Identification and epitope mapping of swine acute diarrhea syndrome coronavirus accessory protein NS7a via monoclonal antibodies

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ABSTRACT

Swine acute diarrhea syndrome coronavirus (SADS-CoV) is an emerging swine enteric coronavirus that causes vomiting, severe diarrhea, dehydration and death in suckling piglets. NS7a is putative accessory protein that is predicted to be encoded by SADS-CoV, but still to be confirmed experimentally. In the present study, recombinant NS7a protein was expressed in a prokaryotic expression system and used as an antigen to prepare monoclonal antibodies (mAbs) specific to NS7a protein. We obtained two anti-NS7a mAbs, termed AH5 and EH3, that were shown by western blotting to react with the natural NS7a protein in Vero E6 cells infected with SADS-CoV. Using the produced mAbs, we observed by confocal microscopy that NS7a protein was expressed in the cytoplasm. Further studies revealed that the motif [31]VNTWQEFA[38] was the minimal unit of the linear B-cell epitope recognized by mAb AH5, and the motif [82]FDLFERF[88] was the minimal unit of the linear B-cell epitope recognized by mAb EH3. Alignment of amino acids showed that these two epitopes were highly conserved among different SADS-CoV strains and SADS-related coronaviruses from bats, but with one substitution in these two motifs in bat coronavirus HKU2. In summary, we generated and characterized two mAbs against SADS-CoV NS7a protein, and demonstrated NS7a expression in SADS-CoV-infected cells for the first time.

1. Introduction

Swine acute diarrhea syndrome coronavirus (SADS-CoV), also named swine (or porcine) enteric alphacoronavirus, is a newly emerged coronavirus that causes SADS, manifesting as acute vomiting, acute watery diarrhea and rapid weight loss in newborn piglets within 1 week postnatally (Fu et al., 2018; Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). SADS-CoV was first identified in a pig farm in Qingyuan, Guangdong Province, Southern China in early December 2016 in suckling pigs with severe diarrhea, after commonly known etiological agents were ruled out in the deceased animals (Zhou et al., 2018). Thereafter, outbreaks of SADS were documented in three adjacent pig farms in the following 5 months that led to the deaths of approximately 25,000 piglets and significant economic losses (Zhou et al., 2018). Subsequently, Li et al. described the isolation and genome sequencing of

SADS-CoV strain CH/FJWT/2018 from fecal and small intestinal samples collected from Fujian Province in 2018 (Li et al., 2018). Zhou and colleagues demonstrated that the re-emerged SADS-CoV was responsible for a large-scale outbreak of piglet diarrhea in another farm near the origin of the initial SADS pandemic in February 2019 (Zhou et al., 2019).

SADS-CoV is an enveloped, single-stranded, positive-sense RNA virus belonging to genus *Alphacoronavirus* within the subfamily *Orthocoronavirinae* of family *Coronaviridae* in order *Nidovirales*. SADS-CoV is considered to be of bat origin and shares ~95% nucleotide identity at the genome level with the reported bat HKU2 strain (Gong et al., 2017; Pan et al., 2017; Yang et al., 2019, 2020). The genome of SADS-CoV is ~27.2 kb, whose organization is arranged by 5′ untranslated region (UTR), open reading frame (ORF)1a/1b, spike (S), ORF3, envelope (E), membrane (M), nucleocapsid (N), NS7a, NS7b, and 3′ UTR in order (Xu et al., 2019; Zhou et al., 2018; Yang et al., 2020). Similar to other

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coronaviruses, ORF1a and ORF1b occupy two-thirds of the genome and encode two viral replicase polyproteins, pp1a and pp1ab, which are proteolytically cleaved into 15 mature nonstructural proteins (Woo et al., 2010). While the last one-third of the genome encodes S, E, M and N, as well as three putative accessory genes, ORF3, NS7a and NS7b (Zhou et al., 2018).

Accessory proteins are genus-unique for coronavirus. Each coronavirus contains a varied number of accessory proteins, and the predicted sequences of these proteins do not share high levels of homology, even within the same genus (Fang et al., 2016; Liu et al., 2014; Tan et al., 2006). Most of coronavirus accessory proteins are not essential for viral replication *in vitro* (Liu et al., 2014; Yount et al., 2005), but multiple studies have revealed that some accessory proteins are functional, either involving in immune modulation (Siu et al., 2014) or affecting viral pathogenesis *in vivo* (Redondo et al., 2021; Silvas et al., 2021).

Bioinformatics analysis indicate that there are three putative accessory genes, ORF3, NS7a and NS7b in the SADS-CoV genome. However, these hypotheses remain largely to be demonstrated at the protein expression level, despite determination of subgenomic mRNA for these accessory genes (Yang et al., 2019). Here, we describe the generation of monoclonal antibodies (mAbs) specific to accessory protein NS7a of SADS-CoV, and we characterize the epitopes of the generated mAbs. Based on the NS7a mAbs, we demonstrated that the predicted NS7a protein was expressed and localized in the cytoplasm of SADS-CoV-infected Vero E6 cells, providing critical biological evidence for the existence of SADS-CoV NS7a for the first time. These data indicate that the mAbs characterized here could be useful tools to investigate the function of NS7a.

