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Development and Diagnostic Application of Monoclonal Antibodies Against Swine Acute Diarrhea Syndrome Coronavirus (SADS-CoV)

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Abstract

Swine Acute Diarrhea Syndrome Coronavirus (SADS-CoV) is a member of the *Coronaviridae* family. The virus is associated with severe small intestine inflammation and diarrhea in suckling piglets. In 2017, SADS-CoV was first detected and identified as the causative agent of a devasting swine disease outbreak in southern China. Routine monitoring and early detection of the source of infection is therefore integral to the prevention and control of a SADS-CoV outbreak in the United States. However, the United States does not currently have any diagnostic or surveillance tests to identify this emerging disease. To address these industry needs, we developed monoclonal antibodies against SADS-CoV. To start with, the nonstructural SADS-CoV, papain-like protease 2 region (PLP2) was cloned into an *E.coli* plasmid expression vector Next, the nonstructural protein SADS-CoV PLP2 was expressed, purified and used as antigen. Then, the purified antigen was used to immunized mice to produce monoclonal antibodies and rabbits were immunized to produce antisera containing polyclonal antibodies also for diagnostic test development. Mouse spleen cells and a mouse tumor cell line (NS-1 myeloma) were fused together to produce hybridoma clones. The resulting monoclonal antibodies were characterized and evaluated for their ability to specifically recognize SADS-CoV PLP2 epitope in cell culture. In total, nine monoclonal antibodies and approximately 105 milliliters of rabbit antisera were produced. The immunoreactivity of each monoclonal antibody and antisera was tested by enzyme linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), and Western blot assays. The monoclonal antibodies will be used to develop validated diagnostic ELISA, IFA, Western blot tests and to better understand virus replication. Overall, the monoclonal antibodies, reagents, and assays will be vital in establishing the first early detection and surveillance tests thus preventing major economic losses to the swine industry.

Introduction

Swine Acute Diarrhea Syndrome Coronavirus (SADS-CoV) is an enveloped, positive-sense, single-stranded RNA virus that belongs to the genus *Alphacoronavirus* in the family *Coronaviridae* [1]. Coronaviruses are separated into four distinct genera based on genotypic and serological characterization: alpha-CoV, beta-CoV, gamma-CoV, and delta-CoV. SADS-CoV is an alpha coronavirus [2]. Several coronaviruses are known to cause diarrhea in swine and cause

substantial economic losses to the industry. Transmissible gastroenteritis (TGEV), porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) have been identified to induce clinical diarrhea in young pigs. PEDV caused high fatalities to newborn piglets in China in late 2010 and in the United States in 2013 and posed a serious threat to the pork industry [3]. Additionally, several emerging human coronaviruses have caused respiratory illness over the past 20 years. Since 2002, 8,098 cases of Severe Acute Respiratory Syndrome (SARS) have been reported with a 10% death rate [4]. Additionally, 2,494 cases of Middle East Respiratory Syndrome (MERS) have been confirmed with a 34% death rate since 2012 [5]. Today, MERS has remained endemic in the Middle East. Most recently in December 2019, a novel coronavirus originating in Wuhan, China has been linked to a related respiratory disease and has been detected in 32 locations internationally including the United States. The novel coronavirus has been named SARS-CoV 2 as of February 2020. The pandemic outbreaks and disease have been named Coronavirus Disease 2019 (COVID-19). As of February 7, 2020, 31,528 people have been infected and 638 people have died [6].

Coronaviruses can jump species, which makes them a concern to animal and public health. In addition to extreme diversity among coronaviruses, many circulate in wildlife species [7]. Diversity is most notable in bats. More specifically, SARS like viruses and bat-CoV-HKU2 have been discovered in horseshoe bats. Novel delta-CoVs have also been identified in birds and swine. In February 2017, SADS-CoV contributed to a devasting swine disease outbreak in Guangdong province, China. During the outbreak, more than twenty thousand piglets across four farms died, resulting in huge economic losses. The piglets infected with SADS-CoV mainly exhibited the clinical manifestations of acute vomiting, severe intestinal inflammation, diarrhea, and death. Clinical onset occurred a few days later than those infected with PEDV. Additionally, the swine herds were vaccinated against PEDV [8]. Initial reverse transcription PCR tests used primers specific for PEDV, TGEV, and PDCoV. None of these viruses were detected in all clinical samples. Furthermore, the recovered sows showed no seroneutralizing antibodies against PEDV [9]. The genome sequence of the causative agent of this large-scale outbreak shared remarkable homology with a bat coronavirus detected in a cave near the pig farm [8]. SADS-CoV had 95% nucleotide identity at the full-genome level with the previously reported bat-HKU strains [10]. This implies SADS-CoV most likely originated in bats. To address the origin and evolutionary history, Bayesian analysis indicate the virus emerged approximately 91 years ago and may have circulated in swine herds for several decades [3]. From May 2017 to January 2019, no new SADS-CoV cases were reported in the Guangdong province. In February 2019, another large scale outbreak of approximately two thousand piglet deaths was reported from a farm near the origin of the SADS-CoV pandemic in 2017 [11]. The re-emergence of SADS-CoV and high lethality solidifies the need for diagnostic test validation.

SADS-CoV consists of a 27.1 kilobasepair genome and is organized similarly to the bat-like HKU2 strains of coronavirus (Figure 1) [12]. The genome consists of a 5’ untranslated region (UTR) followed by at least seven open reading frames (ORF1a, ORF1b, and ORF2-6). ORFs 1a and 1b occupy the 5’-proximal two-thirds of the genome and code for nonstructural proteins (nsps) [13]. The translation of ORF1a yields a replicase polyprotein (pp) 1a. Similarly, ORF1b is expressed into pp1ab. These pp1a and pp1ab are post-translationally cleaved by

internal proteases generating 16 processing end products named nsp1-16. The remaining ORFs in the 3'-proximal genome region encodes for the following structural and nonstructural proteins expressed from the respective 3'-co-terminal set of subgenomic mRNAs: spike (S), nonstructural protein 3a (NS3a), envelope (E), membrane (M), nucleocapsid (N), nonstructural protein 7a (NS7a), and 3' UTR [12].

