig 2). We further successfully established a specific indirect ELISA method for the detection of SADS-CoV antibodies by ELISA reaction conditions optimizing, threshold determination, repeatability and specificity analysis. IFA is another common serological method, which is also widely used to identify viruses (Xu et al., 2018). To evaluate the practicality of the S-iELISA, we used IPI-FX, a stable porcine ileum epithelial cell line (X. Wang et al., 2019), to perform IFA to test the same samples tested by S-iELISA. We did not use Vero cells for IFA due to some pig virus vaccines like PEDV vaccine were made with Vero cells (Won et al., 2019), and antibodies against Vero cells might exist in the pig serum, leading to false positive results. Our results showed that the overall coincidence rate between IFA and S-iELISA was 97.3% when IFA as standard evaluation method, indicating that S-iELISA can accurately detect whether the body is infected with SADS-CoV. It is known that the S protein of CoVs plays an important role in virus attachment and entry, and induction of neutralizing antibodies *in vivo*(Wen et al., 2018). The titer of neutralizing antibodies often indicates the protection rate (Q. Li et al., 2018), but the detection of neutralizing antibody is time-consuming. Combining virus neutralization results with 7 different OD_{450nm} values sera, we found that OD_{450nm} values obtained by S-iELISA were positively correlated with SADS-CoV neutralization. This will help to assess the protective effect of the vaccines, but more samples need to be tested to reinforce this conclusion.

Pathogens are detected mainly by nucleic acids and specific antibodies (Thachil, Gerber, Xiao, Huang, & Opriessnig, 2015; Xu et al., 2018). A positive test for enterovirus nucleic acid in diarrhea samples usually indicated that the virus has infected the body, but sometimes some hosts infected with the viruses are in a non-detoxification state or the target organs are not easy to collect, which required serological tests compensate for nucleic acids test at this time. In addition, serological tests can also track whether a healthy body has been infected with pathogen, which could help choose the right time to immunize vaccines. Although only Guangdong and Fujian reported SADS-CoV infection so far (K. Li et al., 2018; L. Zhou, Li,

et al., 2019), we used S-iELSA to test the pig sera from eleven different provinces in China and confirmed that up to 81.7% of the sera were positive, indicating that these places had existed SADS-CoV infection and certain measures should be taken to prevent the SADS-CoV outbreak. In addition, PEDV, TGEV, PDCoV were also tested by ELISA showed that these viruses all had different degrees of infection in China, and there were co-infections of multiple viruses, which there is a risk of outbreak, indicating that the importance of virus surveillance. Together, all these results confirmed that indirect ELISA for SADS-CoV was established in this study is specific, sensitive, repeatable and can be applied for detection the SADS-CoV infection in swine industry. However, there are still several important questions remain for us to do. For instance, how to improve the expression of S protein in *baculovirus* -HEK 293F cells expression system? Whether the specificity of detection can be improved by selecting part of S protein as coating antigen? Elucidation of these questions will help us to develop a specific ELISA method to detect SADS-CoV infection. In addition, IgA induced by mucosal immunity play an key role against enteroviruses (Wen et al., 2018), indicating that further development of an ELISA method based on S protein to detect IgA will help to more accurate evaluate of the protection of vaccines.

In conclusion, an indirect ELISA method with strong specificity, high sensitivity and good repeatability was established in the present study by using a recombinant S protein from SADS-CoV, which might facilitate the development of a reliable tool for the large-scale detection of SADS-CoV in pig farm and evaluate the effectiveness of vaccines.

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Author Contributions

ZCX conceived and designed the experiments; PP, ZCX performed the experiments; ZCX analyzed the data; YCC, ZCX, CYX, YPG, THJ, QFZ, SMZ and MYH contributed reagents/materials/analysis tools; ZCX wrote the paper. YCC checked and finalized the manuscript. All authors read and approved the final manuscript.

Conflict of Interest : The authors declare that they have no conflict interest.

Ethical Statement

Authors have declared that Ethical Statement is not applicable in the current manuscript as the serum samples were collected from the pig farms.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

References

Doyle L.P., Hutchings L.M. (1946). A transmissible gastroenteritis in pigs. *Journal of the American Veterinary Medical Association* , *108* , 257–259.