2. Materials and methods

2.1. Cells, virus, animals and main reagents

Vero E6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin, at 37 °C in a humidified incubator under 5% CO_2. The SP2/0 myeloma cell line was maintained in DMEM supplemented with 20% heat-inactivated FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. SADS-CoV (GenBank accession No. MF094681) was isolated from intestinal contents of infected piglets in Guangdong Province, Southern China, which has been investigated extensively in our laboratory (Han et al., 2019; Zhang et al., 2020). Rabbit polyclonal antibodies against recombinant N protein of SADS-CoV were also prepared and preserved in our laboratory (Unpublished data). Six-week-old female BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. and housed in SPF isolators ventilated under negative pressure. The prokaryotic expression vector pGEX-6p-1 was maintained in our laboratory. Complete and incomplete Freund's adjuvants, HAT (hypoxanthine, aminopterin and thymidine) and HT (hypoxanthine and thymidine) supplements, and 50% polyethylene glycol 4000 (PEG4000), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Alexa-Fluor-488-conjugated donkey anti-mouse IgG antibody ($H + L$), donkey anti-mouse IgG ($H + L$) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 594 and goat anti-rabbit IgG ($H + L$) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 594 were purchased from Invitrogen. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG ($H + L$) antibody was purchased from Beijing Zhongshan Goldenbridge Biotechonology CO., LTD. (China). IRDye 800CW goat anti-mouse IgG H&L (926–32,210), and IRDye 680RD goat anti-rabbit IgG H&L (926–68,071), were purchased from LiCor Bio-Sciences, Lincoln, NE, USA.

2.2. Expression and purification of recombinant NS7a protein

To obtain recombinant NS7a protein in a prokaryotic expression system, the NS7a gene of the SADS-CoV was amplified and inserted into prokaryotic expression vector pGEX-6p-1. Recombinant plasmids pGEX-6p-NS7a were identified by sequencing, and then the positive recombinant plasmids were transformed into *Escherichia coli* Rosette (DE3) cells and induced with 1 mM isopropyl β-ᴅ-1-thiogalactopyranoside (IPTG) for 6 h in Luria–Bertani medium. Expression conditions including induced temperature and concentration of IPTG were optimized to yield the soluble form of fusion protein GST-NS7a. Expressed recombinant protein was analyzed with SDS-PAGE and detected by staining with Coomassie blue. The expressed recombinant GST-NS7a fused protein was purified using Glutathione Sepharose 4B affinity chromatography column (GE Healthcare, Bio-Sciences, Uppsala, Sweden). Purified proteins were used to immunize mice. Fusion protein was analyzed by western blotting using mouse mAb against the GST tag (Sigma, St. Louis, MO, USA) to confirm that the NS7a protein had been incorporated into the recombinant protein.

2.3. Preparation of mAbs against NS7a

Animal care and all procedures were conducted in accordance with animal ethics guidelines and approved protocols. The animal experiment was approved by the Animal Care and Ethics Committee of Harbin Veterinary Research Institute, and approval number was Heilongjiang-SYXK-2020–032.

Four 6-week-old female BALB/c mice were immunized with 100 µg purified recombinant NS7a protein three times via subcutaneous injection at 2-week intervals. The antigen was emulsified with complete Freund's adjuvant for the first-dose immunization, and emulsified with incomplete Freund's adjuvant for the two following booster immunizations. The antibody levels were examined by indirect ELISA based on recombinant NS7a. An intraperitoneal booster with the same dose of protein without adjuvant was administered at 3 days before cell fusion. Mice were killed 3 days later and spleen cells were isolated and fused with SP2/0 myeloma cells according to standard procedures (Galfre and Milstein, 1981). The fused cells were seeded in 96-well plates and cultured in presence of HAT (Sigma) for hybridoma selection. One week later, the medium was discarded and replaced with fresh medium containing HT. The antibodies against NS7a secreting from the fused cells in culture supernatants were identified by NS7a indirect ELISA. The positive hybridoma were subcloned three times via limiting dilution, to obtain a purified hybridoma cell strain. Ten-week-old female BALB/c mice were pretreated with 500 µL sterile liquid paraffin, then positive hybridoma cells were inoculated by intraperitoneal injection, and ascites was harvested 10 days later. mAbs were isotyped using an SBA Clonotyping™ System-HRP kit (SouthernBiotech, Birmingham, AL, USA). The mAbs were characterized by western blotting and indirect immunofluorescence assay (IFA).

2.4. Indirect elisa

The development of indirect ELISA based on NS7a protein was used to select the positive clones. The 96-well microtiter plates were coated with 1 µg/mL purified NS7a protein at 4 °C overnight. After washing with phosphate-buffered saline containing 0.05% Tween 20 (PBST), the plates were blocked with PBS containing 5% skimmed milk at 37 °C for 2 h. Plates were washed three times and then supernatant of hybridoma cultures was added to the plates at 37 °C for 1 h incubation. Serum from immunized and mock-immunized mice served as positive or negative controls, respectively. After three washes, 100 µl HRP-labeled goat anti-mouse IgG antibody (diluted 1:3000) was added and incubated at 37 °C for 45 min. Subsequently, the plates were washed several times to remove unconjugated antibody, 100 µl 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and reacted in the dark at room temperature

for 10 min, followed by 2 M H_2SO_4 to stop the reaction. The absorbance was measured at 450 nm.

2.5. IFA and confocal microscopy

Vero E6 cells were seeded in 96-well plates or glass-bottomed cell culture dishes and inoculated with SADS-CoV at a multiplicity of infection (MOI) of 0.1 when the cells reached 100% confluence. After a cytopathic effect (CPE) appeared at about 30 h postinoculation, the cells were fixed with 4% paraformaldehyde (Sigma–Aldrich) for 30 min at room temperature, and permeabilized with 0.3% Triton X-100 (Sigma-–Aldrich) for 15 min. After being washed three times with PBST, the cells were blocked with 5% skimmed milk in PBS for 2 h at 37 °C. After washing three times with PBST, cells were incubated with the mAbs (diluted 1:500) generated in this study at 4 °C for overnight. After three washes with PBST, cells were incubated with Alexa Fluor 488 donkey anti-mouse IgG ($H + L$) antibody (diluted 1:200) for 1 h at 37 °C. The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Following several washes, the fluorescent images were obtained with an inverted fluorescence microscope (EVOS FL, AMG, USA) or collected with a confocal laser scanning microscope (Zeiss, JENA, Germany).

