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Letter

Identification of two novel B-cell epitopes on the nucleocapsid protein of porcine deltacoronavirus

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Dear Editor,

Porcine deltacoronavirus (PDCoV) is a novel discovered swine enteric coronavirus which can cause diarrhea and dehydration in pigs, particularly in neonatal piglets (Jung et al., 2016). At present, there are no commercial vaccines available for PDCoV. To control PDCoV transmission and perform antiviral therapy efficiently, a rapid and accurate diagnostic method to detect PDCoV is needed. PDCoV nucleocapsid (N) protein is the most abundant protein in the virus particle and plays essential roles in several stages of the viral lifecycle. It can produce high levels of antibodies at the early stage of PDCoV infection (Xu et al., 2013; Dinesh et al., 2020; Van Elslande et al., 2021). However, the characterization of epitopes on PDCoV N protein remains largely unknown.

In this study, the complete PDCoV *N* gene from HNZK-02 strain (MH708123) was amplified by RT-PCR and cloned into the pET-32a (+) prokaryotic expression vector. The target protein with size of 45 kDa was obtained after induced expression (Supplementary Fig. S1A). Western Blot assay (WB) demonstrated that the recombinant protein was specifically recognized by PDCoV positive serum (Supplementary Fig. S1B). Subsequently, the protein was purified using a Ni-NTA agarose column, and the concentration of the purified PDCoV N protein was 0.5 mg/mL (Supplementary Fig. S1A).

The purified PDCoV N protein was used as an immunogenic antigen to generate N-specific monoclonal antibodies (Mabs) with the hybridoma technique. Two hybridoma cell lines, namely 6B7 and 7F8, were acquired through three times of subcloning. To further confirm the specificity of these two Mabs, immunofluorescence assay (IFA) and ELISA assays were performed with the obtained Mabs. Specific fluorescence could be observed in the PDCoV-infected cells, while no fluorescence was observed from the negative controls. Moreover, the fluorescence of Mab 6B7 binding was in the cytoplasm, while the fluorescence of Mab 7F8 binding mainly existed in the nucleus with a small amount of distribution in the cytoplasm (Fig. 1A). From the ELISA results, we can see the two strains of Mabs can react with N protein from the lysates of cells infected with PDCoV (Fig. 1B). The Mab 6B7 subtype was IgG2b and the Mab 7F8 subtype was IgG2a, and both light chains were Kappa, which identified by mouse monoclonal antibody isotyping ELISA kit (Fig. 1C).

To identify the specificity to PDCoV of the generated Mabs, the PDCoV, porcine epidemic diarrhea virus (PEDV), and porcine transmissible gastroenteritis virus (TGEV) infected cells were detected by Mabs using IFA and WB, respectively. From the WB results, we could see that Mabs 6B7 and 7F8 had specificity to PDCoV, while these Mabs could not react with PEDV or TGEV (Fig. 1D). IFA results showed that the two Mabs only reacted with the PDCoV infected cells, but not with PEDV and TGEV infected cells (Fig. 1E).

To verify the cross-reactivity of the two Mabs with the N proteins of other different deltacoronaviruses, the N proteins of Asian leopard cat coronaviruses, Sparrow coronaviruses, Wigeon coronaviruses and Chinese ferret badger coronaviruses were expressed in *E. coli* strain Rosetta and verified by WB. Our results showed both two Mabs can recognize the N protein of Asian leopard cat coronaviruses, but not with the other avian deltacoronaviruses (Fig. 1F). By comparing the amino acid sequences of the N protein of Asian leopard cat coronavirus with 21 PDCoV strains in different regions worldwide available from the NCBI, we found that the amino acid sequence of N protein of the Asian leopard cat coronavirus reported in Guangxi had the highest homology (99.4%) with the two PDCoV strains detected in Thailand in 2016 (Supplementary Fig. S3), which showed that these two viruses may be geographically closed.

To identify the epitopes recognized by two Mabs, as depicted in Fig. 2A, the N protein were further truncated into small discrete groups and expressed in *E. coli*. The acquired proteins were detected by Mabs 6B7 and 7F8 using WB assays (Fig. 2B). The results indicated that the minimum antigenic epitopes that could be recognized by Mabs 6B7 and 7F8 were N5-13 (amino acids 326-QDWEWDDA-333) (Fig. 2C) and N4-15 (amino acids 251-NFQAGAITLTFSYSITVKEGSPDYER-276) (Fig. 2D), respectively.