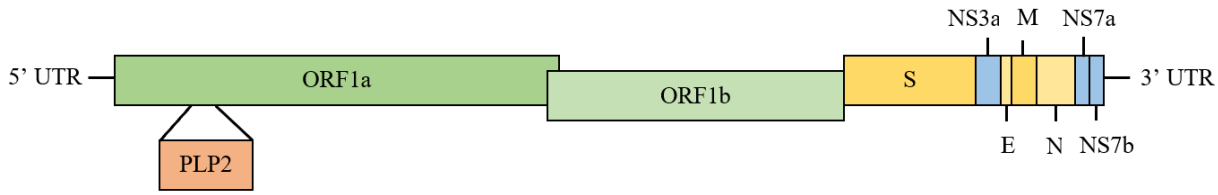

Figure 1. SADS-CoV genomic arrangement. The 27.1 kilobase pair genome consist of a 5' untranslated region followed by open reading frame (ORF) 1a, ORF1b, spike (S), nonstructural protein 3a (NS3a), envelope (E), membrane (M), nucleocapsid (N), nonstructural protein 7a (NS7a), and 3' UTR. ORF1a yields a replicase polyprotein 1a which is post-translationally cleaved by internal proteases. The result is 16 processing end products named nsp1-16. We targeted a region of nsp3 called papain-pike protease 2 (PLP2) to produce monoclonal antibodies against SADS-CoV.

Considering the high lethality of this new emerging virus and the similar clinical signs caused by other porcine enteric coronaviruses, there is an urgent need to establish a sensitive and reliable diagnostic method to aid in early discovery and identification of a SADS-CoV infection in the United States. Our major objective was to develop anti-SADS-CoV monoclonal antibodies. Specifically, we targeted the Papain-like Protease 2 (PLP2) region of within the nsp3 region of ORF1a. The PLP2 is part of the replicase machinery of the virus and is produced very early during infection. This provided the rationale behind using PLP2 gnomic region as our diagnostic target. Since the PLP2 region is a part of the SADS-CoV replicase, these monoclonal antibodies will also lead to a better understanding of how the virus replicates.

Monoclonal antibodies are a monovalent antibody which bind to the same epitope. Monoclonal antibodies are produced from a single B-lymphocyte clone. In 1975, Georges Kohler and Cesar Milstein produced the first monoclonal antibodies by using a hybridoma technique. Hybridomas are generated by immunizing a certain species against a specific antigen epitope. Then, the B-lymphocytes are harvested from the animal spleen and are fused with an immortal myeloma cell line lacking the hyoxanthine-guanine-phoshoribosyltransferase (HGPRT) gene. Additionally, the myeloma cell line does not contain any immunoglobulin-producing cells. Next, the hybridomas are cultured *in vitro* in selective medium which contains hypoxanthine-aminopeterin-thymidine. Only the hybridomas survive as they have inherited immortality from the myeloma cells and selective resistance from the primary B-lymphocytes. Myeloma cells which have not undergone fusion with primary B-lymphocytes lack HGPRT. As a result, the myeloma cells cannot synthesize nucleotides in the selective media. The initial culture of hybridomas contains a mixture of antibodies derived the result of many different primary B-

lymphocytes. Each primary B-lymphocyte secrets its own specific antibody yielding a polyclonal supernatant. To resolve this issue, individual clones are separated by dilution into multiple culture wells. The supernatant of hundreds of individual wells can be screened to determine specific antibody activity against the desired antigen epitope. Once specificity of individual clones is recognized, positive wells are recloned and retested for activity. The generated positive hybridoma clones and monoclonal antibodies are stored long tern in liquid nitrogen. Likewise, hybridoma clones can be thawed from liquid nitrogen and expanded to produce more monoclonal antibodies [14].

Monoclonal antibodies have a wide range of applications. Monoclonal antibodies can be used in the context of diagnostic tests such as enzyme-linked immunosorbent assays (ELISA), immunofluorescence assay (IFA), and Western blot (WB). In each of these assays, the monoclonal antibody detects the presence of a specific epitope in the antigen. After detection, the sample can be concluded positive or negative for the antigen. An ELISA is used to detect the presence of antibodies against a specific protein. The antigen is attached to a surface and a matching antibody is applied over the surface to bind to the antigen. A secondary antibody is added to bind to the primary antibody. This secondary antibody is linked to an enzyme. In the final step, a substance containing the enzyme's substrate is added resulting in a detectable signal or a color change (Figure 2a). In an IFA, antibodies bind to a specific antigen within a cell. The location binding is visualized using fluorescent microscopy. First, cells are grown and transfected with a specific pathogen. Antibodies against the pathogen will bind. After the addition of fluorescent dyes, binding of the antibody to the cell is visualized with a fluorescent microscope (Figure 2b). A Western blot is used to detect specific proteins in a sample of a tissue homogenate or extract. First, the protein is denatured and undergoes separation of molecular weight by gel electrophoresis. An antibody is added to bind to the specific target protein. A secondary antibody binds to the primary antibody. Lastly, the secondary antibody can be visualized by a variety of detection techniques including staining, chemiluminescence, and radioactivity (Figure 2c) [15]. Beyond diagnostics, monoclonal antibodies can be used to purify their target compounds from mixtures using the immunoprecipitation method. More recently, therapeutic treatments of cancer and autoimmune disease have been explored using monoclonal antibodies [14].

Since SADS-CoV is a foreign animal disease to the United States, the live virus could not be used and no serum containing virus could not be obtained from pigs. Accordingly, we synthesized the nonstructural gene of SADS-CoV PLP2. The gene was cloned into an *E. coli* plasmid expression vector. The SADS-CoV PLP2 was expressed, purified and used as antigen for antibody production and ELISA test development. After completion of the mouse immunization regimen, the mouse spleen cells, and mouse tumor line cells were chemically fused together to produce hybridoma clones. The resulting monoclonal antibody-producing clones were characterized and evaluated for their ability to specifically recognize SADS-CoV epitopes. The immunoreactivity of each monoclonal antibody and antisera was tested by ELISA, IFA, and Western blot assays. The monoclonal antibodies will be used to develop validated diagnostic ELISA, IFA, Western blot tests and to better understand virus replication.