Edwards, C. E., Yount, B. L., Graham, R. L., Leist, S. R., Hou, Y. J., Dinnon, K. H., 3rd, ... Baric, R. S. (2020). Swine acute diarrhea syndrome coronavirus replication in primary human cells reveals potential susceptibility to infection. *Proceedings of the National Academy of Sciences of the United States of America* , *117* (43), 26915–26925. https://doi.org/10.1073/pnas.2001046117

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., ... Cao, Y. (2017). A New Bat-HKU2-like Coronavirus in Swine, China, 2017.*Emerging infectious diseases* , *23* (9), 1607–1609. https://doi.org/10.3201/eid2309.170915

Guan, H., Wang, Y., Perčulija, V., Saeed, A., Liu, Y., Li, J., ... Ouyang, S. (2020). Cryo-electron Microscopy Structure of the Swine Acute Diarrhea Syndrome Coronavirus Spike Glycoprotein Provides

Insights into Evolution of Unique Coronavirus Spike Proteins. *Journal of virology* , *94* (22), e01301-20. https://doi.org/10.1128/JVI.01301-20

Hu, B., Guo, H., Zhou, P., & Shi, Z. L. (2020). Characteristics of SARS-CoV-2 and COVID-19. *Nature reviews. Microbiology* , 1–14. Advance online publication. https://doi.org/10.1038/s41579-020-00459-7

Knuchel, M., Ackermann, M., Müller, H. K., & Kihm, U. (1992). An ELISA for detection of antibodies against porcine epidemic diarrhoea virus (PEDV) based on the specific solubility of the viral surface glycoprotein. *Veterinary microbiology* ,*32* (2), 117–134. https://doi.org/10.1016/0378-1135(92)90100-8

Li, K., Li, H., Bi, Z., Gu, J., Gong, W., Luo, S., Zhang, F., Song, ... Tang, Y. (2018). Complete Genome Sequence of a Novel Swine Acute Diarrhea Syndrome Coronavirus, CH/FJWT/2018, Isolated in Fujian, China, in 2018. *Microbiology resource announcements* , *7* (22), e01259-18. https://doi.org/10.1128/MRA.01259-18

Li, Q., Xu, Z., Wu, T., Peng, O., Huang, L., Zhang, Y., ... Cao, Y. (2018). A flagellin-adjuvanted PED subunit vaccine improved protective efficiency against PEDV variant challenge in pigs. *Vaccine* , *36* (29), 4228–4235. https://doi.org/10.1016/j.vaccine.2018.05.124

Li, Y., Zheng, F., Fan, B., Muhammad, H. M., Zou, Y., & Jiang, P. (2015). Development of an indirect ELISA based on a truncated S protein of the porcine epidemic diarrhea virus. *Canadian journal of microbiology* , *61* (11), 811–817. https://doi.org/10.1139/cjm-2015-0213

Lin, H., Zhou, H., Gao, L., Li, B., He, K., & Fan, H. (2018). Development and application of an indirect ELISA for the detection of antibodies to porcine epidemic diarrhea virus based on a recombinant spike protein. *BMC veterinary research* , *14* (1), 243. https://doi.org/10.1186/s12917-018-1570-5

Lu, M., Liu, Q., Wang, X., Zhang, J., Zhang, X., Shi, D., ... Feng, L. (2020). Development of an indirect ELISA for detecting porcine deltacoronavirus IgA antibodies. *Archives of virology* , *165* (4), 845–851. https://doi.org/10.1007/s00705-020-04541-6

Ma, L., Zeng, F., Cong, F., Huang, B., Huang, R., Ma, J., & Guo, P. (2019). Development of a SYBR green-based real-time RT-PCR assay for rapid detection of the emerging swine acute diarrhea syndrome coronavirus. *Journal of virological methods* , *265* , 66–70. https://doi.org/10.1016/j.jviromet.2018.12.010

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y. L., ... Huang, Y. W. (2017). Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. *Veterinary microbiology* , *211* , 15–21. https://doi.org/10.1016/j.vetmic.2017.09.020

Pan, Z., Lu, J., Wang, N., He, W. T., Zhang, L., Zhao, W., & Su, S. (2020). Development of a *Taq* Man-probe-based multiplex real-time PCR for the simultaneous detection of emerging and reemerging swine coronaviruses. *Virulence* , *11* (1), 707–718. https://doi.org/10.1080/21505594.2020.1771980

Pensaert, M. B., & de Bouck, P. (1978). A new coronavirus-like particle associated with diarrhea in swine. *Archives of virology* , *58* (3), 243–247. https://doi.org/10.1007/BF01317606

Sestak, K., Zhou, Z., Shoup, D. I., & Saif, L. J. (1999). Evaluation of the baculovirus-expressed S glycoprotein of transmissible gastroenteritis virus (TGEV) as antigen in a competition ELISA to differentiate porcine respiratory coronavirus from TGEV antibodies in pigs. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* , *11* (3), 205–214. https://doi.org/10.1177/104063879901100301