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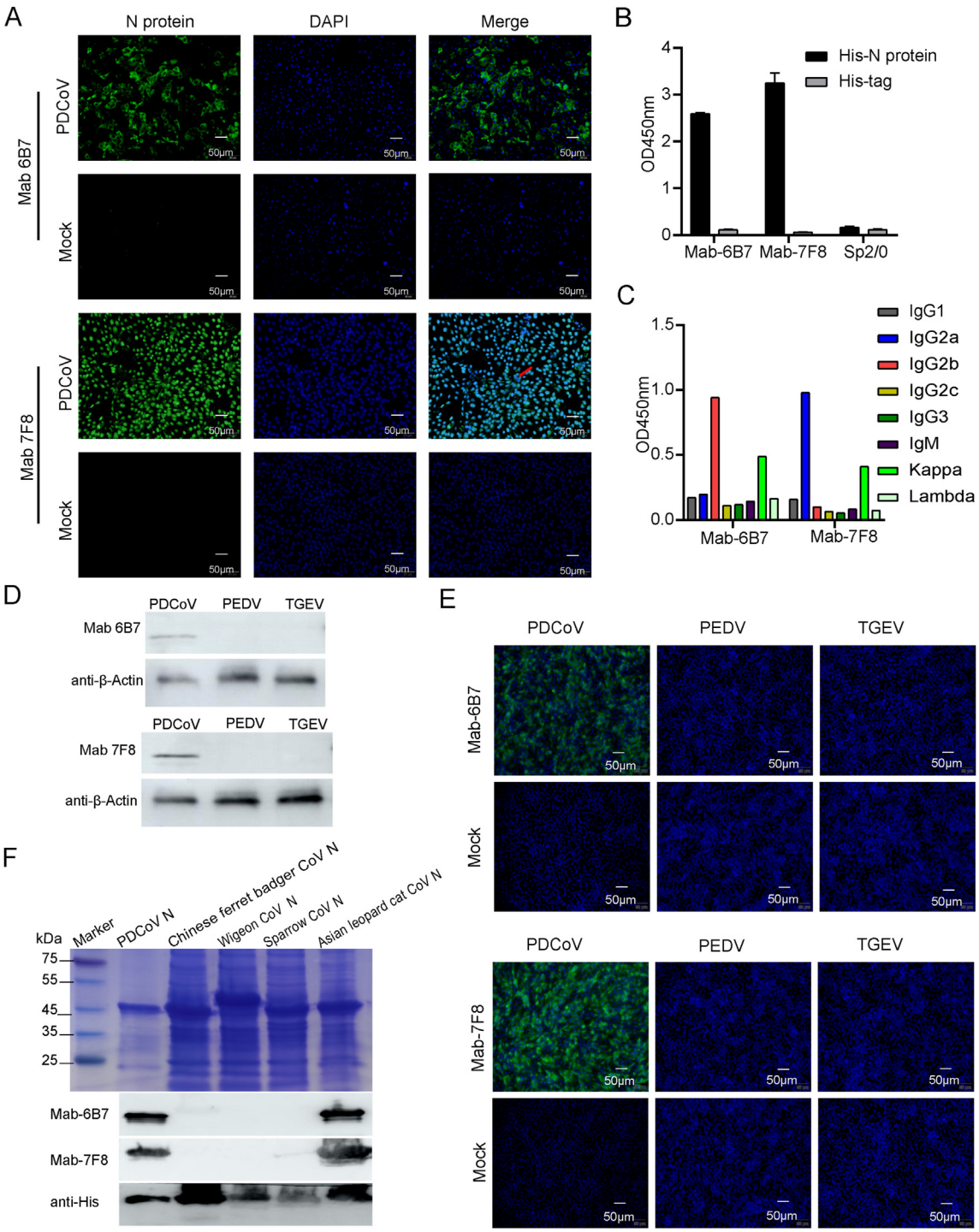

Fig. 1. Selection and identification of monoclonal antibodies (Mabs) against PDCoV N protein. **A** Identification of the two Mabs with PDCoV-inoculated cells by IFA. The area indicated by the red arrow is the area where the nucleus and cytoplasm of the cell fluoresce. **B** Analysis of the two Mabs react with His-N protein by ELISA. **C** Analysis of the two Mabs subtypes. **D** Analysis of reactivity of Mabs 6B7 and 7F8 with PDCoV, TGEV, and PEDV-infected cells determined by western blot. **E** Reactivity of Mabs 6B7 and 7F8 with PDCoV, TGEV and PEDV infected cells determined by IFA. **F** WB results of two Mabs cross-reacted with the N proteins of Asian leopard cat CoV, Sparrow CoV, Wigeon CoV, and Chinese ferret badger CoV.