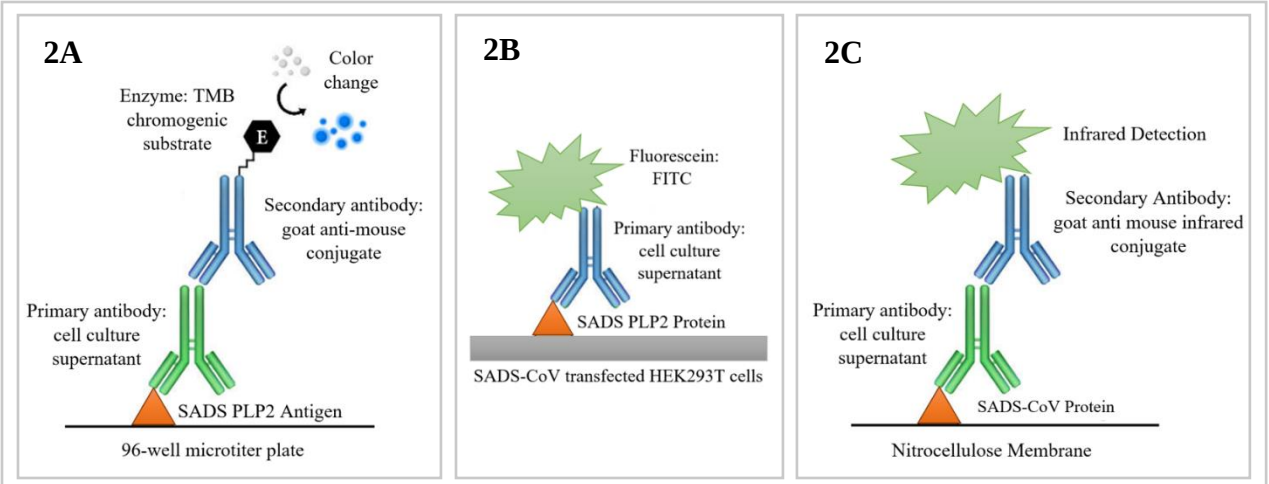

Figure 2. Diagnostic application of monoclonal antibodies by ELISA, IFA, and Western Blot. Anti-SADS-CoV PLP2 monoclonal antibodies were detected by ELISA with the addition of a secondary goat anti-mouse conjugate and TMB enzymatic chromogenic substrate to SADS PLP2 antigen coated 96-well plates (Figure 2A), by IFA with the addition of Fluorescein isothiocyanate (FITC) to SADS-CoV transfected HEK293T cells (Figure 2B), and by western blot with the addition a secondary goat anti-mouse infrared conjugate to the SADS-CoV protein transferred by electroblot to a nitrocellulose membrane (Figure 2C).

Materials and Methods

Cells

HEK293T cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 20 g/ml streptomycin). Cells were maintained at 37˚C in a humidified 5% CO_2 incubator.

Antigen production and expression of the recombinant SADS-CoV PLP2 protein

The antigen used for the immunization of mice and rabbits was a recombinantly expressed, full length, SADS-CoV, PLP2. The 1014 nucleotide SADS-CoV PLP2 gene sequence codon optimized for *E. coli expression* and the full Kozak sequence was added (GeneScript; Piscataway, NJ). Then, the gene was cloned into the pET-28a plasmid expression vector (Novagen; Madison,WI) and then transformed into BL21-Codon Plus (DE3)-RP competent cells (Stratagene; La Jolla, CA) for protein expression.

Next, 20 µL of transformed cells were plated onto Luria-Bertani agar plates containing 50 µg of kanamycin/mL and incubated at 37˚ C with constant shaking at 200 RPM overnight. The following morning colonies from the agar plates were added to 1 L of pre-warmed 2X yeast extract tryptone (YT) culture medium containing 50 µg of kanamycin/mL. Bacterial cultures grew to an approximate OD_{600} of 0.5 at 37˚ C. SADS-CoV PLP2 expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1.0 mM to induce

transcription of both the Lac operon and our downstream PLP2 protein. Additionally, the *E. coli* bacterial culture was incubated for 8 hours at 37˚ C with shaking at 200 RPM. The agar was strained out and bacteria was pelleted by centrifugation at 12,000 x g for 10 minutes at 4˚ C. The pellet was resuspended in 40 mL of lysis buffer solution (B-PER, Pierce, Rockford, IL) and then incubated for 15 minutes at 20-22˚ C. The suspension was centrifuged at 12,000 x g to separate the soluble from insoluble proteins. The resulting 138 amino acid recombinant protein was denatured using 8 M urea, and subsequently purified using nickel-NTA affinity column chromatography and refolded back to its native conformational state. Individual affinity column elutions were collected, pooled and confirmed by SDS-PAGE. Elutions were aliquoted and frozen at -80˚ C. The 41 kDa recombinant protein was confirmed by western blotting using a 6X histidine specific monoclonal antibody (Novagen; Madison, WI). The SADS-CoV PLP2 antigen was scaled up in large batch cultures and purified to a degree of approximately 95%. Only the insoluble, linear form of PLP2 was used to immunize mice and rabbits for hybridoma production and antisera for the development of anti-SADS-PLP2 monoclonal antibodies [16].

Development and diagnostic application of rabbit antisera and mouse monoclonal antibodies

Mice were immunized with the recombinant SADS-PLP2 protein to produce hybridoma clones and monoclonal antibodies to be used in the context of IFA, ELISA, and Western blot assays. Hybridomas were produced as previously described [17, 18]. A group of 3, 10-week-old specific pathogen-free BALB/c AnN mice were immunized intraperitoneally with 250 µg of the recombinant antigen mixed in equal volumes with a water-in-oil adjuvant (Seppic Monanide ISA 50 V2; Seppic, France). A 60-day immunization schedule is common for the immunization of viral, nonstructural proteins. Three identical booster immunizations were given at three-week intervals. Fusion of mouse splenocytes and myeloma cells was performed using NS-1 myeloma cells and 50% polyethylene glycol (GIBCO, Grand Island, NY) and then cultured in the presence of hypoxanthine-aminopterin-thymidine for the selection of viable hybridoma clones. Cell culture supernatants were screened by a PLP2 ELISA and IFA to identify hybridoma clones that will recognize homologous PLP2 antigen in the context of IFA, ELISA, and Western blot. Positive hybridoma clones were subcloned at least twice by limiting dilution. A commercial lateral-flow immunoglobulin typing kit (Serotec; Raleigh, NC) using hybridoma culture fluids determined the immunoglobulin isotypes of the monoclonal antibodies.