2.6. Western blotting

Western blotting was performed to analyze the reactivity of mAbs with natural NS7a derived from SADS-CoV-infected Vero E6 cells and GST-fused NS7a polypeptides expressed in *E. coli* Rosette (DE3) cells. Whole cell lysates were subjected to separate by 12.5% SDS-PAGE and then transferred to nitrocellulose membranes. After blocking with 5% (w/v) skimmed milk in PBS for 2 h at room temperature, membranes were incubated with mAbs against SADS-CoV NS7a (1:500 dilution) overnight at 4 °C. After washing three times with PBST, the membranes were then incubated with 1:5000-diluted IRDye 800CW goat anti-mouse IgG ($H + L$) (LiCor BioSciences) for 45 min at room temperature. Membranes were washed with PBST and the specific protein bands were visualized by an Odyssey infrared imaging system (LiCor BioSciences).

2.7. Epitope mapping

To map the epitopes of the NS7a protein, a panel of 28 partially overlapping polypeptides (A1–A14 and B1–B14) spanning the NS7a protein (Fig. 1) were expressed in *E. coli* Rosette (DE3) cells. For A1–A5 and B1–B5, 10 pairs of primers were designed to amplify the genes of the fragments from plasmid pGEX-6p-NS7a, and those truncated proteins A1–A5 and B1–B5 were expressed with a GST tag. For each polypeptide A6–A14 and B6–B14, a pair of oligonucleotide strands was synthesized. Each pair of oligonucleotide strands was annealed, and the resultant double-stranded DNA containing a *Bam*HI and a *Xho*I cohesive terminus at the 5′ and 3′ ends, respectively. The annealed fragments were inserted into the expression vector pGEX-6p-1 to generate recombinant plasmids and the constructs were identified by sequencing. Finally, the confirmed recombinant plasmids were transformed into *E. coli* Rosette (DE3), and the target polypeptides were expressed as GST fusion proteins in prokaryotic expression system. A series of recombinant fusion peptides were induced by IPTG and the recombinant proteins determined by staining with Coomassie blue after SDS-PAGE or western blotting using the anti-GST mAb as primary antibody. Epitope mapping was performed based on the above polypeptides and generated mAbs against to NS7a protein by western blotting.

2.8. Sequence and homology analysis of NS7a protein epitopes

To better determine the conservation of the identified epitopes among SADS-CoV reference strains and SADS-related coronaviruses (SADSr-CoVs), the epitope motif and flanking sequences of NS7a protein were aligned with selected SADS-CoV strains using DNAMAN software (Lynnon BioSoft Inc., San Ramon, CA, USA). Alignment analysis was performed for the defined epitope and corresponding regions of other associated coronaviruses using the DNASTAR Lasergene software (DNASTAR Inc., Madison, WI, USA).

3. Results

3.1. Expression and purification of recombinant NS7a protein

The full-length NS7a gene of SADS-CoV was inserted into prokaryotic vector pGEX-6p-1 and expressed in *E. coli* Rosette (DE3) as a
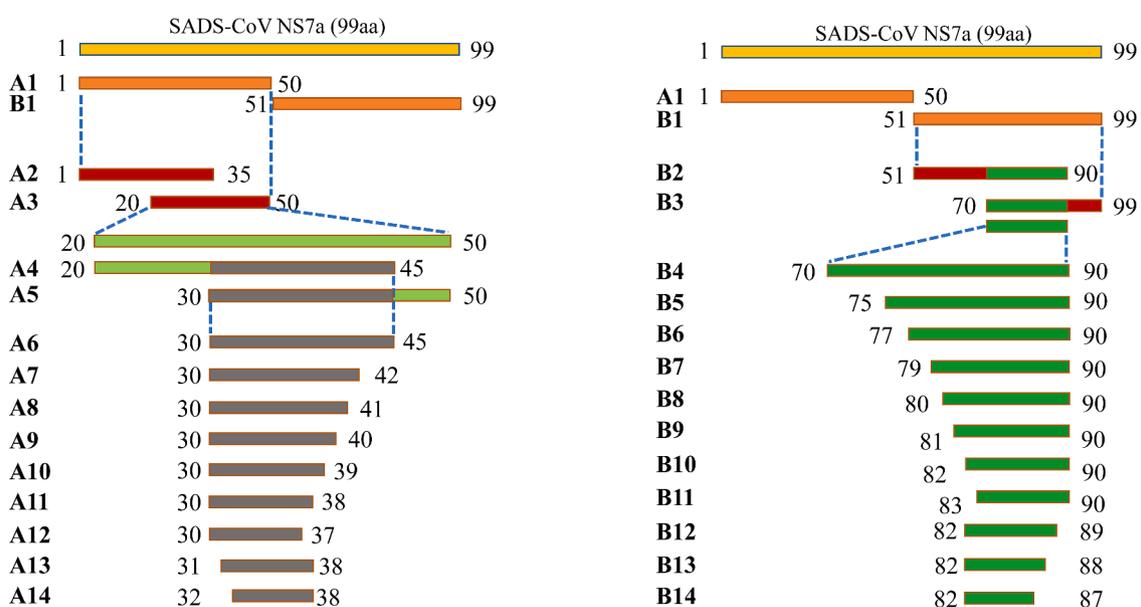
Fig. 1. Schematic diagram of truncated SADS-CoV NS7a fragments used for B-cell linear epitope mapping. To identify the epitopes of mAbs against SADS-CoV NS7a protein, the NS7a gene was divided into 28 truncated fragments and cloned into pGEX-6p-1 for recombinant protein expression. The bars represent the different truncated NS7a proteins. The numbers represent the initial and final amino acid positions for each fragment within the NS7a protein.

