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Characterization of two neutralizing monoclonal antibodies with conformational epitopes against porcine deltacoronavirus

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Abstract

Porcine deltacoronavirus (PDCoV) is a globally distributed swine enteropathogenic virus that emerged in the last decade. A recent report of PDCoV infection in Haitian children also highlights potential public health implications. In this study, two monoclonal antibodies (mAbs), 1C2 and 5H5, were generated and showed high specificity for the PDCoV S protein. Both mAbs displayed high-titer neutralizing capabilities, suggesting their potential for passive immunotherapy. Epitope mapping revealed that the mAbs likely recognized conformational epitopes in the S1 subunit domains A and B of the native S protein, thereby blocking the interaction between the S1 receptor-binding domain and the cellular receptor, which could inhibit viral entry into host cells. This study offers new biological tools for PDCoV detection and lays the groundwork for the future development of porcine-specific antibodies for the prevention and treatment of PDCoV in piglets.

Keywords Porcine deltacoronavirus (PDCoV), Spike, Neutralizing antibody, Monoclonal antibody, Epitope

Introduction

Coronavirus (CoV) is an enveloped, single-stranded positive-sense RNA virus characterized by their crown-like appearance due to spike proteins on their envelope (Barcena et al. 2009; Masters 2006). CoVs primarily infect birds and mammals, causing diseases that affect the respiratory, gastrointestinal, hepatic and central nervous systems (Chen et al. 2020; Ge et al. 2013; Wang et al. 2006). They are classified into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (Adams and Carstens 2012). *Alphacoronaviruses* and *Betacoronaviruses* predominantly infect mammals, while *Gammacoronaviruses* are more likely to infect avian species and marine mammals. Deltacoronaviruses (DCoVs), however, can infect both birds and pigs (Woo et al. 2012; Yang et al. 2020).

The *Deltacoronavirus* genus was officially classified in 2011, with porcine deltacoronavirus (PDCoV) being first detected in Hong Kong in 2012 (Woo et al. 2012).

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Initially, no clinical symptoms were associated with PDCoV, but its pathogenic potential was recognized in 2014 following outbreaks of porcine diarrhea in the United States (Li et al. 2014; Wang et al. 2014). Since then, PDCoV has been reported in several Asian, North American, and South American countries (Ma et al. 2015; Marthaler et al. 2014; More-Bayona et al. 2022).

PDCoV's genome is approximately 25.4 kb, making it the smallest among known coronaviruses. Its structure comprises four main structural proteins: spike (S), small envelope (E), membrane (M), and nucleocapsid (N). The S protein, crucial for viral entry and infection, is anchored to the virus surface in trimers (Bosch et al. 2003; Shang et al. 2018). The S protein is divided into S1 and S2 subunits. The S1 subunit is further divided into four domains (S1A to S1D) from the N-terminus to the C-terminus according to structural and functional analysis (Liu et al. 2021; Xiong et al. 2018). The S1A domain displays sialic acid-binding activity, whereas the S1B domain is the receptor-binding domain (RBD) that is critical to the PDCoV host range and tissue tropism (Hulswit et al. 2016; Liu et al. 2021). Although the receptor for PDCoV has not been fully elucidated, porcine aminopeptidase N (pAPN) is identified as the primary receptor for PDCoV entry into cells (Wang et al. 2018). The S1-RBD binds to the conserved domain II of APN (Ji et al. 2022; Li et al. 2018). However, PDCoV may also utilize other receptors, as evidenced by infection in pAPN-knockout swine intestinal epithelial cells (Zhu et al. 2018). Moreover, lung fibroblast-like cells derived from pAPN-knockout porcine alveolar macrophage (PAM) cultures support high levels of PDCoV replication (Stoian et al. 2020).

The origin of PDCoV remains unknown, but it is hypothesized to have originated from sparrows (Woo et al. 2009). It is postulated that avian DCoVs may serve as the genetic source for mammalian DCoVs, with the potential for cross-species transmission from birds to mammals (Ma et al. 2015). PDCoV has been shown to infect a variety of host species, including calves (Jung et al. 2017), chickens (Liang et al. 2019), and turkeys (Boley et al. 2020), and has been detected in human plasma samples (Lednicky et al. 2021). Additionally, PDCoV has successfully infected cells from a wide range of species, including human, porcine, bovine, avian and primate cells (Fang et al. 2021; Hu et al. 2015; Jung et al. 2018, 2020; Li et al. 2018; Wang et al. 2019), which raises concerns about its potential threat to public health. Therefore, there is an urgent need to develop efficient antibodies and antiviral drugs to control PDCoV infection in humans and various animal species.

This study characterized two murine monoclonal antibodies (mAb), 1C2 and 5H5, produced by immunizing with the whole PDCoV virus. The isotypes and specific reactivity of the mAbs to PDCoV were identified, revealing their potent neutralizing activity as determined by neutralization assays. The epitopes of two mAbs were mapped to the linker region between the S1A and S1B domains. These findings are significant for the development of PDCoV detection methods and the formulation of prevention strategies, including the design of subunit vaccines and the application of passive immunotherapy.

