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A novel neutralizing monoclonal antibody recognizes a linear antigenic epitope of the spike protein of swine acute diarrhoea syndrome coronavirus

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Abstract
Swine acute diarrhoea syndrome coronavirus (SADS-CoV) causes vomiting, severe diarrhoea and death in newborn piglets. The spike (S) protein plays a crucial role in promoting virus invasion and inducing neutralizing antibody production. In this study, the extracellular region of the S protein was used as an immunogen to immunize BALB/c mice. After immunization, B cells were collected, fused with SP2/0 myeloma cells, cultured and subcloned, and a cell line capable of secreting neutralizing antibodies was obtained and named as 5D6. Additionally, it was determined that the 5D6 mAb could be used as the primary antibody for western blotting and indirect immunofluorescence assay (IFA) to detect SADS-CoV. Further studies indicated that the 5D6 mAb binds to the ^{136}STSHAAD^{142} motif, which located in the N-terminal domain (NTD) of the spike protein. This result suggested that the NTD of the S protein can induce the production of neutralizing antibodies. Amino acid sequence alignment revealed that the epitope of the 5D6 mAb was conserved among SADS-CoV strains. This study helps elucidate the S protein function of SADS-CoV, and the 5D6 mAb may be used to develop diagnostic and treatment tools for detecting SADS-CoV infection.

Keywords Swine acute diarrhoea syndrome coronavirus (SADS-CoV), Neutralizing antibodies, Antigenic epitope

Introduction

Swine acute diarrhoea syndrome coronavirus (SADS-CoV), also known as swine enteric alphacoronavirus (SeACoV) [1] or porcine enteric alphacoronavirus (PEAV) [2], belongs to the genus Alphacoronavirus, family Coronaviridae, and order Nidovirales. SADS-CoV was first discovered in Guangdong Province, China, in 2017 and has caused the death of approximately 25,000 piglets, resulting in great economic losses in the pig industry [1, 3]. SADS-CoV is a highly contagious infectious pathogen that can cause clinical symptoms, including acute vomiting, watery diarrhoea and rapid weight loss in newborn piglets. Furthermore, SADS-CoV can infect a variety of cell lines [4, 5]. In particular, SADS-CoV has a greater

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replication efficiency in human cell lines than in porcine cell lines [6, 7], indicating that SADS-CoV is potentially at greater risk of cross-species transmission, especially transmission to humans.

The SADS-CoV genome length is approximately 27 kb, and it encodes four structural proteins, namely, the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins, and various nonstructural and accessory proteins [8, 9]. In coronaviruses, the S protein covers the surface of the virus in the form of trimers and plays crucial roles in determining the host range and tissue tropism of the coronavirus [10, 11]. The S protein of SADS-CoV (1130 amino acids) is smaller than other coronavirus S proteins [12]. Although SADS-CoV is classified as an alphacoronavirus, the SADS-CoV S protein belongs to the betacoronavirus group, as indicated by phylogenetic analyses [12–14]. SADS-CoV may have originated from a hybrid infection involving both an alphacoronavirus and a betacoronaviruses [2, 15]. Therefore, further research on the SADS-CoV S protein is essential.

Neutralizing antibodies that recognize coronaviruses primarily target the S glycoprotein, which plays a crucial role in virus entry. The S protein of coronaviruses has been demonstrated to elicit immune responses [16, 17]. However, to date, fewer neutralizing antibodies that target the SADS-CoV S protein have been identified [18].

In the present study, for the purpose of obtaining neutralizing antibodies and further understanding the SADS-CoV S protein, BALB/c mice were immunized with the SADS-CoV S protein. Through cell fusion, subcloning and identification, we obtained a cell line named as 5D6, that was able to secrete neutralizing antibodies against SADS-CoV. The use of mAbs will contribute to further understanding the characteristics and functions of the SADS-CoV S protein.

Materials and methods

Cells and viruses

The SP2/0 myeloma Vero cells used to culture the SADS-CoV were stored at Guangdong Laboratory Animals Monitoring Institute and cultured at 37 °C with 10% foetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM). Expi293F cells were obtained from Thermo Fisher Scientific (catalogue # A14527) and cultured in Expi293F expression medium (Thermo Fisher Scientific, USA) in an orbital incubator shaker at 37 °C with 5% CO_2.

SADS-CoV was isolated from the intestinal tissue homogenates of infected piglets and stored in Guangdong Laboratory Animals Monitoring Institute Guangzhou (GenBank accession MG557844) [2].

Protein expression and purification

The extracellular domain of the gene encoding the SADS-CoV S protein (GenBank accession MG557844) was codon optimized, cloned and inserted into the pCDNA3.1 vector with a C-terminal T4 trimerization fold and a 6-His tag. The spike protein was expressed by transient transfection of 1 mg of DNA into 1 L of Expi293 F cells using PEI transfection reagent. Three days after transfection, the cell culture supernatant was collected. The S protein of SADS-CoV was purified as previously described by He et al. [19]. The purified S protein was identified by SDS–PAGE and western blotting and stored at −80 °C.

Production of mAbs against the SADS-CoV S protein

The animal experiments were approved by the Guangdong Laboratory Animals Monitoring Institute (animal research proposal number: IACUC2021167). Three six-week-old BALB/c female mice were intramuscularly injected with 100 μg of purified S protein with Addavax (InvivoGen, USA) as an adjuvant (1:1 ratio) for immunization. After three immunizations at 2-week intervals, the mice were boosted with the same antigen in the same formulation. The antibody titre was measured by the indirect ELISA method based on recombinant S protein generated in the laboratory. Indirect ELISA was performed as previously described [20]. When the titre of the antibody test was greater than 50,000, the mice were intraperitoneally administered 100 μg of protein without adjuvant. Vaccinated mice were euthanized 3 days later, and spleen cells fused with SP2/0 cells were isolated using standard procedures [21] with modifications by Cong et al. [20].