Rabbits were immunized with the recombinant SADS-PLP2 protein to produce antisera to be used as a positive control in the context of ELISA, IFA, and Western blot assays. A group of 2, 4-month-old rabbits were immunized subcutaneously and intramuscularly with the recombinant antigen mixed in equal volumes with a water-in-oil adjuvant (Seppic Monanide; Seppic, France). A 150-µg dose was administered to 3 separate subcutaneous locations and 1 intramuscular location. Three booster immunizations were given at three-week intervals. Following immunizations, rabbits were euthanized, and blood was collected by cardiac puncture. Resulting antisera was evaluated for specific recognition of SADS-CoV PLP2 by IFA, ELISA, and Western blot. Animal studies were approved by the South Dakota State University Institutional Animal Care and Use Committee (approval numbers 17-066A and 17-067A)

Transfection and development of immunofluorescent assay for hybridoma antibody screening

For hybridoma and antibody screening, the full length PLP2 region (GeneScript; Piscataway, NJ) was cloned into a mammalian expression vector pcDNA3.1. The full Kozak sequences was added for enhanced Eukaryotic translation. The Kozak sequence contains the AUG start codon and aids in the scanning translational initiation process of the 16S subunit of the small ribosome. Then, the gene transformed into BL21-Codon Plus (DE3)-RP competent cells (Stratagene; La Jolla, CA). Bacterial cultures were grown according to the expression of the recombinant SADS-CoV PLP2 protein. DNA was purified from cellular proteins according to the Qiagen Plasmid Plus Midi Kit (Qiagen; Hilden, Germany). Nanodrop determined DNA concentration and 260/280 absorbance ratio. Additionally, the 1014 base pair SADS-CoV PLP2 region was confirmed by a restriction enzyme endonuclease digestion reaction. The purified plasmid DNA was cut with restriction enzymes BamH1 and Xho1 overnight at 37˚ C. The following morning, the 41 kDa region was analyzed on a 1% agarose gel in modified tris-acetate-EDTA (TAE) buffer. The SADS-CoV PLP2 DNA was scaled up in large batch cultures and purified. Subsequently, the purified SADS-CoV PLP2 DNA was used to transfect HEK293T cells for hybridoma antibody screening and the IFA assay.

HEK293T cells were seeded at a concentration of $3x10^6$ cells per plate in a Immulon 1B, medium binding, 96 well micro-titer plate (Thermo Labsystems; Franklin, MA). Cells were incubated at 37˚ C for 48 hours to 80% confluency. Then, the cells were transfected with 0.4 ug of SADS-PLP2 DNA per well in serum-free medium. The Invitrogen Lipofectamine 3000 Transfection Reagent (Fisher Scientific; Hampton, NH) was used at the following quantities and ratios to transfect two Immulon 1B, medium binding, 96 well micro-titer plates (Thermo Labsystems; Franklin, MA). First, 135 µL Opti-MEM (ThermoFisher Scientific; Waltham, MA) was added to each well. In a sterile microcentrifuge tube, 87 µL of Lipofectamine 3000 was homogenized by gentle vortex in 725 µL of Opti-MEM. In a separate sterile microcentrifuge tube, 58 µL of the P3000 reagent and 58 µL of the SADS-PLP2 DNA was homogenized by gentle vortex in 725 µL of Opti-MEM. 812 µL of diluted Lipofectamine was added to 812 µL of diluted DNA/P3000 (1:1 ratio). The transfection mixture was incubated for 20 minutes at room temperature. 15 µL of the transfection mixture was added to alternating wells of the 96 well microtiter plate. The protein was expressed *in vivo* for 72 hours.

After 72 hours of expression, media was gently dumped out from the plates. The cells were fixed with 80% acetone for 45 minutes to one hour and dried for 15 minutes. The anti-HA tag monoclonal antibody (ThermoFisher Scientifi, Waltham, MA) was diluted at 1:5000 in PBS-BN + 1% Sodium Azide. 100 µL per well of diluted antibody was added. Plates were incubated at 37˚ C for 1-1.5 hours and washed gently three times with PBS. Fluorescein isothiocyanate (FITC; MP Bio; Santa Ana, CA) was diluted in PBS-BN + 1% Sodium Azide. 100 µL per well of diluted FITC conjugate was added. Plates were incubated for 1 hour at 37˚ C. Plates were washed four times gently with PBS and approximately 100 µL per well of PBS was added. With microscope, plates were observed with UV fluorescent filter.

Immunofluorescent antibody assay for hybridoma antibody screening

Immulon 1B, medium binding, 96 well micro-titer plates (Thermo Labsystems, Franklin, MA) were seeded, transfected, and fixed as outlined in the transfection and development of

immunofluorescent assay for hybridoma antibody screening. After cell fixing, 50 µL per well of undiluted cell culture supernatant was added. The plate was incubated at 37˚C with CO_2 for 1 hour and washed three times with PBS. FITC (MP Bio; Santa Ana, CA) was diluted in PBS-BN + 1% Sodium Azide. 100 µL per well of diluted FITC conjugate was added. Plates were incubated for 1 hour at 37˚ C. Plates were washed four times gently with PBS and approximately 100 µL per well of PBS was added. The cells were examined for specific fluorescence with an inverted microscope and a UV light source (Nikon Eclipse TS100).

Enzyme-linked immunosorbent assay for hybridoma antibody screening

For coating plates, SADS PLP2 antigen was diluted 1:2000 in Antigen Coating Buffer (ACB). For heterologous HIS-tag control, the SVS VP2 12-15-15 antigen was diluted 1:2000 in ACB. 100 µL per well of diluted SADS-PLP2 antigen was added in alternating odd-wells to an Immulon 1B, medium binding, 96 well micro-titer plate (Thermo Labsystems, Franklin, MA). 100 µL of diluted HIS-antigen control was added to even-wells of the plate. The plate was incubated at 37˚ C for 1 hour. Then, the plate was incubated overnight at 4˚ C. On the following day, the plate was washed three times with PBS + 0.05% Tween 20 (PBST). 200 µL per well of blocking antibody diluent buffer (BAD) was added to each well. The plate was incubated at 37˚ C for 1 hour. The plate was washed four times with PBST. 50 µL per well of undiluted cell culture supernatant was added to each well and incubated at room temperature for 1 hour. The goat anti-mouse IgG, IgA, IgM-HRP conjugate (MP-Bio; Santa Ana, CA) was diluted 1:5000 in BAD. The plate was washed five times with PBST. 100 µL per well of the diluted secondary antibody conjugate was added and incubated at room temperature for 1 hour. TMB chromogenic substrate (VMRD; Whitman Country, WA) was warmed to room temperate and tested by performing a 1:100 dilution of the enzyme-containing secondary. A clear to blue color should be observed in this test. The plate was washed five times with PBST. 100 µL per well of the chromogenic substrate was added. The reaction was stopped with the addition of 100 µL per well of the 2N H_2SO_4. Optical densities were quantified spectrophotometrically at 450 nm with a ELx800 microplate reader (BioTek Instruments Inc.; Winooski, VT).