Results

Generation of two PDCoV mAbs used to detect PDCoV in infected cells

BALB/c mice were immunized with PDCoV virion particles that had been purified using ultracentrifugation to produce the mAbs. Consequently, two candidate mAbs from the hybridoma cell lines, designated 1C2 and 5H5, were selected for further identification. The mAbs 1C2 and 5H5 were characterized to be the subclass IgG1 and IgG2b, respectively.

In order to determine the reactivity between the PDCoV antigen and two specific mAbs, 1C2 and 5H5, a variety of detection methods, IFA, flow cytometry, and ELISA, were used in PDCoV-infected cells. LLC-PK1 cells were infected with PDCoV and incubated for 48 h followed by test with 1C2 or 5H5 as the primary antibodies Mock-infected cells served as the negative control. The PDCoV antigen could be stained with either 1C2 or 5H5 in PDCoV-infected cells but not in mock-infected cells (Fig. 1A). In addition, the flow cytometry results reveal that either 1C2 or 5H5 could bind to the surface PDCoV protein with similar binding affinities in PDCoV-infected but not in mock-infected cells (Fig. 1B). Furthermore, when purified PDCoV virions were used to coat the plates in ELISA detection, and a two-fold serial dilution of 1C2 or 5H5, or mouse pre-immune serum was applied as the primary antibody, PDCoV showed significant reactivity with both 1C2 and 5H5, but not with the pre-immune serum (Fig. 1C). The results collectively demonstrated that the mAbs 1C2 and 5H5 are efficient antibodies against PDCoV.

The PDCoV S protein is the target antigen of two mAbs

Mabs 1C2 and 5H5 were generated from mice immunized with the PDCoV viral particles. To determine which specific structural protein of PDCoV serves as the target antigen, expression plasmids carrying Myc-tagged S, E, M and N genes of PDCoV were constructed and individually transfected into BHK-21 cells. IFA was conducted at 48 h post-transfection, using 1C2 or 5H5 as primary antibodies. Both mAbs exclusively bound to cells expressing the S protein, with no reactivity observed in cells expressing the E, M, or N proteins (Fig. 2A). Furthermore, the flow cytometry analysis indicated that