The mAb preparation was performed according to standard procedures [22]. In brief, the fused cells were first cultured in hypoxanthine-aminopterin-thymidine (HAT, Sigma, USA) medium for six days, after which the medium was changed to hypoxanthine-thymidine (HT, Sigma, USA) to select fused cells. Hybridoma supernatants were analysed by indirect immunofluorescence assay (IFA). Then, positive hybridoma cells were cloned three times using the limiting dilution method. The supernatant of positive hybridoma cells was purified using HiTrap Protein G Sepharose columns (Cytiva, Uppsala, Sweden), and the subtype of the mAb was determined with a Mouse Monoclonal Antibody Isotype Elisa Kit (Southern Biotech Co., China) according to the manufacturer's protocol.

IFA and confocal microscopy

Vero cells and ST cells were plated in 96- or 12-well plates. The cells were subsequently infected with SADS-CoV, PEDV, PDCoV or TGEV at a multiplicity of infection (MOI) of 0.1 after they formed monolayers. When

a cytopathic effect (CPE) appeared, the cells were fixed with 4% paraformaldehyde for 10 min at 4 °C, and 2% bovine serum albumin was used as a blocking agent for 2 h. The cells were subsequently incubated with the mAb (5D6) for 1 h at 37 °C, after which they were washed three times with Alexa Fluor 488-conjugated donkey anti-mouse IgG (diluted 1:2000) for 45 min at 37 °C. Finally, the nuclei of the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, USA). Images of the cells were acquired with a confocal laser scanning microscope (Zeiss, JENA, Germany).

Identification of the mAb by Western blotting

Western blotting was performed to identify the reactivity of the mAbs with the purified S protein expressed in Expi293 F cells, the natural S protein derived from SADS-CoV-infected Vero cells and the GST-fused truncated S protein polypeptides expressed in *E. coli* Trasseta (DE3). The experimental methods were performed as previously described [20] with minor modifications. In brief, proteins and cell lysates were separated by 12% SDS–PAGE and transferred to a PVDF membrane (Merck Millipore, USA). The membranes were blocked with 5% skim milk in PBS and then incubated with the mAb (5D6) as the primary antibody and goat anti-mouse IgG-HRP at room temperature as the secondary antibody. Finally, the specific proteins were visualized using electrochemiluminescence (ECL) reagents with an Azure c600 bioanalytical imaging system (Azure Biosystems, USA).

Virus neutralization assay

To determine whether the antibody had neutralization activity, a neutralization test was conducted with mAb 5D6 according to a previously described method [23] with minor modifications. In brief, the purified mAb was serially diluted 2-fold in culture medium, starting at a concentration of 2 mg/mL, and 50 µL of each dilution was mixed with 50 µL of SADS-CoV (200 $TCID_{50}$) and incubated for 1 h at room temperature. The antibody-virus mixture was then added to Vero cell monolayers in 96-well plates and incubated for 1 h at 37 °C. After incubation, the cells were washed and further incubated in medium supplemented with trypsin (each millilitre of solution contained 8 µg of trypsin). Then, we observed the CPE in these cells for 3–5 days.

Determination of the minimal antigenic epitope

To identify the epitopes of the mAbs that bind to S proteins, a series of overlapping truncated S protein polypeptides were expressed in this study (Fig. 1). The truncated gene segments in the first, second, third and fourth rounds were designed with primers to amplify gene fragments from the viral gene, and these fragments were subsequently cloned and inserted into the pGEX-6p-1 vector and expressed as fusion proteins containing GST tags. For the fifth round, a pair of oligonucleotide strands was synthesized for each truncated gene segment. After annealing, each pair of oligonucleotide strands was cloned and inserted into the pGEX-6p-1 vector and expressed as fusion proteins containing GST tags. The correctly sequenced recombinant plasmids were transformed into *E. coli* Transetta (DE3), expression was induced by incubation with IPTG for 8 h at 16 °C, and the proteins were detected by western blotting with an anti-GST mAb and mAb 5D6.

S protein structure and epitope distribution

To determine the spatial position of the epitope of 5D6, the structure of the SADS-CoV S protein (PDB: 6M39) was used as a model. Chimera was used to generate the images.

Biological information analysis for the minimal epitope

To determine the conservation of the mAb 5D6 epitope peptide, the sequences of 15 SADS-CoV strains from 2016 to 2023, four SADS-related coronaviruses and the bat coronavirus HKU2 were downloaded from GenBank. Alignment analysis was performed for the defined epitope and corresponding regions using the Jotun Hein method in the software program MegAlign.

Results

Expression and purification of the SARS-CoV S protein

The sequence encoding the extracellular domain of the S protein of SADS-CoV was inserted into the eukaryotic expression vector pCDNA3.1 and expressed in Expi293 F cells. The protein was purified from the supernatant. As shown in Fig. 2, the recombinant pCDNA3.1-SADS-CoV-S protein was successfully expressed, as verified by SDS–PAGE and western blotting analyses (Fig. 2A and B).

Preparation of mAbs against the SADS-CoV S protein

After the mice were immunized, their spleen cells were fused with SP2/0 myeloma cells, and positive clones of hybridoma cells were selected. Ultimately, mAb 5D6, which recognizes the SADS-CoV S protein, was obtained by western blotting (Fig. 3A). The heavy and light chain subclasses were determined using the SBA Clonotyping System-HRP (Southern Biotech, Birmingham, AL, USA). As shown in Fig. 3B, the subclass of the heavy chain of mAb 5D6 was classified as IgG1, and the light chain was classified as κ.