recombinant GST fusion protein, which was determined by SDS-PAGE (Fig. 2A) and western blotting (Fig. 2B). The molecular mass of recombinant NS7a protein was ~38.7 kDa upon staining with Coomassie brilliant blue R250 after SDS-PAGE (Fig. 2A), which was in accordance with its predicted molecular weight. The recombinant NS7a protein of SADS-CoV was mainly expressed in *E. coli* Rosette (DE3) in soluble form (Fig. 2A, lane 4), when the recombinant bacteria were induced under low temperature at 15 °C. It was purified by GST affinity column chromatography and yielded a calculated concentration of ~25 mg recombinant NS7a/liter of LB medium and had excellent purity (Fig. 2A, lane 5). Western blotting showed that purified GST-fused NS7a protein could be recognized by anti-GST-tag mAb (Fig. 2B, lane 8).

3.2. Production and characterization of sads-cov NS7a protein-specific mAbs

Purified recombinant NS7a protein was used as antigen to immunize 6-week-old female BALB/c mice. After four immunizations at 2-week intervals, spleen cells were isolated and fused with SP2/0 cells to prepare hybridomas. The supernatant of hybridoma cells was collected and assessed by indirect ELISA based on SADS-CoV NS7a protein, and positive hybridoma clones were screened. Ultimately, two hybridoma cell lines (AH5 and EH3) secreting antibodies specific to SADS-CoV NS7a protein were obtained and subcloned three times via limiting dilution. Isotype determination showed that mAb AH5 was subclass IgG1/κ-type, while mAb EH3 was subclass IgA/κ-type.

In order to determine the specificity of mAbs, western blotting was carried out. mAbs AH5 and EH3 reacted specifically with the recombinant NS7a protein expressed in the prokaryotic system (Fig. 3A), while they could not react the GST-tag expressing by the empty vector pGEX-6p-1. To further test the specificity of mAbs, HEK 293T cells transfected with pCAGGS-HA-NS7a plasmid were analyzed by western blotting using mAbs AH5 and EH3 as the primary antibody. AH5 and EH3 specifically reacted with eukaryotically expressed NS7a protein but did not react with cell lysed samples from empty plasmid pCAGGS-HA transfections (Fig. 3B). To confirm whether NS7a is indeed expressed in SADS-CoV-infected Vero E6 cells, western blotting was conducted using the generated mAbs as the primary antibody. SADS-CoV-infected Vero E6 cells with obvious CPE or mock-infected cells were lysed with lysis buffer RIPA at about 30 h postinfection. Western blotting showed that

specific bands similar to the expected size of NS7a protein were detected by mAbs AH5 and EH3 in lysates of SADS-CoV-infected Vero E6 cells, while no specific bands were observed in the mock-infected cells (Fig. 3C).

### 3.3. Localization of sads-cov NS7a protein in infected and transfected cells

To examine localization of the NS7a protein in SADS-CoV-infected Vero E6 cells, the cells were fixed and incubated with mAbs AH5 and EH3, respectively, stained with Alexa-Fluor-488-conjugated donkey anti-mouse IgG (H + L) antibody and DAPI. NS7a protein was localized with confocal laser scanning microscopy. As seen in Fig. 4A, NS7a protein was localized predominantly in the cytoplasm of SADS-CoV-infected Vero E6 cells. Also, NS7a protein and N protein were colocalized in the cytoplasm of virus infected Vero E6 cells when stained with NS7a protein specific mAb AH5 and N protein polyclonal antibodies (Fig. 4A). To further confirm the localization of NS7a protein, eukaryotic construct pAcGFP-NS7a (encoding GFP-NS7a fusion protein) was transfected into Vero E6 cells, and then IFA was performed based on the mAb AH5 as primary antibody and the Alexa Fluor-594 donkey anti-mouse IgG (H + L) as secondary antibody. Results indicated that the native green fluorescence from GFP-NS7a fused protein was only observed in the cytoplasm of transfected Vero E6 cells (Fig. 4B). Moreover, red fluorescence signal was also only seen in the cytoplasm of Vero E6 cells, which overlapped the green fluorescence completely (Fig. 4B).