We used I-TASSER to predict the 3D structure model of PDCoV N protein based on several basic sequences. The predicted structures were displayed with PyMol software (PyMOL 2.0). The results showed that the epitope 326-333 AA was located in close proximity and exposed at a vertex on the surface of the N protein, indicating that the epitope 326–333 AA might be a linear epitope. By contrast, epitope 251–276 AA was much more complex, most of the amino acids were located on the surface, and a small fraction of its residues were buried inside the N protein. The epitope peptide chain was folded at both ends and brought together in the middle to form a specific spatial structure, indicating that the epitope 251–276 might be a conformational epitope (Fig. 2E). The epitope 326–333 AA has 8 AAs and can be recognized by Mab 6B7 using WB, a method that imposes protein denaturation, indicating that epitope 326–333 AA maybe a linear epitope, which agreed with the result that epitope 326–333 AA is located on the surface of the predicted 3D structure of N protein. However, WB suggested that epitope

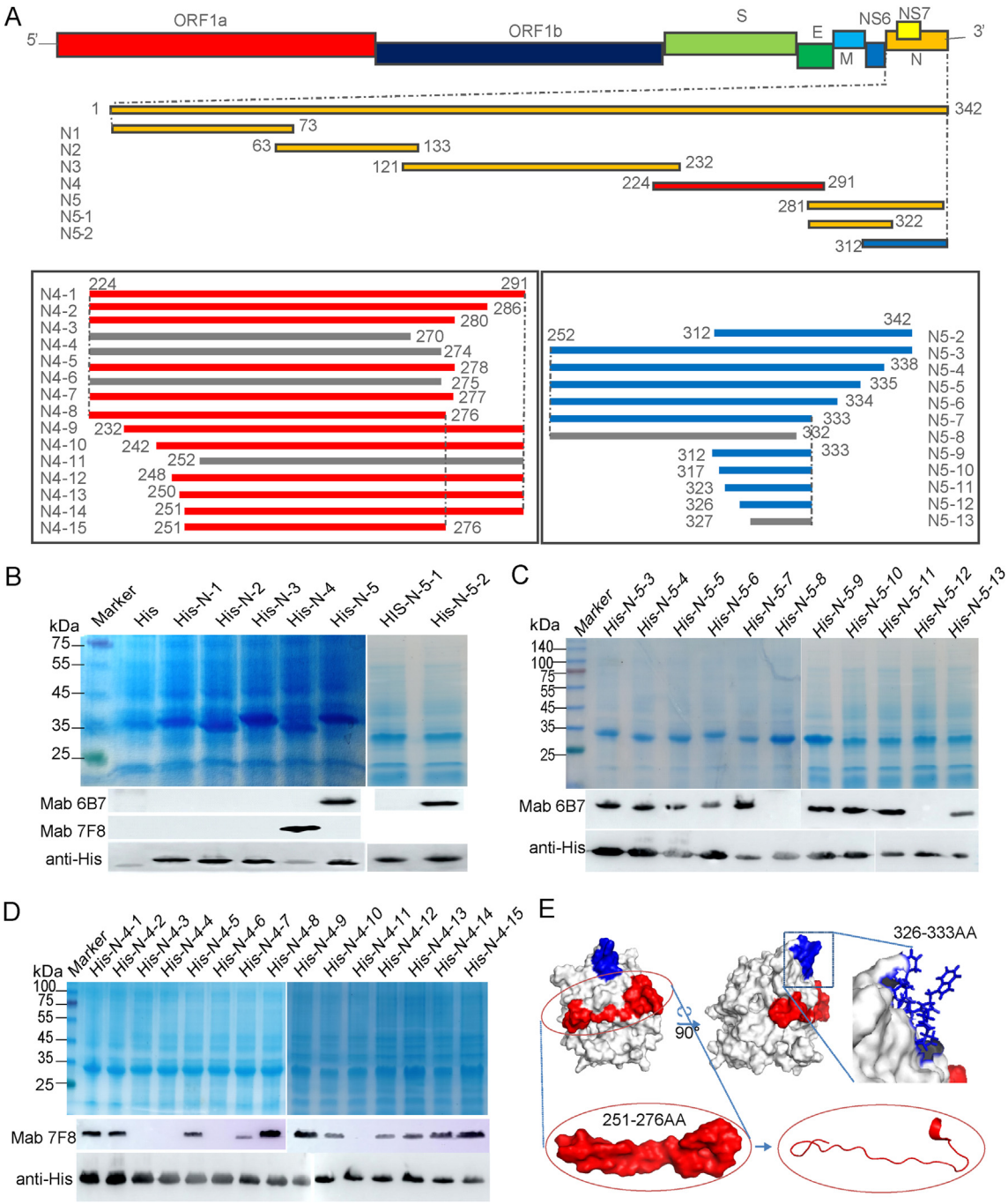

Fig. 2. Identification and analysis of epitopes recognized by Mabs against PDCoV N protein. PDCoV N gene was divided into mutually overlapping N1, N2, N3, N4, N5, N5-1, N5-2fragments. And the segments were expressed using the pET-32a (+) vector and detected by the screened Mabs 7F8 and 6B7. **A** Schematic diagram of the epitope mapping. The segments that could only be recognized by Mab 7F8 are highlighted in red; the segments that could only be recognized by Mab 6B7 are highlighted in blue; and the segments in gray are those that could not be recognized by either Mab 7F8 or Mab 6B7. **B** The segments of PDCoV N protein (namely His-N1, His-N2, His-N3, His-N4, His-N5, His-N5-1 and His-N5-2) were expressed in *E. coli* and analyzed using SDS-PAGE and Western blot. (**C** and **D**) After the first round of identification, N4 and N5-2 were further divided and expressed in *E. coli* and analyzed by western blot using Mab 7F8 and 6B7 (1:4000 dilution), respectively. **E** Model of the I-TASSER predicted 3D structure of the N-protein, visualized using the PyMOL molecular graphics and modeling system. The blue area represents the epitope 326–333 AA, which is displayed in surface and sticks mode respectively, and the red area represents the epitope 251–276 AA, which is displayed in surface and cartoon mode respectively.

251–276 AA is a linear epitope, and the 3D model shows that the epitope 251–276 AA has a certain spatial structure. Studies on the N proteins of SARS-CoV and SARA-CoV-2 have demonstrated that the inherently disordered Serine/Arginine (SR)-rich region between the N-terminal RNA binding domain and the C-terminal dimerization domain anchors the ribonucleoprotein complex to the viral membrane (Dinesh et al., 2020; Korn et al., 2020; Tseng et al., 2021). At the same time, there are indeed multiple serines and arginines in epitopes 251–276 AA. Therefore, we speculate that the epitope 251–279 AA may be an internally disordered conformational epitope or a longer linear epitope. The exact structure of this Mab need to be further verified by analysis the nucleotide sequences of light chain and heavy chain of the Mabs,