Neutralization capacity of the mAb in vitro

To evaluate the neutralization capacity of the generated mAbs, the purified mAb 5D6 (diluted to 2 mg/ml) was used to test the neutralization of SADS-CoV in Vero cell culture. The mAb 5G12, which targets the SADS-CoV N

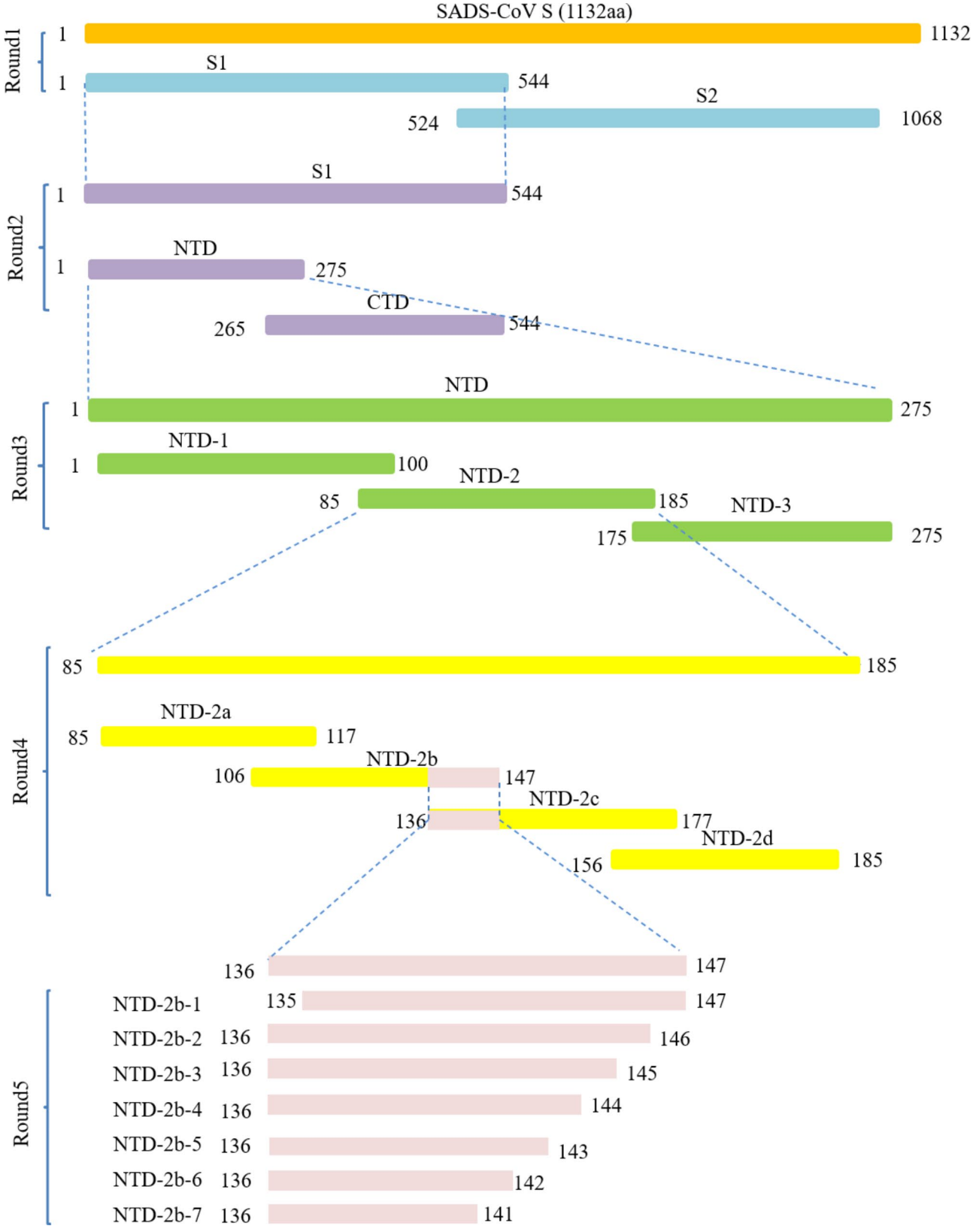

Fig. 1 Schematic diagram of the truncated SADS-CoV S protein fragments used to map B-cell epitopes. Five rounds of S peptides were conducted to investigate the epitopes of the generated mAb. The original whole S (1135 aa) was marked with orange, the first round was marked with blue, the second round was marked with purple, the third round was marked with green, the fourth round was marked with yellow, and the fifth round was marked with pink. The numbers indicate the initial and final amino acid positions for each SADS-CoV S fragment