3.4. Epitope mapping of mAbs

To determine epitopes recognized by anti-NS7a mAbs AH5 and EH3, a panel of truncated polypeptides (Fig. 1) fused with GFP-tag were constructed and expressed in *E. coli* Rosette (DE3) and subjected to western blotting. Firstly, NS7a protein was divided into two fragments, peptide A1 (1–50 aa) and peptide B1 (51–99 aa). mAb AH5 reacted with peptide A1 and mAb EH3 recognized peptide B1 (Fig. 5A). In the second round, two overlapping fragments A2 (1–35 aa) and A3 (20–50 aa) were expressed for peptide A1, along with two overlapping fragments B2 (51–90 aa) and B3 (70–99 aa). Western blotting showed that mAb AH5 only reacted with peptide A3 (Fig. 5B), while mAb EH3 reacted with both B2 and B3 (Fig. 5D). Peptide A3 (20–50 aa) was further divided into
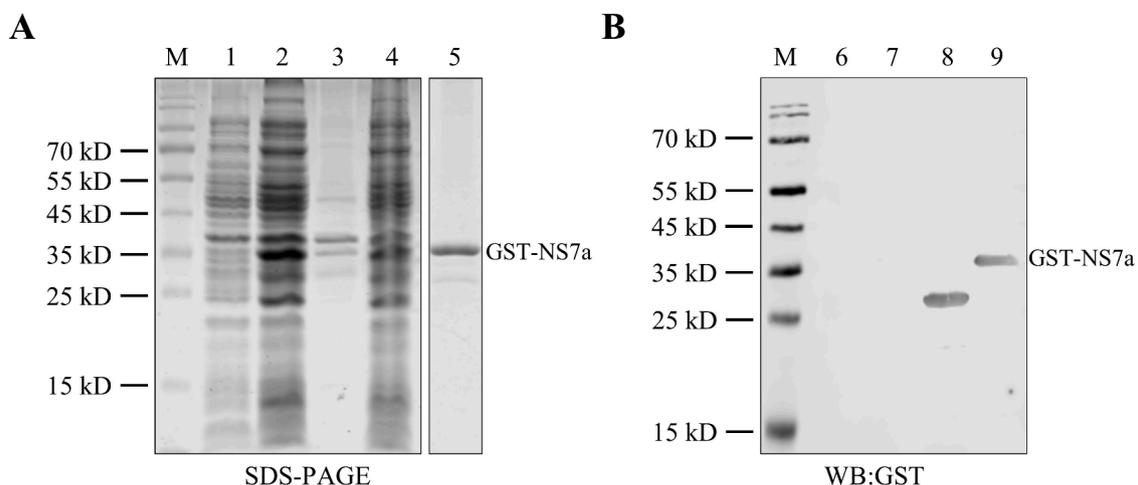
Fig. 2. Expression, purification and identification of recombinant fusion NS7a protein with GST tag. Bacterial lysates from *E. coli* Rosette (DE3) with recombinant plasmid pGEX-6p-NS7a were subjected to SDS-PAGE (A) and western blotting (B) with anti-GST mAb. Anti-GST mAb was used as primary antibody and IRDye 680RD goat anti-rabbit IgG H&L as secondary antibody in western blotting. (A and B) Lane 1, whole *E. coli* Rosette (DE3) with pGEX-6p-NS7a without induction; Lane 2, whole *E. coli* Rosette (DE3) with pGEX-6p-NS7a induced by IPTG; Lane 3, precipitation of *E. coli* Rosette (DE3) with pGEX-6p-NS7a induced by IPTG after sonication; Lane 4, supernatant of *E. coli* Rosette (DE3) with pGEX-6p-NS7a induced by IPTG after sonication; Lane 5, purified fusion NS7a protein; Lane 6, whole *E. coli* Rosette (DE3) with pGEX-6p-1 empty vector without induction; Lane 7, whole *E. coli* Rosette (DE3) with pGEX-6p-NS7a without induction; Lane 8, whole *E. coli* Rosette (DE3) with pGEX-6p-1 empty vector induced by IPTG; Lane 9, purified fusion NS7a protein; M: Protein marker.
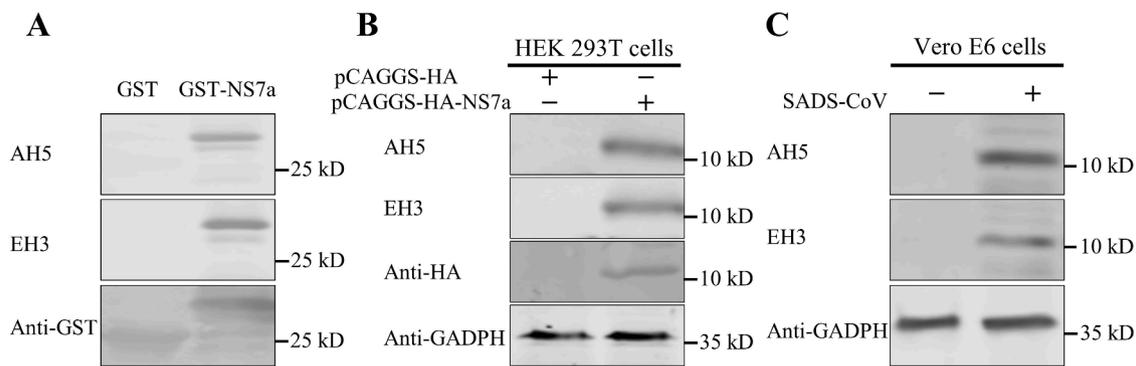
Fig. 3. Characterization and identification of the generated mAbs against NS7a. (A) Recombinant GST-NS7a protein expressed in *E. coli* Rosette (DE3) was subjected to SDS-PAGE, then transferred onto nitrocellulose membrane for western blotting, using mAbs AH5 and EH3 as primary antibody. *E. coli* Rosette (DE3) expressing GST protein was used as a control. (B) HEK 293T cells were plated in six-well plates following transfection with pCAGGS-HA-NS7a or empty vector. At 36 h post-transfection, cells were harvested for western blotting with mAbs AH5 and EH3. (C) Western blotting of expression of natural NS7a protein in SADS-CoV-infected Vero E6 cells. Vero E6 cells were plated in six-well plates and mock-infected or infected with SADS-CoV at MOI 0.1. At about 30 h post-infection, western blotting was performed using mAbs AH5 and EH3 as primary antibody.
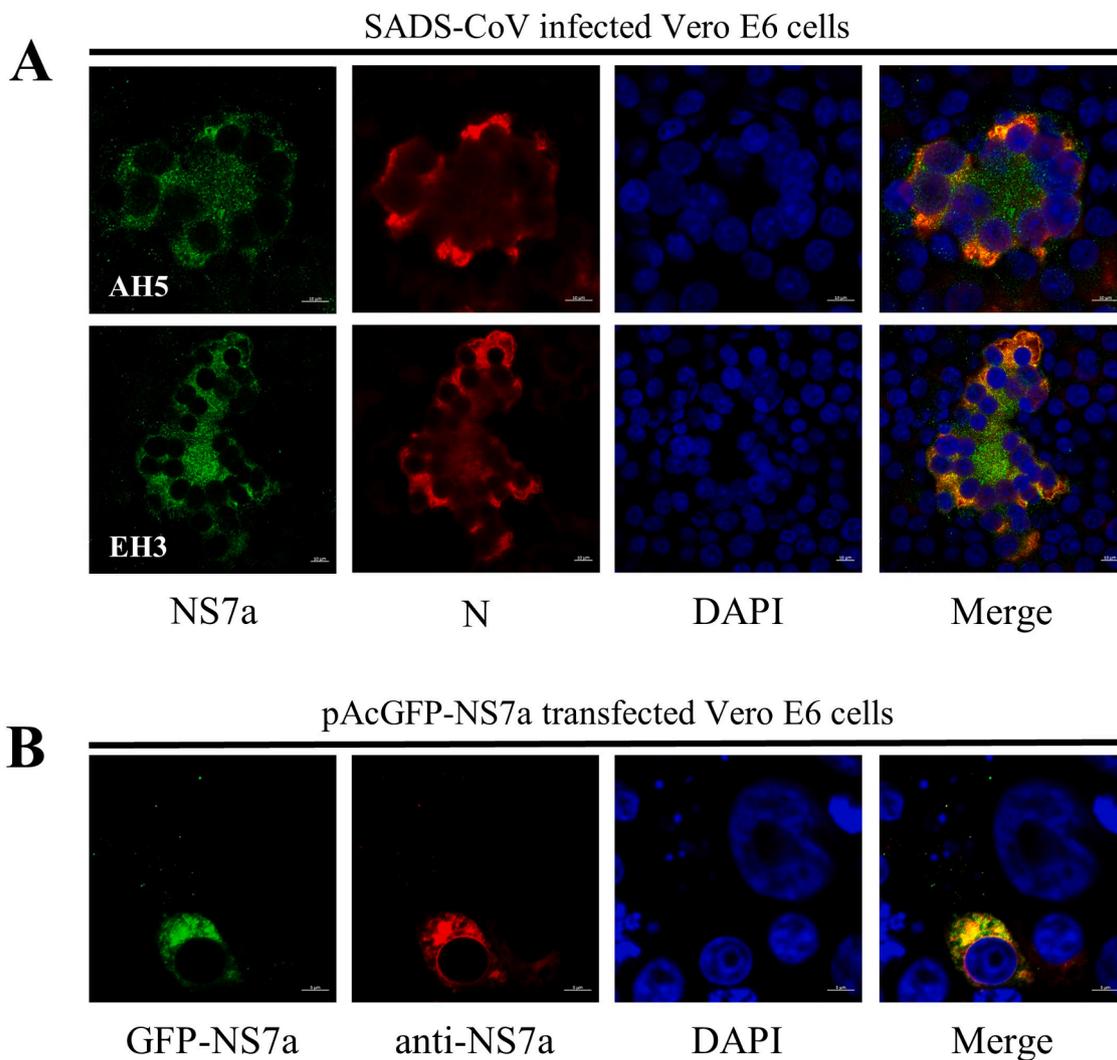
Fig. 4. Localization of SADS-CoV NS7a protein. (A) Detection of NS7a protein in Vero E6 cells infected with SADS-CoV by IFA using mAbs AH5 and EH3 as primary antibody. (B) Localization of GFP-tagged NS7a protein in Vero E6 cells transfected with pAcGFP-NS7a.