Western Blot for hybridoma antibody screening

The recombinant SADS-CoV PLP2 protein was detected by color chromogen development and by infrared. For the color chromogen development Western blot, the recombination SADS-CoV PLP2 protein was diluted at a 1:6 ratio in Laemlli sample buffer. Samples were heat treated for 5-10 minutes at 97˚ C. Samples were loaded into an 8% polyacrylamide gel (Invitrogen; Carlsbad, CA) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 120 Volts for 75 minutes. Next, the protein gel was transferred to nitrocellulose membrane (Fisher Scientific; Hampton, NH) by electroblot at 125 Volts for 1 hour. The membrane was blocked in 10% nonfat dry milk (Shurefine; Skukie, IL) in PBS overnight at 4˚ C with constant agitation. The next day, the cell culture supernatant was diluted 1:25 in 5% nonfat dry milk in PBST plus 0.1% Bovine Serum Albumin (BSA) for 1 to 3 hours at room temperature. The membrane was washed three times for 5 minutes each in PBST. The goat anti-mouse HRP secondary antibody (Seracare; Milford, MA) was diluted 1:5000 in 5% nonfat

dry milk in PBST and incubated at room temperature for 1 hour with constant agitation. The membrane was washed three times for minutes each in PBST. The protein was detected using 4-chloro- 1-naphol as the chromogenic substrate.

For the Licor infrared detection method, the recombination SADS-CoV PLP2 protein was diluted at a 1:8 ratio in Laemlli sample buffer. Samples were heat treated for 5-10 minutes at 97˚ C. Samples were loaded into an 8% polyacrylamide gel (Invitrogen; Carlsbad, CA) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 120 Volts for 75 minutes. Next, the protein gel was transferred to nitrocellulose membrane (Fisher Scientific; Hampton, NH) by electroblot at 125 Volts for 1 hour. The membrane was blocked in 5% nonfat dry milk (Shurefine; Skukie, IL) in PBS overnight at 4˚ C with constant agitation. The next day, the cell culture supernatant was diluted 1:150 in PBST plus 0.1% Bovine Serum Albumin (BSA) for 2 hours at room temperature. The membrane was washed three times for 5 minutes each in PBST. The goat anti-mouse IR secondary antibody (LiCOR Biotechnology; Lincoln, NE) was diluted 1:15000 in 1% nonfat dry milk in PBST plus 0.1% sodium dodecyl sulfate (SDS) for 1 hour at room temperature with constant agitation. The membrane was washed three times for minutes each in PBST. The protein was detected using the Odyssey IR detection system.

Results

Development of immunofluorescent assay for hybridoma screening

The full length PLP2 region was cloned into a mammalian expression vector pcDNA3.1 (Genescript) for hybridoma and antibody screening. Cutting the DNA by a restriction enzyme endonuclease digestion reaction and visualization of resulting DNA fragments on an agarose gel confirmed the presence of the SADS-CoV PLP2 insert in the purified DNA (Figure 3). The restriction enzyme endonuclease digested SADS-CoV PLP2 insert band appeared as expected around 1000 base pairs. The restriction enzyme endonuclease digested PEDV PLP2 control was known to be 984 bp and was chosen as a control due to the similarity in number of base pairs as the SADS-CoV PLP2 insert. Bacterial transformants were isolated from the construct was used to prepare DNA for mammalian cell transfection and antibody screening. The IFA assay showed bright and diffuse immunofluorescent staining of individual transfected cells which expressed the PLP2-HA fusion protein (Figure 4). Some toxicity issues were resolved by experimentation with differing transfection chemistries and optimization of assay conditions.

Expression of recombinant full-length PLP2 protein for antigen production

Coomassie blue staining and Western blotting verified the integrity of the 40.5 kDa protein antigen (Figure 5). The protein was overexpressed at a concentration of 3.0 mg/ml and was purified to a level of ~95%.

Development of reagents for detection of SADS-CoV antigen for diagnostic tests

Two different primary wells were identified as secreting anti-SADS-CoV PLP2 antibodies for ELISA and/or IFA assays after performing the hybridoma fusion. The hybridomas within each well of a 96-well microtiter plate were cloned to limiting dilution. Multiple subclones were screened and then selected for expansion. Subclones were selected based upon IFA fluorescent intensity or ELISA optical density. After expansion, a final screening of each clone's reactivity was assessed by preforming an ELISA, IFA, and Western blot using cell culture supernatant. The results of the nine monoclonal antibodies are summarized in Table 1, where the isotype and relative immunoreactivity of each of the antibodies is identified. Additionally, the cytoplasmic, immunofluorescent staining of SADS-CoV PLP2 transfected HEK293T cells using the cell culture supernatant of six anti-SADS-PLP2 monoclonal antibodies are pictured (Figure 6). Intense, cytoplasmic, immunofluorescent staining of SADS-CoV PLP2 transfected HEK293T cells using rabbit anti-SADS-CoV PLP2 polyclonal antisera was observed (Figure 7). In total, approximately 105 mLs of rabbit anti- SADS-CoV PLP2 polyclonal antisera was harvested from Rabbit A and B. Results of the Western blot detection of rabbit polyclonal antibodies (antisera) and recombinant expressed SADS-CoV PLP2 antigen for all nine of the monoclonal antibodies (cell culture supernatant) are shown (Figure 8). The 126 series of antibodies comparatively gave a significantly stronger signal and lower background signal by the Western detection than the 163 series of antibodies.