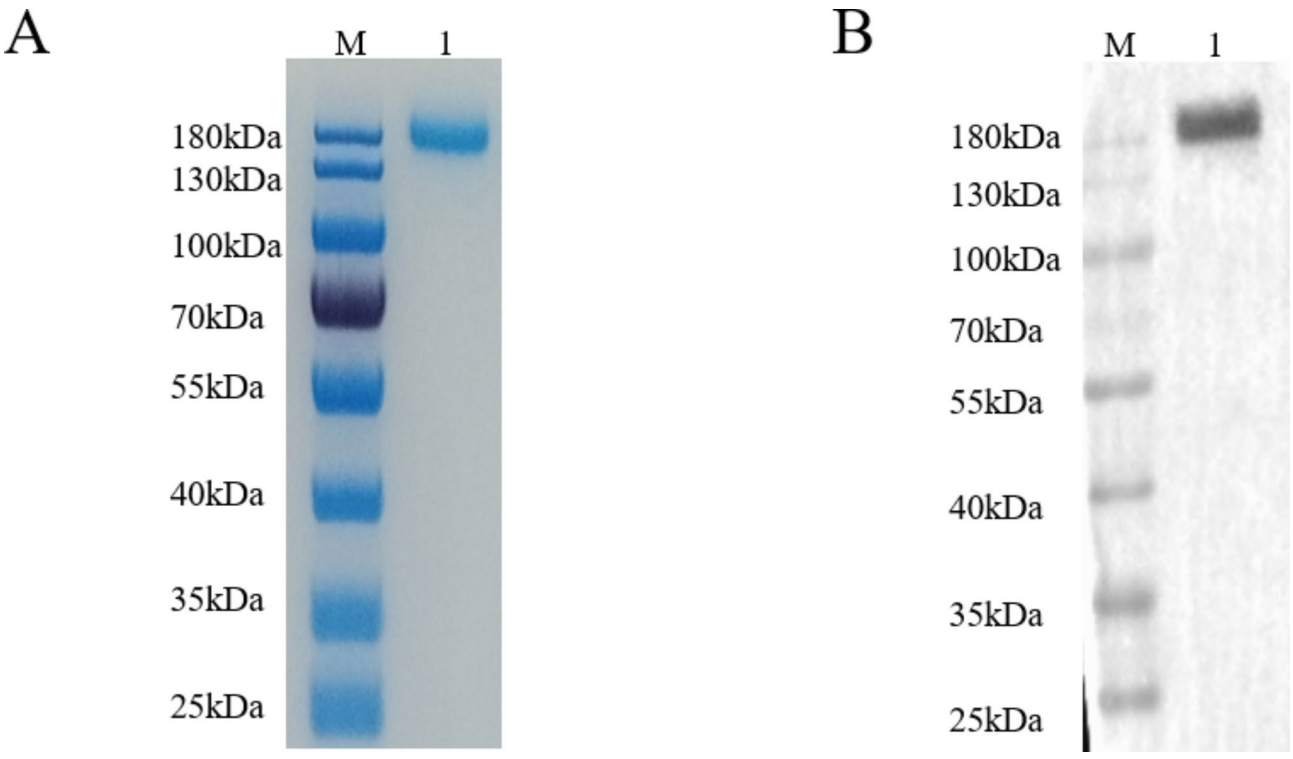

Fig. 2 Identification of the purified SADS-CoV S protein. (**A**) SDS–PAGE, lane 1: the expressed product of the S protein after purification, lane M: protein marker. (**B**) western blotting analysis. An anti-His mAb was used as the primary antibody. Lane 1: purified S protein; lane M: protein marker

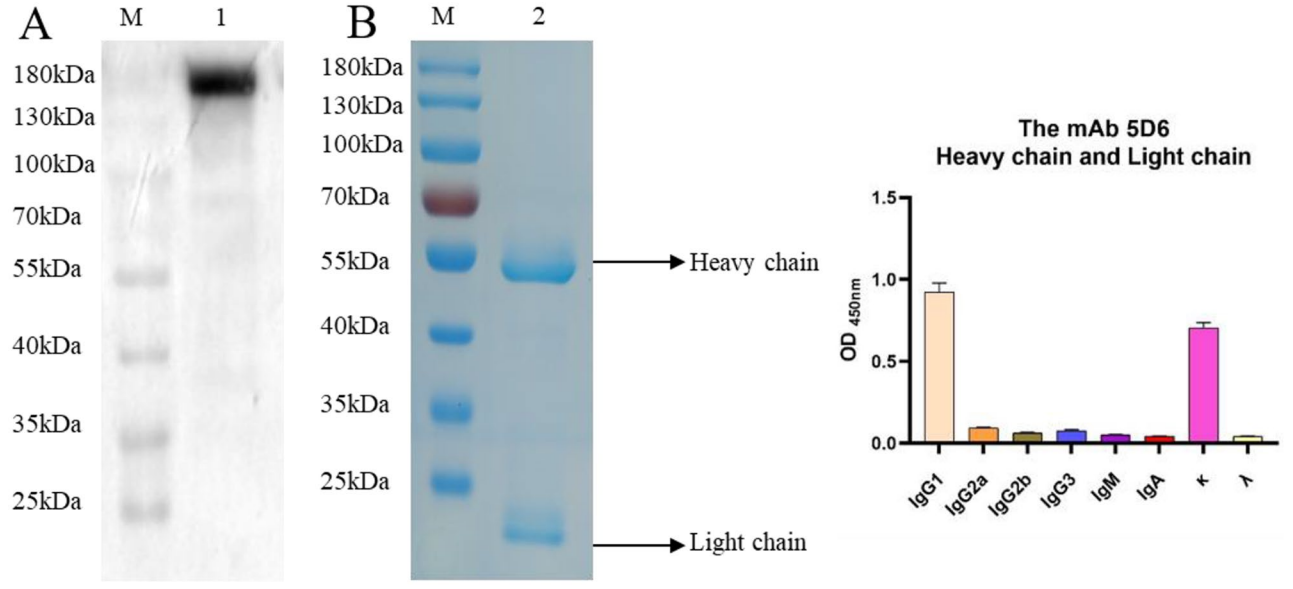

Fig. 3 Identification of the generated mAbs that recognize the S protein. (**A**) western blot. mAb 5D6 was used as the primary antibody. Lane 1: purified S protein; lane M: protein marker. (**B**) The purified monoclonal antibody was identified by SDS–PAGE, and the isotype of mAb 5D6 was determined by isotype ELISA

protein [20], was used as the negative control. As shown in Fig. 4, mAb 5D6 inhibited SADS-CoV infection completely in Vero cells when the mAb 5D6 concentration was greater than 125 μg/ml.

Monoclonal antibody specificity assays

The specificity and immunogenicity of mAb 5D6 were evaluated by western blotting and IFA. ST cells were infected with PDCoV and TGEV, and Vero cells were infected with PEDV and SADS-CoV. When CPE was

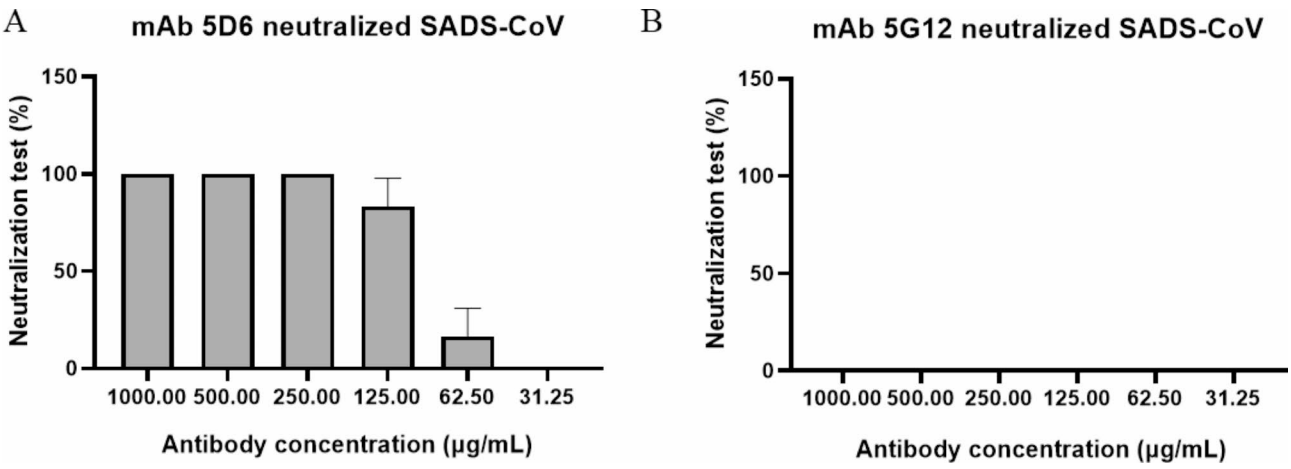

Fig. 4 Neutralization test of mAb. SADS-CoV was neutralized completely when the mAb 5D6 concentration was greater than 125 μg/ml. The mAb 5G12, which is specific for the SADS-CoV N protein, was used as a control (**A**) Neutralization test of mAb 5D6 (**B**) Neutralization test of mAb 5G12