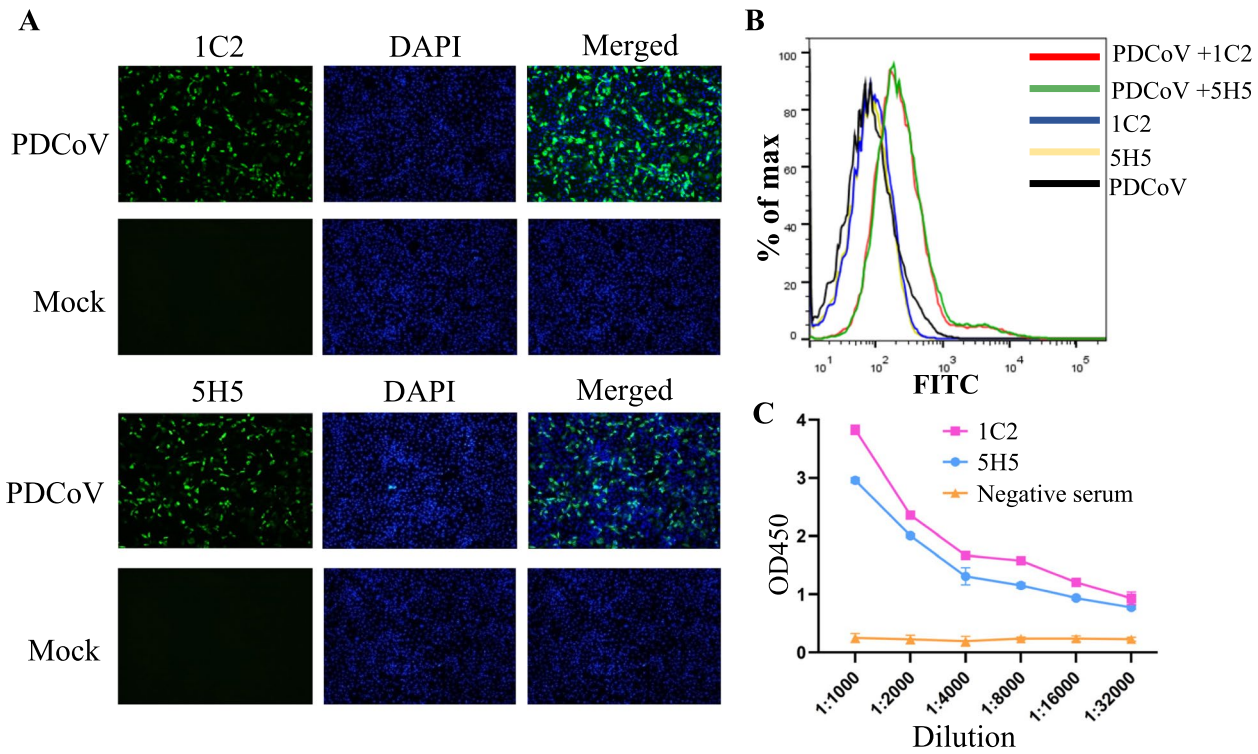

Fig. 1 Analysis of the monoclonal antibodies (mAbs) against PDCoV. (**A**) Antibody binding to PDCoV-infected LLC-PK1 cells, labeled with 1C2 or 5H5. Magnification, × 100. (**B**) mAb reactivity with PDCoV-infected LLC-PK1 cells assessed by flow cytometry. Red: PDCoV-infected cells with 1C2; green: PDCoV-infected cells with 5H5; black: PDCoV-infected cells with only secondary antibody; blue/yellow: LLC-PK1 cells with 1C2/5H5 as negative controls. (**C**) ELISA evaluated mAb reactivity to PDCoV. Negative serum was control. Measured at 450 nm

either 1C2 or 5H5 could block the PDCoV-S1 protein binding to the surface of LLC-PK1 cells or BHK-21 stably expressing pAPN (BHK-pAPN) cells (Fig. 2B). The result indicated that 1C2 and 5H5 are specific to the S protein of PDCoV, which could block the interaction between the S1 and the receptor pAPN.

Potent neutralizing activity of 1C2 and 5H5 against PDCoV

Since the CoV S protein contains neutralizing epitopes, we next investigate whether two mAbs possess the neutralizing activity against PDCoV by virus neutralizing assay. LLC-PK1 cells were infected with equal amount of PDCoV pre-incubated with a two-fold serially diluted 1C2 or 5H5, or the mouse pre-immune serum. At 72 h post-infection (hpi), either 1C2 or 5H5 reduced PDCoV infection in LLC-PK1 cells significantly; in contrast, the negative control serum did not inhibit PDCoV infection (Fig. 3A). The mAb 1C2 suppressed about 10% of PDCoV infection at the 1:16,384 dilutions, whereas 5H5 could inhibit about 20% of PDCoV infectivity at the same dilution (Fig. 3B). Therefore, 1C2 and 5H5 are the potent neutralizing mAbs, with 1C2 having a greater neutralizing activity.

The epitope recognized by two mAbs is located within the S1A to S1B domain (S1AB)

Having established the neutralizing capacity and identified the S1 subunit as the target of two mAbs, we proceeded to map the mAb epitopes. To do this, we constructed a total of nine expression plasmids carrying Myc-tagged truncated S genes corresponding to specific domains, designated as PDCoV-S1, PDCoV-S2, PDCoV-S1AB, PDCoV-S1A, PDCoV-S1B, PDCoV-S1C, PDCoV-S1D, PDCoV-S1A+, and PDCoV-S1B+ (Fig. 4A). After individually transfecting these truncated plasmids into BHK-21 cells, IFA was performed to determine which expressed products reacted with 1C2 or 5H5 staining. The results showed that PDCoV-S1 and PDCoV-S1AB were recognized by both 1C2 and 5H5, whereas PDCoV-S1A, PDCoV-S1B, PDCoV-S1A+, PDCoV-S1B+, PDCoV-S2, PDCoV-S1C, and PDCoV-S1D were not (Fig. 4B; data from PDCoV-S2, PDCoV-S1C, and PDCoV-S1D not shown). This suggests that the antigenic epitopes recognized by the two mAbs are located within the S1AB region.

It is puzzling that none of the constructs PDCoV-S1A, PDCoV-S1B, PDCoV-S1A+, and PDCoV-S1B+ were recognized by 1C2 and 5H5. The expression of these