Thachil, A., Gerber, P. F., Xiao, C. T., Huang, Y. W., & Opriessnig, T. (2015). Development and application of an ELISA for the detection of porcine deltacoronavirus IgG antibodies. *PloS one* , *10* (4), e0124363. https://doi.org/10.1371/journal.pone.0124363

Verma, R., Boleti, E., & George, A. J. (1998). Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. *Journal of immunological methods* , *216* (1-2), 165–181. https://doi.org/10.1016/s0022-1759(98)00077-5

Wang, H., Cong, F., Zeng, F., Lian, Y., Liu, X., Luo, M., ... Ma, J. (2018). Development of a real time reverse transcription loop-mediated isothermal amplification method (RT-LAMP) for detection of a novel swine acute diarrhea syndrome coronavirus (SADS-CoV). *Journal of virological methods* ,*260* , 45–48. https://doi.org/10.1016/j.jviromet.2018.06.010

Wang, X., Fang, L., Liu, S., Ke, W., Wang, D., Peng, G., & Xiao, S. (2019). Susceptibility of porcine IPI-2I intestinal epithelial cells to infection with swine enteric coronaviruses. *Veterinary microbiology* , *233* , 21–27. https://doi.org/10.1016/j.vetmic.2019.04.014

Wen, Z., Xu, Z., Zhou, Q., Li, W., Wu, Y., Du, Y., ... Cao, Y. (2018). Oral administration of coated PEDV-loaded microspheres elicited PEDV-specific immunity in weaned piglets. *Vaccine* , *36* (45), 6803–6809. https://doi.org/10.1016/j.vaccine.2018.09.014

Won, H., Lee, D. U., Jang, G., Noh, Y. H., Lee, S. C., Choi, H. W., ... Lee, C. (2019). Generation and protective efficacy of a cold-adapted attenuated genotype 2b porcine epidemic diarrhea virus. *Journal of veterinary science* ,*20* (4), e32. https://doi.org/10.4142/jvs.2019.20.e32

Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., ... Yuen, K. Y. (2012). Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *Journal of virology* , *86* (7), 3995–4008. https://doi.org/10.1128/JVI.06540-11

Xu, Z., Zhang, Y., Gong, L., Huang, L., Lin, Y., Qin, J., ... Cao, Y. (2019). Isolation and characterization of a highly pathogenic strain of Porcine enteric alphacoronavirus causing watery diarrhoea and high mortality in newborn piglets. *Transboundary and emerging diseases* , *66* (1), 119–130. https://doi.org/10.1111/tbed.12992

Xu, Z., Zhong, H., Zhou, Q., Du, Y., Chen, L., Zhang, Y., ... Cao, Y. (2018). A Highly Pathogenic Strain of Porcine Deltacoronavirus Caused Watery Diarrhea in Newborn Piglets. *Virologica Sinica* , *33* (2), 131–141. https://doi.org/10.1007/s12250-018-0003-8

Yang, Y. L., Qin, P., Wang, B., Liu, Y., Xu, G. H., Peng, L., ... Huang, Y. W. (2019). Broad Cross-Species Infection of Cultured Cells by Bat HKU2-Related Swine Acute Diarrhea Syndrome Coronavirus and Identification of Its Replication in Murine Dendritic Cells *In Vivo* Highlight Its Potential for Diverse Interspecies Transmission. *Journal of virology* , *93* (24), e01448-19. https://doi.org/10.1128/JVI.01448-19

Yin, L., Zeng, Y., Wang, W., Wei, Y., Xue, C., & Cao, Y. (2016). Immunogenicity and protective efficacy of recombinant fusion proteins containing spike protein of infectious bronchitis virus and hemagglutinin of H3N2 influenza virus in chickens. *Virus research* , *223* , 206–212. https://doi.org/10.1016/j.virusres.2016.07.010

Yu, J., Qiao, S., Guo, R., & Wang, X. (2020). Cryo-EM structures of HKU2 and SADS-CoV spike glycoproteins provide insights into coronavirus evolution. *Nature communications* , *11* (1), 3070. https://doi.org/10.1038/s41467-020-16876-4

Zhou, L., Chen, Y., Fang, X., Liu, Y., Du, M., Lu, X., ... Lan, T. (2020). Microfluidic-RT-LAMP chip for the point-of-care detection of emerging and re-emerging enteric coronaviruses in swine. *Analytica chimica acta* , *1125* , 57–65. https://doi.org/10.1016/j.aca.2020.05.034