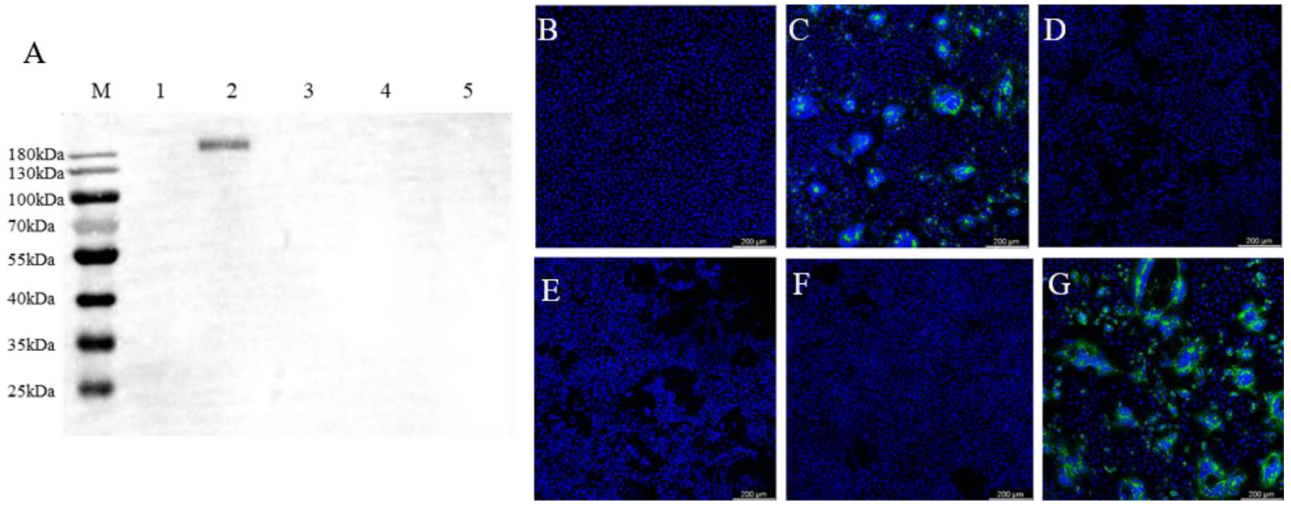

Fig. 5 Identification of the specificity and immunogenicity of mAb 5D6 against SADS-CoV. (**A**) western blot, lane 1, lysate of control Vero cells; lane 2, lysate of Vero cells infected with SADS-CoV; lane 3, lysate of Vero cells infected with PEDV; lane 4, lysate of ST cells infected with PDCoV; lane 5, lysate of ST cells infected with TGEV. (**B**) IFA results of uninfected Vero cells stained with mAb 5D6. (**C**) IFA results of Vero cells infected with SADS-CoV and stained with mAb 5D6. (**D**) IFA results of Vero cells infected with PEDV and stained with mAb 5D6. (**E**) IFA results of ST cells infected with PDCoV and stained with mAb 5D6. (**F**) IFA results of ST cells infected with TGEV and stained with mAb 5D6. (**G**) IFA results of Vero cells infected with SADS-CoV and stained with positive control serum

evident, virus-infected and mock-treated cells were lysed in RIPA lysis buffer for western blotting. As shown in Fig. 5A, mAb 5D6 reacted with SADS-CoV, and the specific bands corresponded to the expected size of the S protein, whereas no specific bands were observed for the other three viruses.

To further determine the specificity of mAbs targeting SADS-CoV, the cells were fixed with paraformaldehyde for IFA detection and incubated with mAb 5D6. The cells were subsequently stained with an Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody and DAPI. The IFA results revealed green fluorescence in Vero cells inoculated with SADS-CoV and positive control serum (Fig. 5C and G). Green fluorescence was not detected in the negative control or in cells infected with TGEV, PEDV or PDCoV (Fig. 5B, D, E and F), indicating that mAb 5D6 had good specificity.

Identification of the antigenic epitope that is recognized by the mAb

The surface accessibility of an epitope is also important for the interaction of the antibody and antigen. Therefore, we evaluated the surface epitope accessibility of spike proteins. As shown in Fig. 6, the S glycoprotein comprises the S1 and S2 subunits, so truncated S1 and S2 segments with GST tags, namely, S1 (1–544 aa) and S2 (524–1068 aa), were expressed in *E. coli.* The western blotting results revealed that the S1 subunit could be recognized by