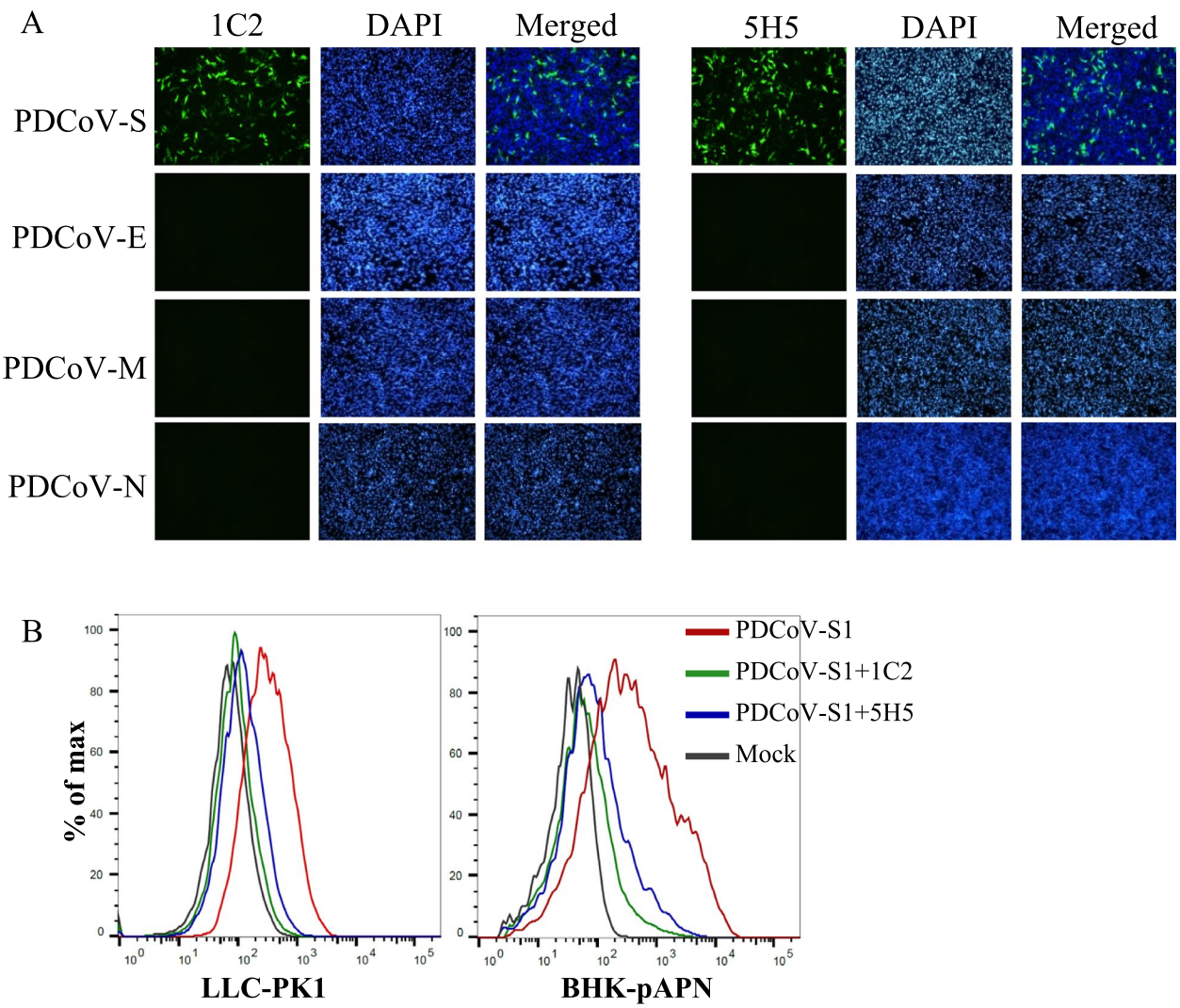

Fig. 2 Specific binding of mAbs to the PDCoV-S protein. (**A**) 1C2 or 5H5 binding to PDCoV proteins (S, -E, -M, and -N) was detected via IFA. Magnification, × 100. (**B**) Flow cytometry analyzed 1C2 (green) and 5H5 (blue) blocking of PDCoV S1 binding to receptor-expressing cells, LLC-PK1 (left) and BHK-pAPN cells (right). Negative serum (red) and secondary antibody (black) were controls

constructs was confirmed by IFA and western blot analysis, using an anti-Myc antibody (Figs. 4C and 4D). Nevertheless, the reactivity between these S-truncated proteins and the mAbs, as well as the anti-Myc antibody, was summarized in Fig. 4E.

mAbs recognize conformational epitopes

Based on the above findings, we hypothesize that the epitopes recognized by the mAbs 1C2 and 5H5 may be conformational, comprising elements from both the S1A and S1B regions. To evaluate whether the antigenic epitopes are indeed conformational, western blot analysis was performed to test the mAbs' ability to recognize the denatured S protein. The purified PDCoV S1-Fc protein (Wang et al. 2018) and the whole cell lysate (WCL) from PDCoV-infected LLC-PK1 cells, which contain the virus-produced S protein, were detectable using a positive control antibody (Qin et al. 2021), a polyclonal antibody against the PDCoV S protein (Fig. 5A). In contrast, the WCL from uninfected cells did not exhibit any specific bands.

The PDCoV S1-Fc protein and the WCL from PDCoV-infected LLC-PK1 cells were also detected by 1C2, but not by 5H5 (Fig. 5A). Western blot analysis suggested that the epitope for 5H5 is conformational, while the nature of the epitope for 1C2 was unclear. To address potential incomplete denaturation before western blotting, protein boiling time before sample loading was extended from