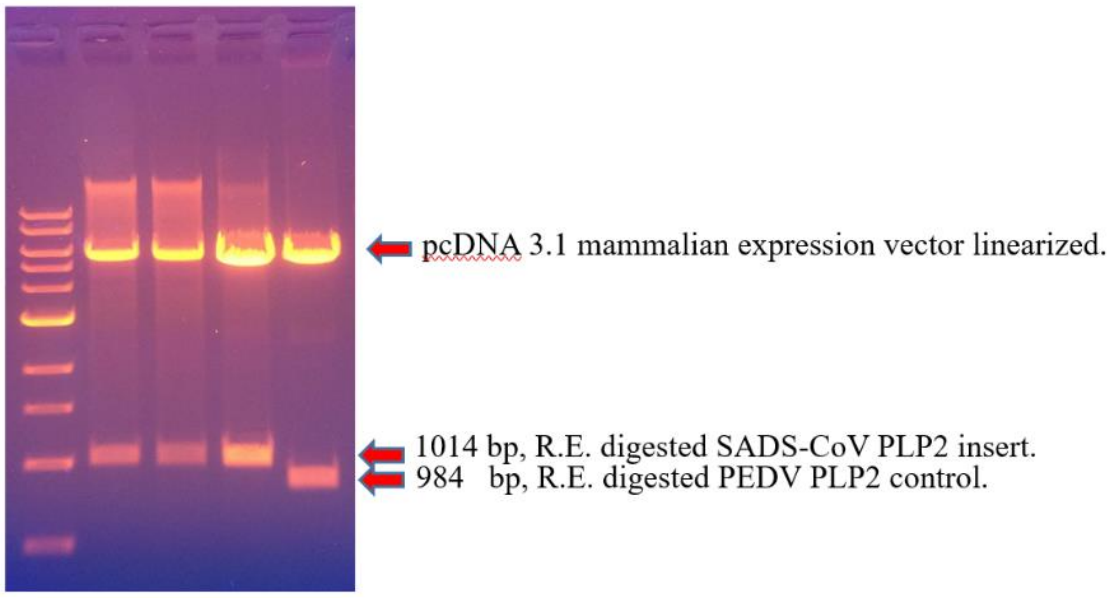

Figure 3. DNA cloning and restriction enzyme endonuclease digestion reaction on an agarose gel. The full length SADS-CoV PLP2 construct was cloned into the mammalian plasmid expression vector pcDNA3.1. The construct was analyzed by an endonuclease digestion reaction on an agarose gel. The restriction enzyme endonuclease digested PEDV PLP2 control was known to be 984 bp and was chosen as a control due to the similarity in number of base pairs as the SADS-CoV PLP2 insert. The restriction enzyme endonuclease digested SADS-CoV PLP2 insert band appeared as expected around 1000 bp. Bacterial transformants were isolated from this construct and were used to prepare DNA for mammalian cell transfection and antibody screening.

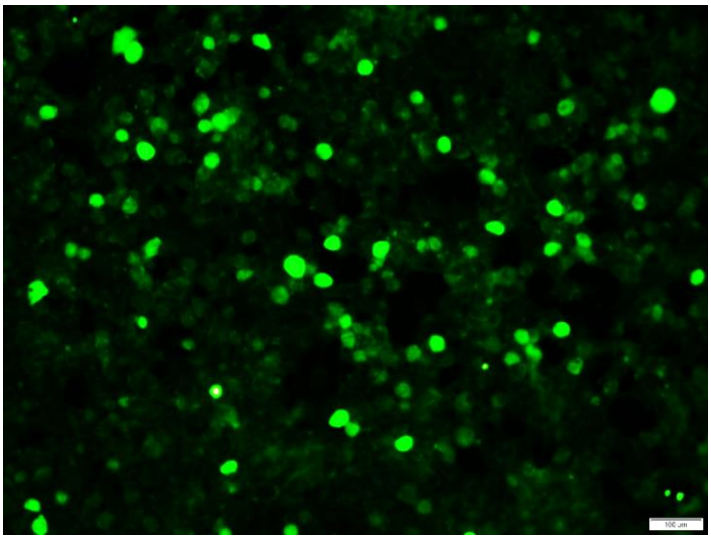

Figure 4. Development of SADS-CoV PLP2 immunofluorescent assay for hybridoma antibody screening at 200X magnification. HEK293T cells were seeded at a concentration of $3x10^6$ cells per plate in a 96 well microtiter plates and were incubated for 48 hours to 95% confluency. Then, the cells were transfected with 0.4 ug of SADS-PLP2 DNA per well in serum-free medium. After 72 hours of expression, the cells were fixed with 80% acetone and stained with an anti-HA monoclonal antibody. The IFA assay showed bright and diffuse immunofluorescent staining of individual transfected cells which expressed the PLP2-HA fusion protein.

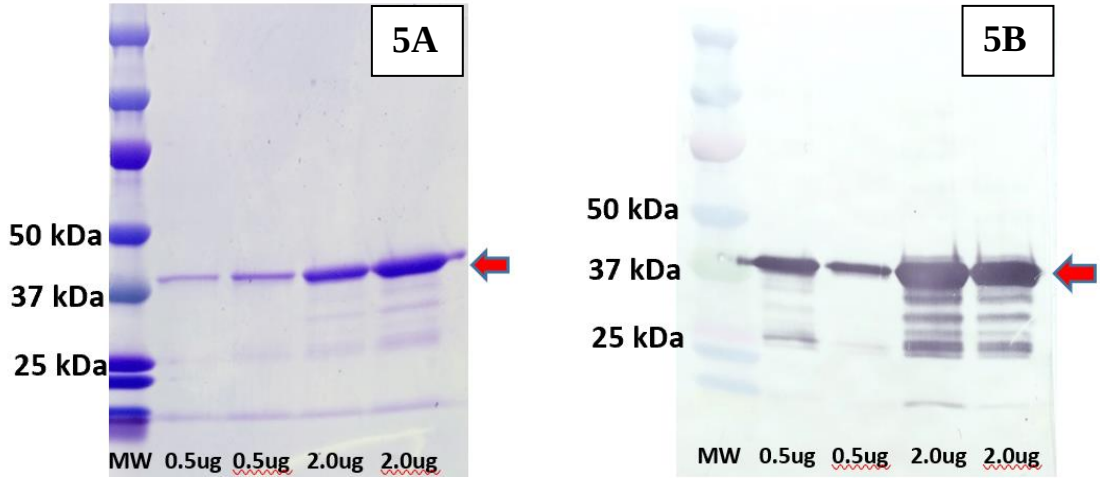

Figure 5. SADS-COV PLPL2 protein detection by Coomassie blue staining and Western blotting. Varying amounts of the SADS-CoV PLP2 protein were electrophoresed on a polyacrylamide gel. After electrophoresis, the gel was cut in half. The left half was stained with Coomassie blue (Figure 3A). The right half was analyzed by Western detection where an anti-HIS monoclonal antibody recognized the specificity of the HIS-tagged SADS-PLP2 fusion protein (Figure 3B). The red arrows confirm the expected molecular weight of the 40.5 kDa protein antigen. The protein was overexpressed at a concentration of 3.0 mg/ml and was purified to a level of ~95%.

Table 1. Isotype and ELISA, IFA, and Western blot reactivity of nine mouse monoclonal antibodies and two rabbit polyclonal antibodies. Monoclonal antibodies were expanded from two separate primary clone isolates. The immunoreactivity of each clone was tested using cell culture supernatant by ELISA, IFA, and Western blot (WB). Rabbit polyclonal antibodies (pAb) were also tested using antisera. The intensity signal is represented by "+/-" on a relative scale. Immunoglobulin isotyping of the resulting monoclonal antibodies was performed using a commercial lateral flow assay.