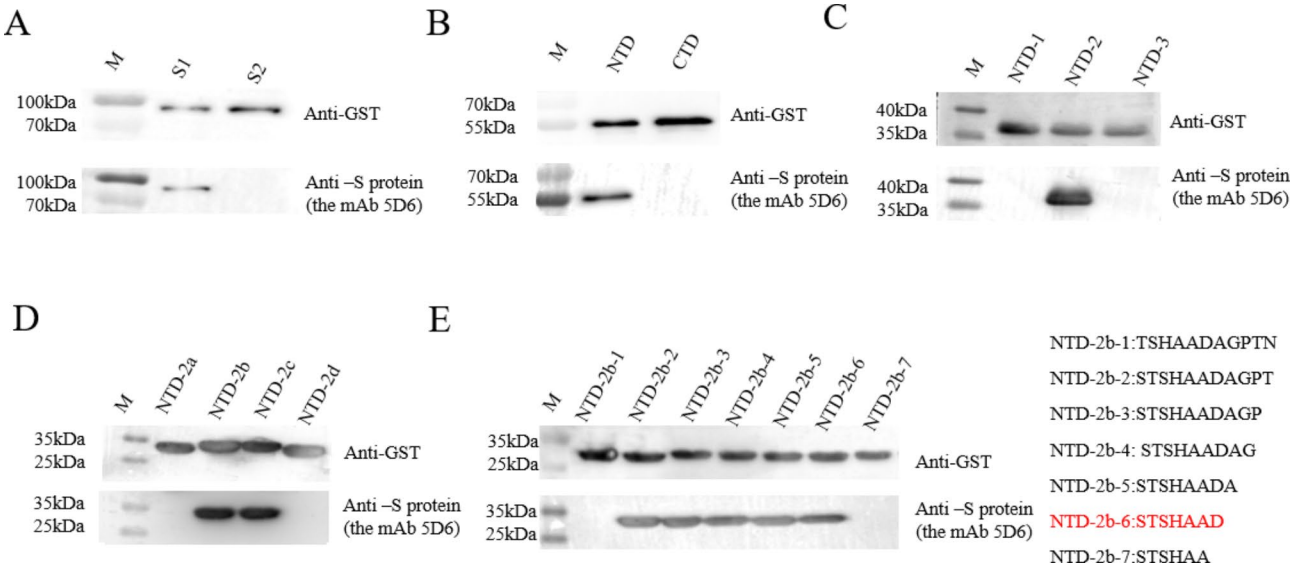

Fig. 6 Identification of B-cell epitopes in the S protein by western blotting. A series of truncated fragments were cloned, inserted into pGEX-6p-1, and expressed as fusion proteins with a GST tag. The expressed proteins were recognized with a GST-tagged mAb by western blotting as positive controls. (**A**) S1, peptide 1–544 aa; S2, peptide 524–1068 aa; (**B**) NTD, peptide 1–275 aa; CTD, peptide 265–544 aa; (**C**) NTD1, peptide 1–100 aa; NTD2, peptide 85–185 aa; NTD3, peptide 175–275 aa (**D**) NTD2a, peptide 85–117 aa; NTD2b, peptide 106–147 aa; NTD2c, peptide 136–177 aa; NTD2d, peptide 156–185 aa. (**E**) Peptide 136–147 aa was divided into 8 overlapping peptides, and the sequence was gradually truncated from both ends until the minimal linear epitope was identified; a minimal peptide ^{136}STSHAAD^{142} was identified as a precise epitope that is recognized by mAb 5D6. Lane M, protein marker

the mAb. S1 can be further divided into an N-terminal domain (NTD) and a C-terminal domain (CTD). Therefore, we divided S1 into an NTD (1–275 aa)-truncated segment and a CTD (265–544 aa)-truncated segment. The western blotting results revealed that the NTD subunit could be recognized by the mAb. The NTD was further divided into three overlapping fragments: NTD-1 (aa 1–100), NTD-2 (aa 85–185), and NTD-3 (aa 175–275). The western blotting results revealed that the NTD-2 subunit could be recognized by the mAb. Then, NTD-2 was further divided into four overlapping fragments: NTD-2a (85–117 aa), NTD-2b (106–147 aa), NTD-2c (136–177 aa) and NTD-2d (156–185 aa). Western blotting revealed that the mAb reacted with NTD-2b and NTD-2c. Thus, we concluded that the antigenic epitope of mAb 5D6 was located between NTD-2b and NTD-2c (136– to 147 aa). To definitively identify the minimal epitope, shorter peptides (Fig. 6E) were prepared for western blotting. The results showed that the mAb could react with the NTD-2b-6 (136–142 aa) segment. Eventually, we demonstrated that ^{136}STSHAAD^{142} was the minimal linear epitope that was recognized by mAb 5D6.

Analysis of the spatial localization of the antigenic epitope

As shown in the PDB model (6M39), the mAb 5D6 binding epitope ^{136}STSHAAD^{143} is located on a loop on the surface of the S protein, and this epitope is lacking because of the low density of the map (Fig. 7A&B).

Analysis of the homology of the antigenic epitope

To explore whether the linear epitope that is recognized by mAb 5D6 is conserved across SADS-CoV isolates, the S protein sequences of fifteen SADS-CoV strains that were made available from 2016 to 2023 were downloaded from GenBank and aligned. As shown in Fig. 7, the epitope 136STSHAAD147 was highly conserved among the SADS-CoV isolates. Additionally, we searched for this defined epitope in homologous sequences from SADS-related CoVs and bat coronavirus HKU2. The results revealed that the identified epitope had high homology with its counterpart in two SADS-rCoVs (MF094687 and MF094688), but it had low homology with bat HKU2 and another two SADS-rCoVs (MF094685 and MF094686).

Discussion

As the S protein of coronavirus on the surface plays a critical role in the virus life cycle, it is the primary immunogenic target for virus neutralization and is a critical component of coronavirus vaccines, diagnostics, and therapeutic targets [24, 25]. SADS-CoV can infect multiple cells and has the potential for cross-species transmission [4, 5]. Compared with other alphacoronavirus, SADS-CoV has unique structural properties. The S protein of SADS-CoV shares structural similarities with those of betacoronaviruses. To elucidate the multiple functions of the SADS-CoV S protein and its role in inducing neutralizing antibodies, mAbs against the S protein are needed.