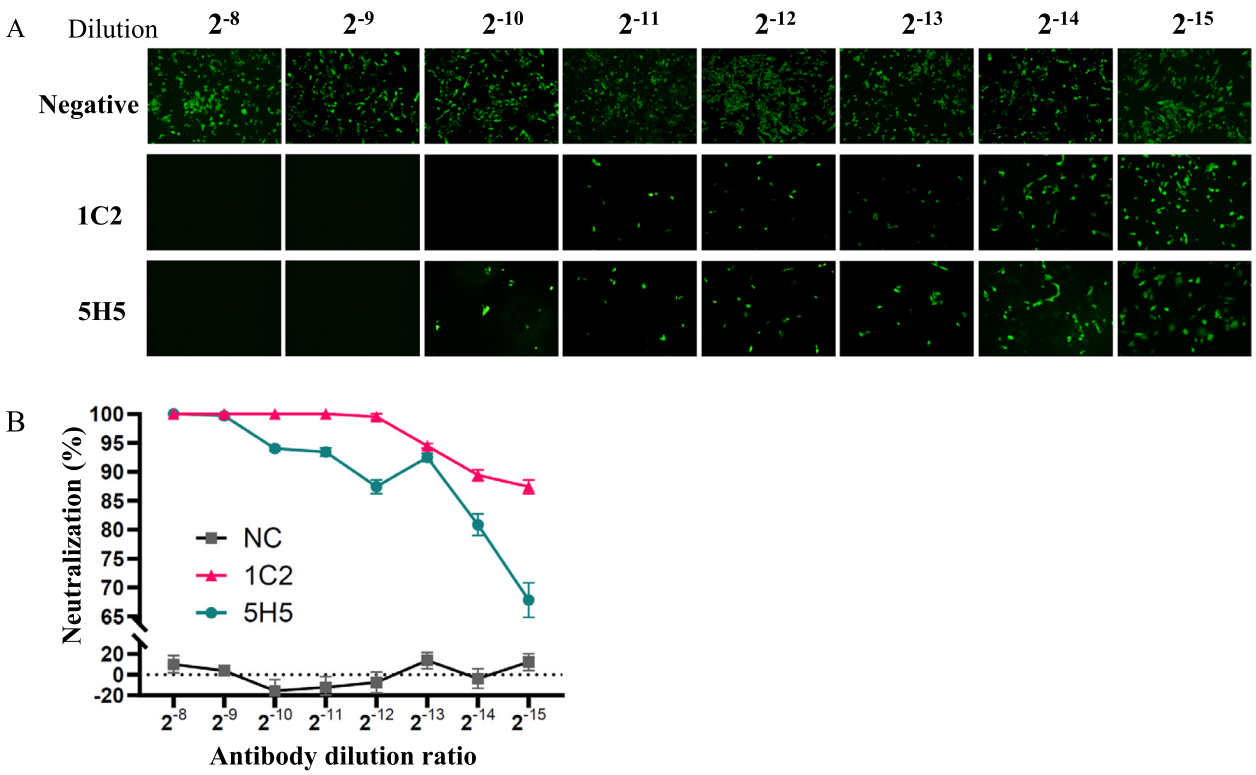

Fig. 3 Neutralizing activity of the mAbs against PDCoV infection. (**A**) PDCoV incubated with diluted 1C2 or 5H5 was used to infect LLC-PK1 cells. Neutralization was evaluated by IFA, using negative mouse serum as a control. Magnification, × 100. (**B**) The fluorescence in Fig. 3A was quantified. The neutralizing activity was calculated as: (1-—average fluorescence of dilution/average fluorescence of negative serum) × 100%

5 to 25 min. This adjustment allowed anti-PDCoV-S to still detect the proteins, but 1C2 could not, suggesting that the epitope for 1C2 might also be conformational (Fig. 5B).

To confirm the effect of protein denaturation on antibody recognition, a comparative denaturing and non-denaturing ELISA was performed as described (Studentsov and Burk 2007). The non-denatured PDCoV S1 protein and a version denatured at 100°C for 20 min were coated and tested for reactivity with the mAbs, with a polyclonal antibody included as a control. The results showed significantly higher reactivity with the non-denatured S1 protein (Fig. 5C), supporting the conclusion that 1C2 and 5H5 recognize conformational epitopes formed by the S1A and S1B steric structure.

Discussion

Two mAbs 1C2 and 5H5 showed high specificity for the S protein in IFA, flow cytometry, and ELISA, making them suitable for PDCoV detection. They exclusively bound to S protein-expressing cells and did not react with cells expressing E, M or N proteins, confirming their S protein specificity. Virus neutralizing assays revealed that both mAb have high-titer neutralizing capabilities, suggesting their potential for passive immunotherapy.

Identification of neutralizing antibody epitopes is crucial for developing safe and effective subunit vaccines. The PDCoV S protein, presented as trimers on the virus surface, is vital for virus-cell binding and mediating entry (Bosch et al. 2003; Shang et al. 2018). It consists of S1 and S2 subunits, and S1 binds to cells via its S1B domain or RBD. Cryo-electron microscopy has detailed the structure of the S trimer, with S1 subunit comprising domains A, B, C and D (Xiong et al. 2018). Our study found that 1C2 and 5H5 specifically recognize conformational epitopes in the S1AB region of the native S protein, as indicated by the green region highlighted in Fig. 4A. As the mAbs did not interact with the denatured S protein in western blot analysis, and indirect ELISA confirmed their binding to native S1 and no reactivity to denatured protein, highlighting the importance of spatial conformation of S1 subunit for antibody interaction.