Zhou, L., Li, Q. N., Su, J. N., Chen, G. H., Wu, Z. X., Luo, Y., ... Ma, J. Y. (2019). The re-emerging of SADS-CoV infection in pig herds in Southern China. *Transboundary and emerging diseases* , *66* (5), 2180–2183. https://doi.org/10.1111/tbed.13270

Zhou, L., Sun, Y., Lan, T., Wu, R., Chen, J., Wu, Z., ... Ma, J. (2019). Retrospective detection and phylogenetic analysis of swine acute diarrhoea syndrome coronavirus in pigs in southern China. *Transboundary and emerging diseases* ,*66* (2), 687–695. https://doi.org/10.1111/tbed.13008

Zhou, P., Fan, H., Lan, T., Yang, X. L., Shi, W. F., Zhang, W., ... Ma, J. Y. (2018). Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature* , *556* (7700), 255–258.

https://doi.org/10.1038/s41586-018-0010-9

Figure legends

Fig.1. The circle map of SADS-CoV-S-Fc fusion expression plasmid.

Fig.2. Expression and purification of the SADS-CoV S-Fc protein.

(A) The expressions of recombinant S-Fc fusion protein were detected by Western Blot using anti-SADS-CoV Ab positive serum, negative serum and anti-Human Fc PcAb as control. (B) SDS-PAGE analysis of the purified S-Fc fusion protein. M represents standard protein markers. The band of purified S-Fc fusion protein was indicated by arrow.

Fig.3. Working conditions optimization of S-iELISA.

(A-B) Coating antigen concentrations and serum sample dilutions. (C-D) Second antibody dilutions and sealing times. (E-F) Sealing buffers and the incubation times of antigen and serum. (G-H) The incubation times of second antibody and exposure times of TMB.

Fig.4. Determination of the cut-off value, repeatability and specificity of S-iELISA.

(A) Forty SADS-CoV-negative serum samples were tested using the S-iELISA and the mean OD_{450nm} value of SADS-CoV-negative serum samples plus three standard deviations (SDs) was used to calculated the cut-off value. SADS-CoV-positive serum as control. (B) Six control serum samples (5 IFA-positive samples and 1 IFA-negative samples) were tested using the S-iELISA and the OD_{450nm} values of serum samples were used to calculate the coefficient of variation (CV) to determine the intra- and inter-assay reproducibility. (C) Porcine positive sera against SADS-CoV, PEDV, PDCoV, TGEV, PCV2, CSFV, PRV, FMDV, PRRSV, and ASFV were tested using the S-iELISA and the mean OD_{450nm} value was calculated to determine whether the sample was positive or negative based on cut-off value. SPF pig serum as control.

Fig.5. Validation of the S-iELISA by IFA.

(A) Identification of SADS-CoV-negative and positive sera by IFA. IPI-FX cells were seeded on 24-well plates and cultured overnight, followed by infection with SADS-CoV at a MOI of 0.01. Mock-inoculated IPI-FX cell culture showing normal cells. SADS-CoV-inoculated IPI-FX cells at 1 d.p.i. showing syncytium (indicated by arrows). At 24 hour postinoculation, cells were fixed and probed with 111 pig serum samples containing SADS-CoV-negative, weakly positive, strongly positive serum samples and 1 × PBS, SADS-CoV Ab (-) serum, SADS-CoV Ab (+) serum as controls, followed by incubation with Cy3-conjugated rabbit anti-pig antibodies (red). Cell nuclei were counterstained with DAPI (blue). Cells were observed with a fluorescence microscope. The scale bar represents 100 μm. (B) The specificity and sensitivity of the S-iELISA were analyzed based on the results of IFA.

Fig.6. Determination of the correlation between SADS-CoV virusneutralization titers and OD_{450nm} values of S-iELISA.

Seven serum samples were tested by S-iELISA and virus neutralization assays, respectively. The OD_{450nm} values and neutralizing antibody titers were compared to determine the relationship between S-iELISA and the neutralizing antibody of SADS-CoV.

Fig.7. Rate of detection of anti-SADS-CoV, PDCoV, TGEV and PEDV IgG antibodies in pig serum samples.

From July 1 to September 30, 2020, a total of 300 serum samples were collected from commercial growing pigs on different farms with reported diarrhea outbreaks from eleven provinces in the China (Shanxi, Yunnan, Guangdong, Jiangxi, Henan, Hubei, Hebei, Hunan, Qinghai, Anhui, Shanxi), and tested for presence of anti-SADS-CoV (A), TGEV (B), PDCoV (C) and PEDV (D) IgG antibodies using the ELISA kits.