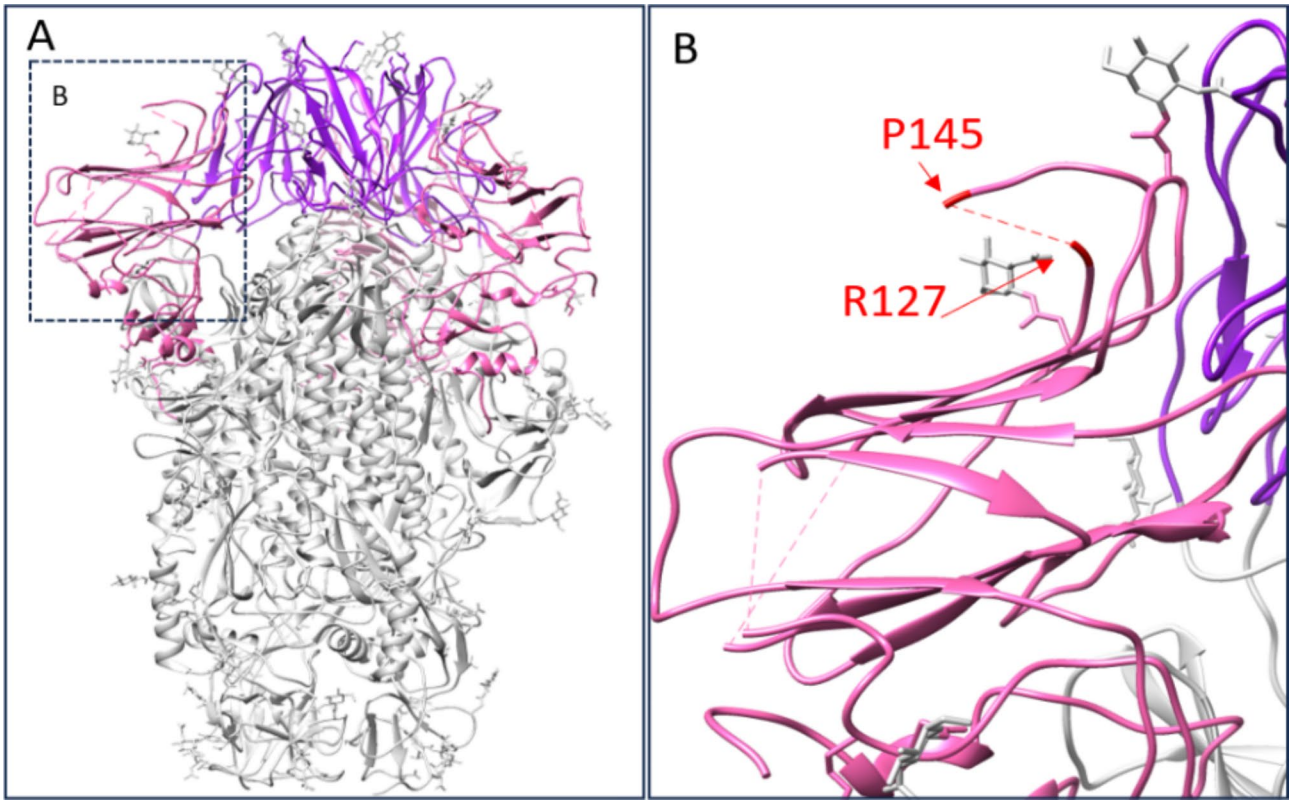

Fig. 7 The mAb 5D6 epitope is located in the spike structure of SADS-CoV. (**A**) The spike structure of SADS-CoV was determined from the PDB model (6M39), with the hot pink region showing the NTD and the purple region showing the CTD. (**B**) The deleted loop was between R127 and P145, and the mAb 5D6 binding epitope ^{136}STSHAAD^{143} was located in this region

In this study, we used a eukaryotically expressed S protein of SADS-CoV as the immunogen to immunize mice, and after cell fusion and screening, the hybridoma cell line 5D6, which was determined to secrete neutralizing antibodies against the SADS-CoV S protein, was successfully generated. However, compared with those of other neutralizing mAbs against other coronaviruses [19, 23, 26], the neutralizing activity of mAb 5D6 was slightly weaker. This result was similar to that of the mAb against SADS-CoV developed by Zhou et al. [18]. This may be due to the unique structure of the SADS-CoV S protein. The epitope in the structure of the SADS-CoV S protein that is recognized by mAb 5D6 located in a loop of the NTD and is a flexible linear epitope; this may be associated with the low neutralizing activity of this antibody.

The neutralization ability of mAb 5D6 was weaker than those of other mAbs [19, 23, 26], maybe cue to its epitope is liner (Figs. 5 and 6). To identify the precise epitope recognized by mAb 5D6, several truncated segments were expressed and identified by western blotting; the results indicate that the epitope recognized by mAb 5D6 located in the NTD of the S protein, and the amino acid sequence ^{136}STSHAADA^{142} was determined to be the minimal epitope. Compared with other researches [18, 27], mAb 5D6 is the first characterized neutralizing antibody against SADS-CoV that was identified to have a linear epitope, which can be a helpful tool for further research on this virus.

The receptor-binding domain (RBD)) of many coronaviruses can bind to receptors on target cells [28–30], and many neutralizing antibodies that bind to RBDs have been discovered [19, 26]. Therefore, subunit vaccines based on the RBDs of coronaviruses have been developed [31–33]. To date, most neutralization antibodies recognize conformational epitopes. However, the mAb 5D6 neutralization antibody was determined to have a linear epitope that located in the NTD, not the RBD (Fig. 7). Another neutralizing antibody against PDCoV, named 4E-3, was also determined to bind a linear epitope [34]. These results suggest that the spike NTD of coronaviruses can also be used as immunogen.

Although we identified the precise neutralization epitope for this mAb, we still do not know the neutralization mechanism, as the receptor of SADS-CoV is still unknown. Based on the structure of the SADS-CoV spike, the epitope region of 5D6 was determined to be in a loop of the NTD of the spike; this region is deleted because of its low density on the map. Why mAb 5D6 binding to this region can neutralize SADS-CoV? Based on researches on other coronaviruses, such as the human CoVs HKU1

and OC43, which use the NTD to bind its sugar receptor 9-O-acetylated sialic acid [35, 36], and Middle East respiratory syndrome coronavirus (MERS-CoV) uses the NTD to bind its sugar receptor Neu5ac [37], we speculate that mAb 5D6 may neutralize the virus by competing with sugar receptor binding sites or causing steric hindrance to the sugar receptor. As 9-O-acetylated sialic acid can trigger the conformation of the HKU1 coronavirus spike to change and open the RBD [36], we can also speculate that mAb 5D6 may maintain the stability of the spike. However, all these hypotheses need further research for confirmation.