The RBD within the S1 subunit is crucial for binding to pAPN, the primary receptor that enables PDCoV entry into host cells. Blocking the S1 RBD-pAPN interaction can significantly inhibit viral cell invasion. The two mAbs showed neutralizing activity against PDCoV through competitively

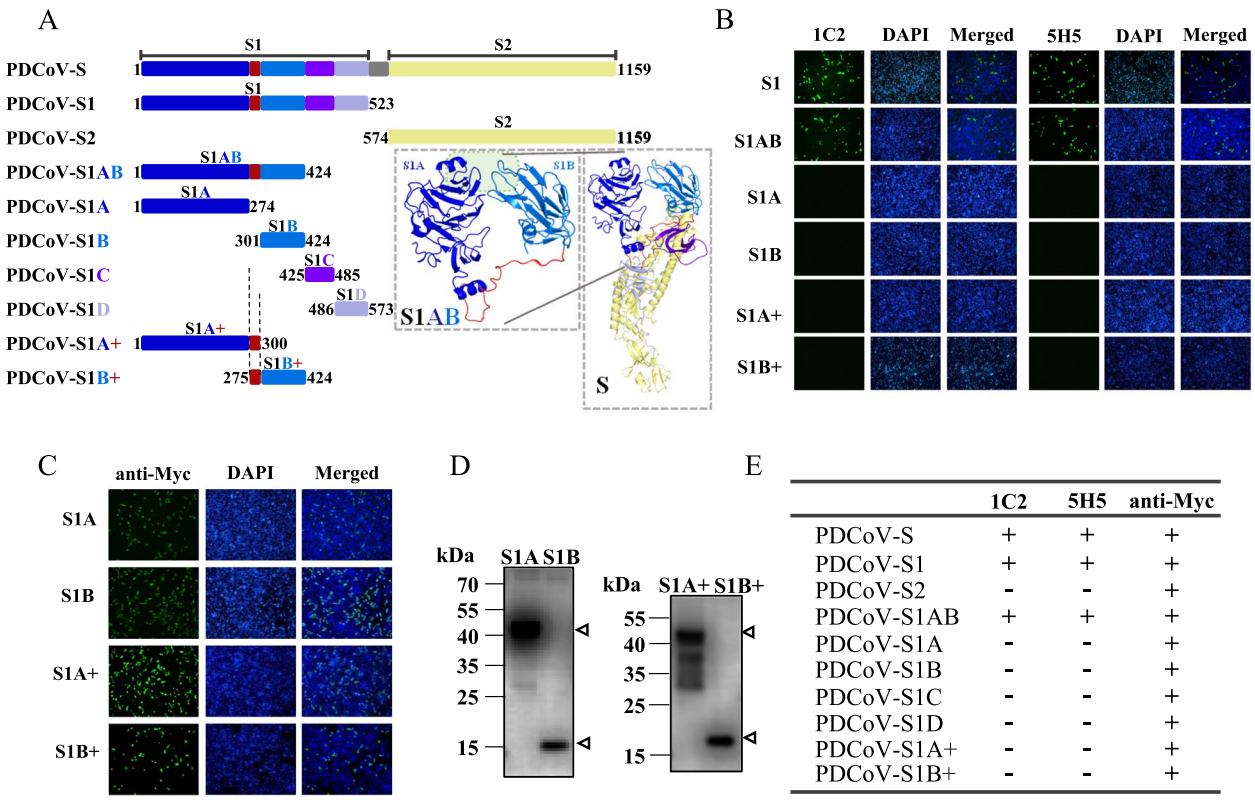

Fig. 4 Epitope mapping of the mAbs. (**A**) Schematic shows PDCoV-S variants for epitope mapping, divided into nine segments. The PDCoV S (PDB 6BFU) structural diagram is displayed, with different domains highlighted by distinct colors. (**B**) IFA detected 1C2/5H5 binding to BHK-21 cells transfected with truncated PDCoV-S plasmids. Magnification, × 100. IFA (**C**) and western blot (**D**) were used to detect the expression of Myc-tagged S1 variants. (**E**) Summary of antibody binding to truncated S proteins: "+" = positive, "-" = negative

binding to the S1 protein. They likely recognize epitopes within the RBD, either by competing with pAPN for RBD binding or by causing steric hindrance upon engagement with S1, thereby inhibiting receptor binding and preventing viral entry into host cells. Future studies based on the structure of the S1 and mAb complex are warranted to elucidate the details of this interaction.

Conclusion

In summary, mAbs 1C2 and 5H5 recognized the native PDCoV S protein and neutralized the virus effectively. The study offers new biological tools for PDCoV detection. Future studies will determine the antibodies' genes to develop porcine-specific modifications for the prevention and treatment of PDCoV in piglets.

Methods

Cells, virus and monoclonal antibodies

LLC-PK1 and BHK-21 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% (wt/vol) antibiotics (penicillin and streptomycin). The PDCoV-HZYH-2019 strain was isolated from pig fecal samples in our lab, which was propagated in LLC-PK1 cells with 5 μg/ml of trypsin at 37°C with 5% CO_2.

The hybridoma cell lines secreting antibodies specifically against PDCoV were generated according the standard mAb procedure as described previously (Zhou et al. 2023). Three rounds of subcloning were then conducted using limited dilution. The mAbs from ascites were purified with protein A beads and characterized for the subclass using an isotyping kit.