two smaller overlapping fragments A4 (20–45 aa) and A5 (30–50 aa). mAb AH5 reacted with peptides A4 and A5 (Fig. 5B). Thus, we concluded that the epitope of mAb AH5 was located within 30–45 aa,

and the epitope of EH3 within 70–90 aa. For more precise epitope determination, shorter peptides (Fig. 1) were prepared for western blotting. Eventually, we demonstrated that [31]VNTWQEFA[38] was the
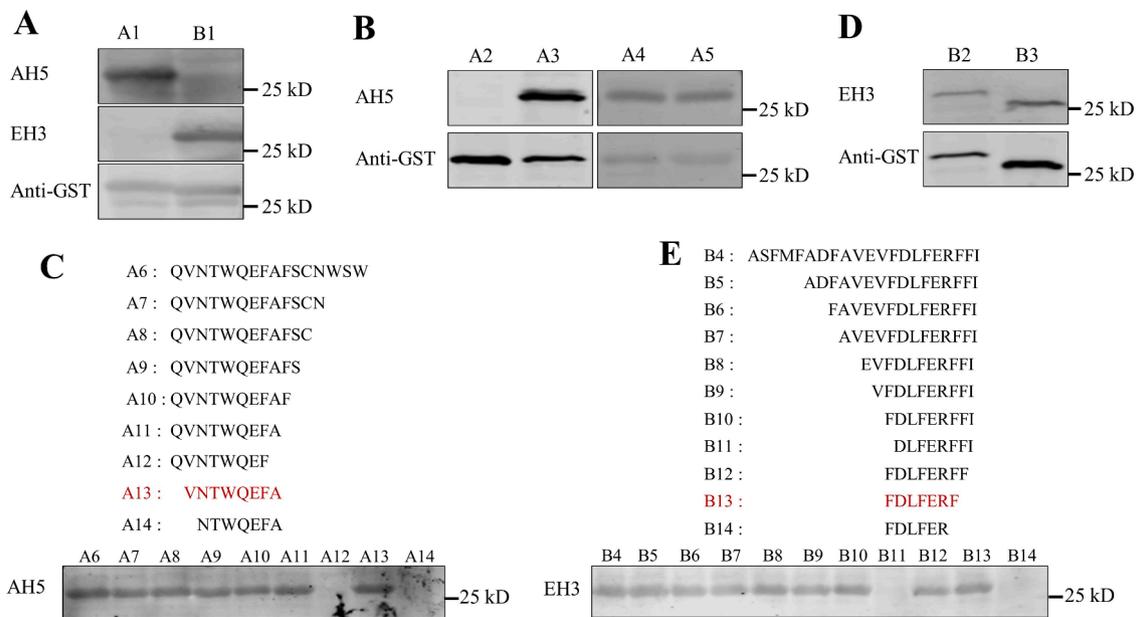
Fig. 5. Identification of B-cell epitopes in NS7a protein by western blotting. A series of truncated fragments of NS7a were cloned into pGEX-6p-1 and expressed as fusion proteins with a GST tag. Two peptides spanning the N protein at aa 1–50 (A1), and aa 51–99 (B1), were expressed. A1 was divided into two, aa 1–35 (A2) and aa 20–50 (A3). A3 was further divided into two peptides, aa 20–45 (A4) and aa 30–50 (A5); peptide B1 was divided into two peptides, aa 51–90 (B2) and aa 70–99 (B3). Western blotting showed that epitopes of mAbs AH5 and EH3 were located within aa 30–45 (A6) (A, B) and aa 70–90 (B4), respectively (A, D). The length of A6 and B4 gradually decreased at both ends until the minimal linear epitope was identified. Ultimately, a minimal peptide ^{31}VNTWQEFA^{38} was characterized as the B-cell epitope recognized by mAb AH5 (C), and a minimal peptide ^{82}FDLFERF^{88} was identified as the minimal epitope recognized by EH3 (E).