which requires further research on the structure and function of the epitope on PDCoV N protein.

To determine the conservation of the two identified epitopes in different PDCoV strains, we compared the most N protein sequences from other PDCoV strains available in GenBank. The results showed that the two epitopes were highly conserved among the PDCoV strains, and the sequence similarities of epitope 326–333 AA (Mab 6B7 recognition) and epitope 251–276 AA (Mab 7F8 recognition) in different PDCoV strains were 100% and 92.3%–100%, respectively (Supplementary Fig. S2A). We further compared the sequence similarities of these two epitopes among the deltacoronaviruses. The results revealed that PDCoV with Asian leopard cat coronavirus, Magpie-robin coronavirus, Thrush coronavirus, Sparrow deltacoronavirus, White-eye coronavirus and Munia coronavirus shared 62.25%–100% sequence similarities in the position of epitope 326–333 AA, and shared 73.1%–96.2% sequence similarities in the position of epitope 251–276 AA. Other deltacoronaviruses had 12.5%–50% sequence similarities in epitope 326–333 AA and 15.4%–61.5% sequence similarities in epitope 251–276 AA with PDCoV (Supplementary Fig. S2B). In addition, sequence alignment among all the known members within subfamily *Coronaviridae* revealed that epitope 326–333 AA and epitope 251–276 AA were still have high specificity, while had low sequence identity with other coronaviruses (Supplementary Fig. S2C).

Several studies have found that N proteins of many coronaviruses have at least one nuclear translocation signal area, which can guide the N protein into the nucleus by interfering with the function of the host cell and thus facilitate virus proliferation. The N protein of PEDV can interact with Vero E6 nucleoprotein B23.1 and then prolongs the initiation of mitosis to delay the cell cycle, which is conducive to the proliferation of the virus (Wurm et al., 2001; Xu et al., 2013). PRRSV N protein can interact with nucleolar protein to inhibit cell division (Yoo et al., 2003). However, there is no report on the effect of PDCoV N protein on host cell function after entering the nucleus. In current study, the Mab 7F8 can recognize the N protein in the nucleus and cytoplasm, while the Mab 6B7 only recognizes the N protein in the cytoplasm. We speculate that the N protein of PDCoV may interact with the protein in the nucleus after entering the nucleus, hindering the recognition site of Mab 6B7. These findings will lay the foundation for future research on the effect of PDCoV N protein on host cell function.

In conclusion, two specific Mabs 6B7 and 7F8 against PDCoV N protein were prepared in this study, and we identified the epitopes targeted by these two Mabs, one of which is a linear epitope (326–333 AA). Another may be an intrinsically disordered conformational epitope or a longer linear epitope (251–276 AA). The two monoclonal antibodies showed strong specificity to PDCoV. Sequence analysis showed that the epitopes of the two antigens were highly conserved. These two Mabs and their targeted epitopes provide tools for the establishment of more accurate detection methods and the study of the viral infection mechanism.

Footnotes

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