Indirect immunofluorescence assay

After infection with PDCoV, LLC-PK1 cells exhibited distinct cytopathic effects at 48 h post-infection (Qin et al. 2019). The cells were then washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Subsequently, the cells were incubated with PDCoV mAbs 1C2 or 5H5 at a 1:1,000 dilution for 1 h at 37 °C, followed by washing with PBS and staining with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific, USA) at a 1:1,000 dilution. After a 1-h incubation at 37°C, the cells were washed again with PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI) at

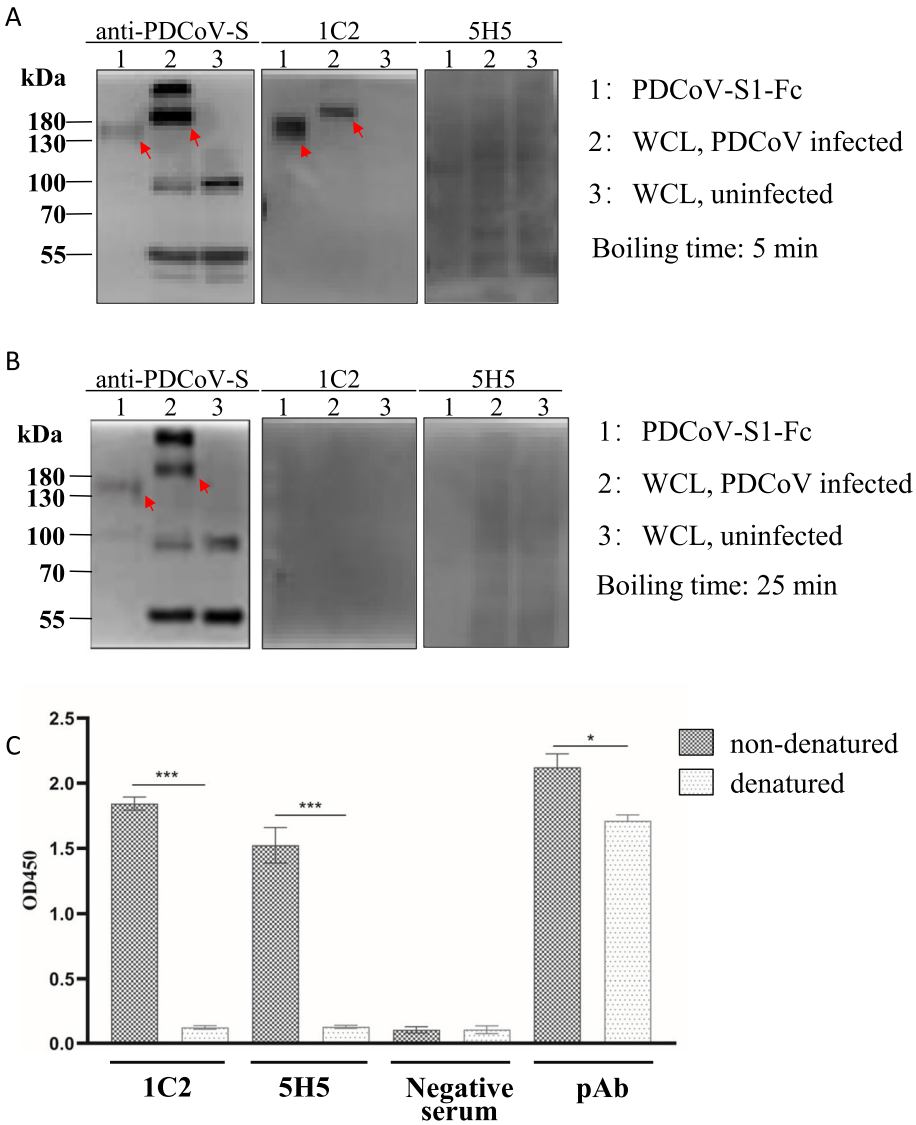

Fig. 5 Identification of conformational epitopes recognized by the mAbs. (**A**, **B**) Western blot analyzed mAb binding to denatured S protein, with anti-PDCoV-S antibody as control. Purified PDCoV S1 proteins (lane 1), PDCoV-infected LLC-PK1 WCL (lane 2), uninfected LLC-PK1 WCL (lane 3). Samples boiled for 5 min (**A**) or 25 min (**B**) before loading. (**C**) 96-well plates coated with native PDCoV S1 (dark bars) and heat-denatured S1 (light bars). Incubated with 1:4000 diluted mAbs, negative mouse serum as control. Absorbance measured at 450 nm

a 1:1,000 dilution, and visualized under a fluorescence microscope.

Flow cytometry analysis

Monocellular suspensions of PDCoV-infected LLC-PK1 cells were obtained 48 h post-infection and incubated with mAbs 1C2 or 5H5 at a 1:1,000 dilution for 1 h at 4°C. After washing three times with PBS, the cells were stained with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific, USA) and incubated for an additional hour at 4°C in the dark. Following three more washes, the cells were resuspended in PBS and analyzed using a flow cytometer.