minimal motif recognized by mAb AH5 (Fig. 6C). Similarly, a minimal peptide, ^{82}FDLFERF^{88}, was characterized as the B-cell linear epitope recognized by mAbs EH3 (Fig. 6E).

3.5. Sequence and homology analysis

Sequence alignment was performed to explore the level of conservation of the epitopes of mAbs AH5 and EH3, among 10 SADS-CoV strains selected from GenBank, using DNA Star. Both epitopes of mAb AH5 (aa ^{31}VNTWQEFA^{38}) and mAb EH3 (aa ^{82}FDLFERF^{88}) were highly conserved among the SADS-CoV strains and SADS-rCoVs from bats, with shared sequence similarity of 100% (Fig. 6). However, we found substitutions ^{31}V to ^{31}D and ^{88}R to ^{88}H in bat coronavirus HKU2 in comparison with the epitopes ^{31}VNTWQEFA^{38}, ^{82}FDLFERF^{88} in SADS-CoV isolates (Fig 0.6). Whether these single amino acid mutations in these two epitopes could decrease or eliminate the antigenicity remains to be established.

4. Discussion

Coronaviruses infect humans and a wide variety of animals, in which they can result in respiratory, enteric, hepatic and neurological diseases of varied severity, and their potential for zoonotic transmission has attracted global public health concerns (Decaro and Lorusso, 2020). Studies in the molecular biology of coronaviruses have shown that they have high frequencies of mutation and recombination due to their distinctive mechanism of viral replication (Masters, 2006), which might allow them to enhance their virulence, change tissue tropism, adapt to new hosts (Pan et al., 2012; Pensaert et al., 1986; Zaki et al., 2012). Severe acute respiratory syndrome coronavirus (SARS-CoV)−1 (Luk et al., 2019), Middle East respiratory syndrome coronavirus (MERS-CoV) (Zaki et al., 2012), and currently pandemic SARS-CoV-2 (Zhu et al., 2020) are notorious human pathogens in the past two decades that originated in wild animals and were transmitted to humans.

In the swine industry, porcine deltacoronavirus (PDCoV) (Hu et al., 2015) and SADS-CoV (Zhou et al., 2018) are emerging viruses with economic importance. To date, six different species of coronavirus have been identified in pigs (Wang et al., 2019), including transmissible gastroenteritis virus (TGEV) together with its natural variant porcine respiratory coronavirus, porcine epidemic diarrhea virus (PEDV), and SADS-CoV in the genus *Alphacoronavirus*, porcine hemagglutinating encephalomyelitis virus in the genus *Betacoronavirus*, and PDCoV in the

Fig. 6. Amino acid sequence alignment of the defined epitopes and flanking region with those of representative SADS-CoV strains, SADSr-CoV strains and bat coronavirus HKU2. The epitopes ^{31}VNTWQEFA^{38} and ^{82}FDLFERF^{88} were highly conservative among SADS-CoV strains and SADSr-CoV, but with few amino acid differences compared to the bat coronavirus HKU2. The black dots represent residues that matched the majority exactly. The homologous regions of different SADS-CoV and SADSr-CoV strains that correspond to the identified epitope are in the box. Abbreviations of each virus and its strain are listed, and the corresponding GenBank accession numbers are shown.

genus *Deltacoronavirus* (Woo et al., 2012). Among them, TGEV, PEDV, PDCoV and SADS-CoV are swine enteric pathogens that cause severe diarrhea, vomiting, dehydration and death, with high mortality in suckling piglets. Diseases caused by these four swine enteropathogenic coronaviruses can hardly be differentiated unless by precise laboratory diagnosis (Wang et al., 2019).