Although the S protein is prone to mutations, sequence analysis of the epitope recognized by mAb 5D6 demonstrated that the epitope was highly conserved among SADS-CoV strains. As shown in Fig. 8, the identification of the epitope further suggested that SADS-CoV originated from SADS-rCoV [2]. Moreover, this study confirmed that the antibody does not cross-react with other porcine enteric coronaviruses, making it suitable for the diagnosis of SADS-CoV among porcine enteric coronaviruses.

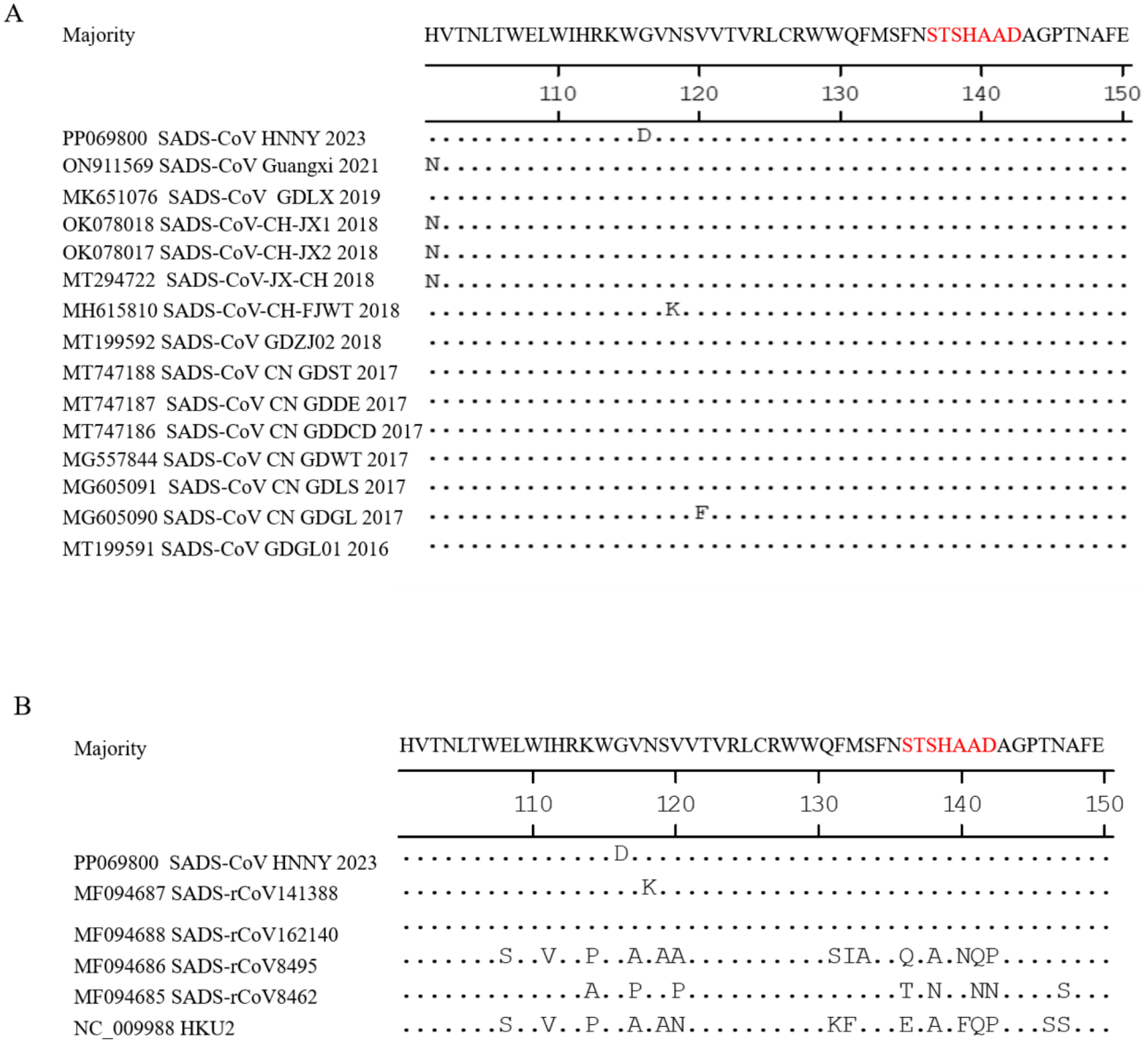

Fig. 8 Alignment of the amino acid sequences of the identified epitopes in the SADS-CoV S protein. (**A**) Comparison of the amino acid sequence of the epitope recognized by mAb 5D6 across fifteen different SADS-CoV strains that were identified from 2016– to 2023. (**B**) Comparison of the amino acid sequence of the epitope recognized by mAb 5D6 among four SADS-rCoV strains and bat coronavirus HKU2. The black dots indicate residues that are exactly matched. GenBank accession numbers are shown at the beginning

Conclusions

In conclusion, we generated a hybridoma cell line, namely, 5D6, which secretes SADS-CoV-specific neutralizing antibodies, and we identified the precise epitope recognized by the antibodies in the ^{136}STSHAAD^{142} region. This epitope is highly conserved across SADS-CoV strains that were identified between 2016 and 2023, and it could be used to identify SADS-CoV strains specifically, which will be useful for clinical applications.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-024-02562-0.

Supplementary Material 1: Table S1. PCR primers used in this study.

Supplementary Material 2

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Author contributions

L.Z and H.L. designed the study, carried out the experiments, L.Z and M.W wrote and reviewed the manuscript. Y.L. performed validation and formal analysis. Y.Z detected and analyzed the data. J.L participated in design and revision. F.C supervised the research, project administration and funding acquisition. All authors revised the manuscript and give their approval of this version to be published.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All animals care and experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Guangdong Laboratory Animals Monitoring Institute. The animal research proposal number is IACUC2021167. The entire procedures were carried out in accordance with the regulations and guidelines established by this committee.

Competing interests

The authors declare no competing interests.

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