Virus purification

PDCoV was purified on sucrose gradients using a modified version of the procedure originally designed for purifying PEDV (Hofmann and Wyler 1990). In brief, PDCoV-infected LLC-PK1 cell cultures were collected at 48 hpi, and intracellular virus was released through three cycles of freezing and thawing, followed by centrifugation at 4000×*g* for 30 min to eliminate coarse cell debris. The virions in the supernatant were pelleted by

high-speed centrifugation at 105,000×*g* at 4°C for 2 h and then resuspended in TNE (20 mM Tris–HCl, pH 7.2, 100 mM NaCl, 2 mM EDTA) to a volume of 5% of the original. The virus preparations were layered onto two sucrose cushions (12 ml of 20% (w/w) and 8 ml of 45% (w/w), prepared in TNE) and centrifuged at 146,000×*g* at 4°C for 16 h. The band formed between the two sucrose concentrations was collected using a syringe and used as the indirect immunofluorescence assay (ELISA) antigen without further processing. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China).

ELISA

Purified PDCoV (0.5 μg per well) was used to coat 96-well plates overnight at 4°C. After washing three times with PBS-T (PBS containing 0.05% Tween 20), the plates were blocked with 5% skim milk for 2 h at 37°C, followed by incubation with various mAbs diluted in PBS-T for 1 h at 37°C. After a washing step, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, diluted 1:1,000, was added, and the mixture was incubated at 37°C for 1 h. Following five washes, the reactions were visualized using TMB (3,3′,5,5′-tetramethylbenzidine; Solabio), and the reaction was stopped by adding 2 mol/L H_2SO_4. The optical density (OD) was measured at 450 nm with a microplate reader.

For denaturing and non-denaturing ELISA, equal amounts of native and denatured S proteins were added to separate wells of an ELISA plate. The S protein was denatured by heating at 95 °C for 20 min.

Virus neutralizing assay

The PDCoV neutralization assay was performed as previously described (Qin et al. 2021). LLC-PK1 cells were seeded in 96-well plates and cultured overnight to achieve a confluent monolayer. The mAbs were heat-inactivated for 30 min at 65°C, followed by two-fold serial dilutions from 1:2 to 1:256 in DMEM. These diluted mAbs were then mixed with 100 $TCID_{50}$ (50% tissue culture infectious dose) of PDCoV and incubated for 1 h at 37°C. Subsequently, the mixtures were used to inoculate LLC-PK1 cell monolayers, which were cultured for 48 h at 37°C. Neutralization activity was assessed by IFA using an anti-S polyclonal antibody.

Construction of recombinant plasmids

The S1 truncated fragments were amplified from the plasmid pRK5-PDCoV-S, which is preserved in our laboratory, and then cloned into the eukaryotic expression vector pRK5 with an N-terminal Myc-tag. The resulting constructs were designated as pRK5-PDCoV-S1, pRK5-PDCoV-S2, pRK5-PDCoV-S1AB, pRK5-PDCoV-S1A, pRK5-PDCoV-S1B, pRK5-PDCoV-S1C, pRK5-PDCoV-S1D, pRK5-PDCoV-S1A+ and pRK5-PDCoV-S1B+, respectively (Fig. 4A).

Expression of recombinant proteins

The recombinant plasmids were transfected into BHK-21 cells, which were grown to 60–70% confluence in 12-well plates, using Lipofectamine™ LTX Reagent with PLUS™ Reagent (Thermo Fisher Scientific) following the manufacturer's protocol. The transfected cells were cultured for 48 h before being subjected to IFA or western blotting to detect protein expression. For IFA, recombinant proteins were detected using anti-Myc antibodies. For western blot analysis, the transfected cells were lysed in lysis buffer (25 mM Tris–HCl, 200 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 1% NP40, and protease cocktail (Biotool, Houston, TX). The samples were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane, which was then blocked with Tris-buffered saline (TBS) containing 3% bovine serum albumin (BSA) overnight at 4 °C. Proteins were detected using an anti-Myc mAb at a 1:1000 dilution, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse IgG at a 1:5000 dilution (Thermo Fisher Scientific).

Abbreviations

CoV	Coronavirus
DCoV	Deltacoronavirus
PDCoV	Porcine deltacoronavirus
mAb	Monoclonal antibody
RBD	Receptor-binding domain
APN	Aminopeptidase N
IFA	Indirect immunofluorescence assay
ELISA	Enzyme-linked immunosorbent assay
WCL	Whole cell lysate
DMEM	Dulbecco's modified Eagle medium
IgG	Immunoglobulin G
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TMB	3,3′,5,5′Tetramethylbenzidine
BSA	Bovine serum albumin
HRP	Horseradish peroxidase
PBS	Phosphate buffered saline

Authors' contributions

W. L, Y.-W. H and B. W designed the experiments; W. L, H.-T. C, Y.-L. Y, Y. S, D. Y, X. L and B. W performed the experiments; P. F. G analyzed the data; W. L, X. L, Y.-W. H and B. W analyzed the data and wrote the manuscript.

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Data availability

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

Declarations

Competing interests

The authors declare no conflicts of interest.

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