SADS-CoV PLP2 Monoclonal/Polyclonal Antibodies	Isotype	Reactivity		
		ELISA	IFA	WB
126-55	IgG_1	+++	-	+++++
126-73	IgG_1	+++++	-	+++++
126-303	IgG_1	+++++	-	+++++
163-133	IgG_1	+++++	+++++	+++
163-134	IgG_1	+++++	+++++	+++
163-137	IgG_1	+++++	++++	+++
163-306	IgG_1	+++++	+++++	+++
163-310	IgG_1	+++++	++++	+++
163-317	IgG_1	+++++	+++	++
Rabbit A	polyclone	+++++	++++	+++++
Rabbit B	polyclone	+++++	++++	+++++

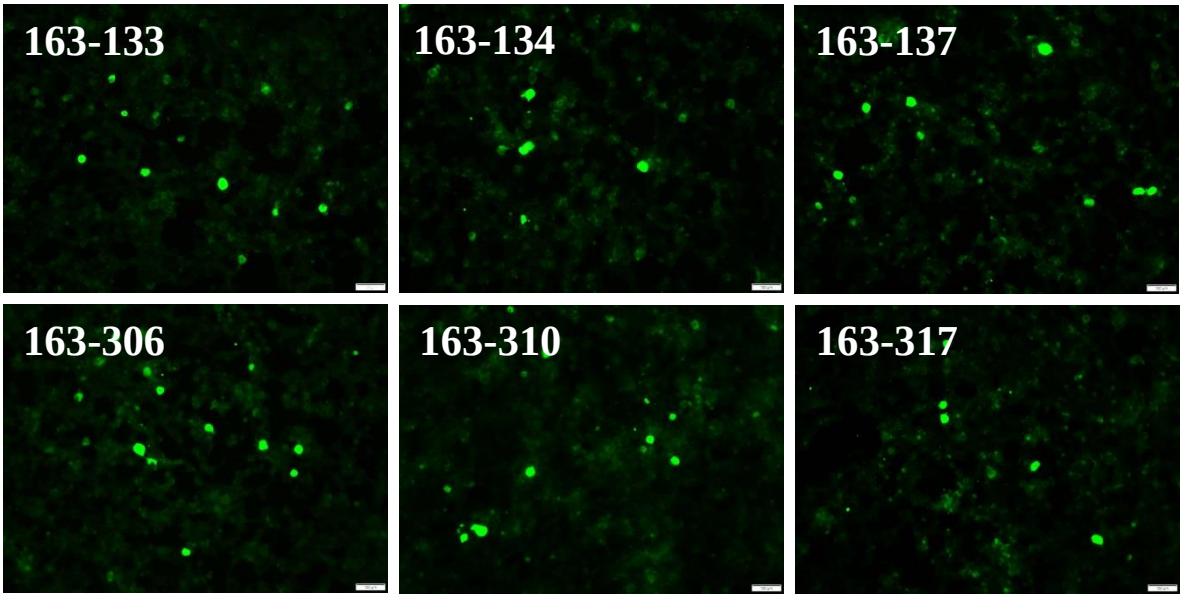

Figure 6. Cytoplasmic, immunofluorescent staining of SADS-PLP2 transfected HEK293T cells by each of six anti-SADS-PLP2 monoclonal antibodies at 200X magnification. After 72 hours of expression, HEK293T cells were fixed with 80% acetone and stained with cell culture supernatant from each of the six hybridoma subclones.

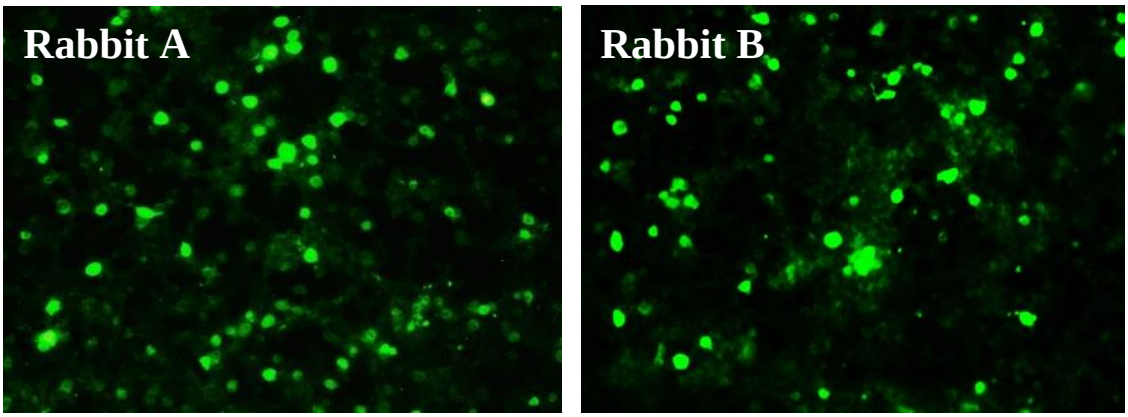

Figure 7. Intense, cytoplasmic, immunofluorescent staining of SADS-CoV PLP2 transfected HEK293T cells using rabbit anti-SADS-CoV PLP2 polyclonal antisera at 200X magnification. After 72 hours of expression, HEK293T cells were fixed with 80% acetone and stained with anti-SADS PLP2 polyclonal antibodies collected from Rabbit A and B. Antisera from both rabbits exhibited anti-PLP2 IFA titers of 1:1200. In total, approximately 105 mLs of rabbit anti- SADS-CoV PLP2 polyclonal antisera was harvested from Rabbit A and B.