The coronavirus genome harbors accessory genes locate downstream of ORF1b, their number, nucleotide sequence and order are diverse among different species of coronaviruses (Decaro and Lorusso, 2020; Redondo et al., 2021). For example, there are five accessory proteins (p3a, p3b, p3c, p7a and p7b) encoded by *alphacoronavirus* feline infectious peritonitis virus, while there is only one accessory protein (pORF3) encoded by PEDV (Liu et al., 2014). SARS-CoV-1 and the most recent SARS-CoV-2 within genus *Betacoronavirus* encode eight and 10 accessory proteins, respectively (Redondo et al., 2021), and three accessory proteins (NS6, NS7, and NS7a) have been identified in PDCoV (Fang et al., 2017). As a newly emerging swine enteric coronavirus, accessory proteins of SADS-CoV have not been fully elucidated yet. Bioinformatics predicts that SADS-CoV encodes three putative accessory proteins ORF3, NS7a and NS7b (Fu et al., 2018; Gong et al., 2017; Pan et al., 2017). Yang et al. examined these predictions by reverse transcription polymerase chain reaction based on leader-body junctions in specific subgenomic mRNAs, and found that NS7a and NS7b were encoded by a bicistronic mRNA (Yang et al., 2019). Subsequently, they attempted to detect the NS7a and NS7b expression in SADS-CoV-infected cells by IFA and western blotting using polyclonal antibodies (pAbs) against these two proteins. Unfortunately, both pAbs failed to react with any antigen in the SADS-CoV-infected cells (Yang et al., 2019).

Preparation of mAb against NS7a is urgent for the biological identification and epitope mapping of SADS-CoV NS7a protein. In this study, the recombinant NS7a protein was expressed in a prokaryotic expression system, mAbs against this protein were successfully produced, and their precise epitopes were further investigated. Prokaryotic expression systems have the advantages of easy operation, high production, and low cost, but lack some modifications present in eukaryotic expression (Fan et al., 2020). To obtain soluble recombinant protein with natural biological activity as an immunogen, we optimized the multiple parameters including temperature, concentration of IPTG, and induction time. Finally, we demonstrated that there was a high yield of recombinant protein expression of soluble form when induction under low temperature at 15 °C. After immunization of mice, cell fusion and screening, two hybridoma clones that secreted mAb specific for the NS7a protein were successfully generated. Western blotting indicated that mAbs AH5 and EH3 reacted with recombinant NS7a protein expressed in *E. coli* Rosette (DE3), and recognized the eukaryotically expressed NS7a protein in pCAGGS-HA-NS7a-transfected HEK 293T cells. Importantly, we demonstrated that the generated mAbs reacted specifically with natural NS7a protein in SADS-CoV-infected Vero E6 cells (Figs 3C), which provided pivotal evidence for the existence of accessory protein NS7a (Fu et al., 2018; Yang et al., 2019; Zhou et al., 2018). Furthermore, subcellular localization showed that NS7a was localized in the cytoplasm of Vero E6 cells (Fig. 4). Mapping epitopes of viral proteins and determining the degree of conservation of identified epitopes can promote our understanding of antigenic structures and virus–antibody interactions. This information is commonly useful and essential in development of diagnostic reagents for clinical applications. As of now, studies about mAbs against SADS-CoV and their epitopes are rarely available, except for a novel mAb specific to N protein, and its epitope has been investigated by our team (Han et al., 2019). MAbs AH5 and EH3 both recognized the denatured NS7a protein by western blotting; therefore, we mapped the precise B-cell linear epitopes of these mAbs against SADS-CoV NS7a protein. A series of peptides covering the NS7a protein were subjected to western blotting, which showed that amino acid ^{31}VNTWQEFA^{38} was determined as the minimal epitope recognized by AH5 (Fig. 5C), while amino acid ^{82}FDLFERF^{88} was identified as the minimal epitope recognized by EH3 (Fig. 6E).

Sequence analysis of the amino acids demonstrated that epitopes identified in NS7a protein were highly conserved among SADS-CoV strains and SADSr-CoVs isolates 8462 and 162,140, but with point mutations in the bat coronavirus HKU2 (Fig. 6). However, NS7a protein of SADSr-CoV isolates 141,388 and 8495 share low similarity (57.6% ~ 61.6%) with SADS-CoV strains and SADSr-CoVs isolates 8462 and 162,140 (Zhou et al., 2018). In addition, NS7a gene had large substitutions in the middle and late stage of SADS-CoV/CN/GDWT/2017 serial passage, and the P83 strain had low virulence in piglets. So, we speculate that NS7a of SADS-CoVs and SADSr-CoVs may not be essential for viral replication, but are probably involved in pathogenesis, however, this remains to be confirmed by further studies.

In summary, we prepared mAbs specific to SADS-CoV NS7a protein and demonstrated that NS7a was expressed in SADS-CoV-infected Vero E6 cells, and identified two precise epitopes recognized by mAbs. Localization analysis suggested that NS7a protein was predominantly located in the cytoplasm of SADS-CoV-infected Vero E6 cells. Our study provides a foundation for further elucidation of the characteristics and function of accessory protein NS7a in the SADS-CoV replication and pathogenicity.

Author statement

Authors' individual contributions: Li Feng, Da Shi, Yibin Qin and Tingshuai Feng conceived and designed the study; Yibin Qin and Tingshuai Feng performed the experiments; Yibin Qin, Tingshuai Feng, Hongyan Shi, Jiyu Zhang, Liaoyuan Zhang, Shufeng Feng, Jianfei Chen, Ying He, Xin Zhang, Zhongwei Chen, Jianbo Liu, Dakai Liu, Da Shi, Li Feng analyzed the data; Yibin Qin, and Da Shi revised the manuscript; Yibin Qin, Da Shi and Li Feng wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval

The animal experiments were approved by Harbin Veterinary Research Institute. The animal Ethics Committee approval number is Heilongjiang-SYXK-2020–032.

Declaration of competing interest

None of the authors have a conflict of interest.

Acknowledgments

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