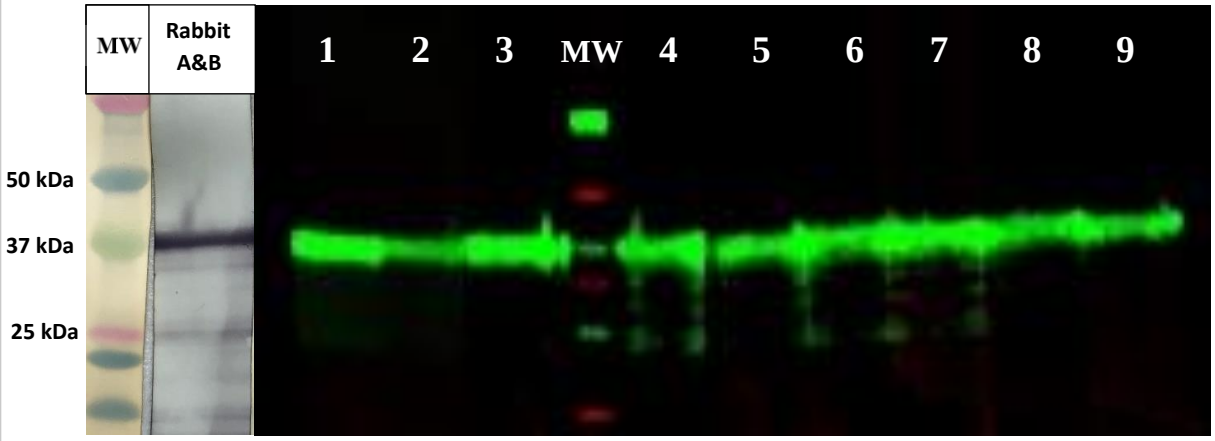

Figure 8. Western blot detection of recombinantly expressed SADS-CoV PLP2 antigen for rabbit polyclonal antibodies and nine mouse monoclonal antibodies. The 40.5 kDa recombinant SADS-PLP2 protein was recognized by Western blotting using two separate detection methods. For the rabbit polyclonal antibodies (left), the Western blot utilized a chromogenic (4-chloro-1-napthol) detection method after staining with a peroxidase-labeled, secondary, anti-rabbit antibody. For the nine mouse monoclonal antibodies (right), the Western blot used an anti-mouse, infrared-labeled secondary antibody and was imaged with a LI-COR Odyssey Fc infrared imaging system. The mouse monoclonal antibodies are designated as follows: (1) mAb 126-55, (2) mAb 126-73, (3) mAb 126-303, (4) mAb 163-133, (5) mAb 163-134, (6) mAb 163-137, (7) mAb 163-306, (8) mAb 163-310, (9) mAb 163-317). The 126 series of antibodies comparatively gave a significantly stronger signal and lower background signal by the Western detection than the 163 series of antibodies.

Discussion

As a recently identified pathogen in China, the possible impact of SADS-CoV on the global swine industry is not fully understood. Clinical manifestations in initial outbreaks included acute vomiting, diarrhea, and death in suckling pigs [8]. Additionally, several other coronaviruses are known to cause diarrhea in swine and cause substantial economic losses to the industry. Specific antibody-based reagents and serological tests are essential for the further study virus replication and the differentiation of SADS-CoV infection from other related coronaviruses such as PEDV, TGEV, and PDCoV.

Therefore, the primary objectives of this study were to develop an initial generation of antibody-based diagnostic reagents and serological assays for the further study of SADS-CoV including IFA, ELISA, and Western blot diagnostic tests. Specific antibody-based reagents have not been previously developed for SADS-CoV. We produced monoclonal antibodies and rabbit antisera against the Papain-like Protease 2 (PLP2) region of within the nsp3 region of ORF1a based on the rationale that it is one of the very first proteins that is produced during its replication cycle.

Nine mouse anti-SADS-CoV PLP2 antibodies from two hybridoma clones were isotyped. Additionally, reactivity was tested by IFA, ELISA, and Western blot. The 126 series were negative by IFA. Therefore, the 126 series can detect linear epitopes of the SADS-CoV PLP2 by ELISA and Western blot, but not the conformational epitope by IFA. In contrast, the 163 series can detect linear and conformational epitopes by IFA, ELISA, and Western blot. The binding of linear and/or conformational epitopes determines the utility of the monoclonal antibodies. By Western detection, the 126 series of antibodies comparatively gave a significantly stronger signal and lower background signal than the 163 series of antibodies. Additionally, approximately 105 mLs of rabbit antisera was collected and tested by IFA, ELISA, and Western blot.

The tools developed during this study can be applied to ongoing and future studies to better understand viral replication and early autoproteolytic activity of SADS-CoV and other coronaviruses. Additionally, the new monoclonal antibody reagents described here should be of substantial value in detection of the SADS-CoV antigen in a variety of applications including the following: early verification of virus isolation attempts and virus titrations; immunohistochemistry staining of fresh tissues; development of field based antigen capture assays such as lateral flow devices; ELISA applications, and development of a Fluorescent Microsphere Immunoassay (FMIA). For validation of IFA, ELISA, and Western blot diagnostic assays, quality control standards that give a high, medium, and low Sample to Positive (S/P) values need to be assigned.

Several drawbacks of relying on antibody-based and serological test for diagnostic purposes have been identified. Cytopathic effect was only observed after several serial passages of SADS-CoV in Vero cells. As a result, isolation and detection of the virus by cell culture was time-consuming [2]. Additionally, to detect SADS-CoV by immunofluorescence staining, the virus-specific antibody and fluorescently labeled antibody are required to react with the SADS-

CoV antigen. This makes the immunofluorescence staining method complex and time consuming [8].

In China, more extensive diagnostic tests beyond antibody-based and serological tests have been developed. A simple, specific, and rapid detection test was developed using real-time reverse transcription loop mediated isothermal amplification (RT-LAMP) based on the conserved nucleocapsid gene of the virus. The method had a detection limit of 1.0×10^1 copies/μL with no cross-reactions with other common swine viruses. However, the RT-LAMP test can't be used for quantitative analysis of the viral copies in the samples [19]. Real-time quantitative PCR (RT-qPCR) is a powerful diagnostic method and has been widely used for detection and quantitation of pathogenic microorganisms because of its high sensitivity and reproducibility. A probe-based real time RT-qPCR for the detection of SADS-CoV with a detection limit of 30 DNA copies/μL [20]. Additionally, a rapid, sensitive, reliable, and cost effective SYBR green-based RT-qPCR assay was established for the detection of SADS-CoV. The SYBR green-based RT-qPCR can be applied to the monitoring of a SADS-CoV infection, epidemiology study, and pathogenicity study [8]. Similar to our objectives, monoclonal antibodies were produced against the SADS-CoV nucleocapsid protein to be used in serological assays [21].

Conclusion

The monoclonal antibody reagents against the SADS-CoV PLP2 region developed here will provide important research and diagnostic tools for the swine industry. Anti-SADS-CoV monoclonal antibodies are a valuable for IFA, ELISA, and Western blot diagnosis. Additionally, they can be further applied to other fluorescence and immunohistochemical staining methods associated with diagnostic and pathogenesis studies. Once validated, the serological assays allow the detection of antibodies developed in response to SADS-CoV infection. Furthermore, the anti-SADS-CoV PLP2 antibodies can be used in an ELISA and FMIA to allow the high-throughput screening of swine serum samples. IFA test may be required for confirmation of individual unexpected results. Further work is needed to validate these anti-SADS-CoV PLP2 monoclonal antibodies in the context of IFA, ELISA, and Western blot diagnostic assays and to adapt them to different samples including milk and oral fluids.

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