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Letter

Identification of an immunodominant neutralizing epitope of porcine astrovirus type 5 capsid protein

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Dear Editor,

Porcine astrovirus (PAstV) is a swine enteric virus which can cause diarrhea and vomiting in pigs, particularly in neonatal piglets (Fang et al., 2019; Su et al., 2020). PAstV was first detected by electron microscopy in the fecal samples from diarrheal piglets in 1980 (Bridger, 1980). Based on viral genomes, PAstV is divided into five distinct genotypes (PAstV1–PAstV5) (Fang et al., 2019; Mi et al., 2020; Su et al., 2020). The PAstV5 was the main genotype prevalent in China. Xiao reported a positivity rate of 24.8% for PAstV5 as the predominant genotype in Hunan Province (Xiao et al., 2017). Cai reported a positive rate of 7.5% for PAstV5 in Sichuan Province (Cai et al., 2016). A study in Yunnan Province revealed a total infection rate of 39.9% for PAstV2 and PAstV5 (Ren et al., 2022). The PAstV genome open read frame 2 (ORF2) encodes for a ~90 kDa structural polyprotein called capsid protein (Cap) (Arias and DuBois, 2017). The region including amino acids (aa) 1–415 (N-terminus) of the Cap is highly conserved, and the region starting at aa 416 (C-terminus) is extremely variable (Ren et al., 2022). Moreover, the Cap can bind to host cell receptors and is the main target of neutralizing antibodies (Ricemeyer et al., 2022). At present, there are no commercial vaccines and medicines available for PAstV. To control PAstV transmit and perform antiviral therapy efficiently, a good neutralizing Cap against PAstV is needed.

In this study, we analyzed the PAstV5 ORF2 protein sequence of the PAstV5-HNPDS-01-P10 strain (GenBank Accession No. OQ781001) which was identified by our laboratory. The signal peptide of the PAstV5 Cap was predicted by SignalP 5.0 (Supplementary Fig. S1A). The antigenicity of the PAstV5 Cap was analyzed using BepiPred-3.0 (Supplementary Fig. S1B) and the Expasy-ProtScale (https://web.expasy.org/protscale/) was used to predict protein hydrophobicity (Supplementary Fig. S1C). The N-terminus of the PAstV5 Cap (aa 35–248) with strong antigenicity and hydrophilicity was selected, and named Cap-H. Then, the PAstV5 Cap-H fragment was amplified by RT-PCR using the specific primers (Supplementary Table S1) and cloned into expression vector pET-32a (+). After IPTG-inducing expression, SDS-PAGE and Western blot analysis results showed the target protein located around 43 kDa, can specifically react with anti-His antibody (Supplementary Fig. S1D and E). All these results indicated successful expression of target recombinant protein.

The PAstV5 Cap-H was purified by Ni-NTA agarose column and the purified protein final concentration was 1 mg/mL. The purified PAstV5 Cap-H was used as an immunogenic antigen to generate specific monoclonal antibodies (mAbs) with the hybridoma technique (Hao et al., 2022). The hybridoma cell line, namely 6C6, was acquired through three times of subcloning. The monoclonal cell line 6C6 was subsequently injected into mouse peritoneal cavity to produce the mAb 6C6 in the ascites. The antibodies in ascites were purified by octanoic acid-ammonium sulfate method. Next, we tested the titer of the mAb 6C6 by coating ELISA plates with purified PAstV5 particles. The indirect ELISA result showed that the titer of the mAb 6C6 in the purified ascites was 1: 5.12×10^4 (Supplementary Fig. S2A). Using a mouse mAb isotype ELISA kit found that the heavy chain subtype of the mAb 6C6 was IgG1, while the light chain subtype was kappa (Supplementary Fig. S2B). The tissues samples were collected from piglets vaccinated with the PAstV5-HNPDS-01 strain and non-infected piglets (Li et al., 2024). Immobilized porcine ileum was detected by immunohistochemistry. The results showed that the PAstV5 antigen was detected in the ileal epithelial cells (red arrow marker) by the mAb 6C6, while the intestines of the uninfected piglets were negative (Supplementary Fig. S2C).

To identify the specificity to PAstV5 of the generated mAbs, the PAstV5, the porcine deltacoronavirus (PDCoV), the porcine sapelovirus (PSV), and porcine transmissible gastroenteritis virus (TGEV) infected cells were detected by the mAb 6C6 using immunofluorescence assay (IFA) (Supplementary Fig. S2D) and Western blot (WB) (Fig. 1A), respectively. The PAstV5 was identified and could be specifically react with the mAb 6C6.

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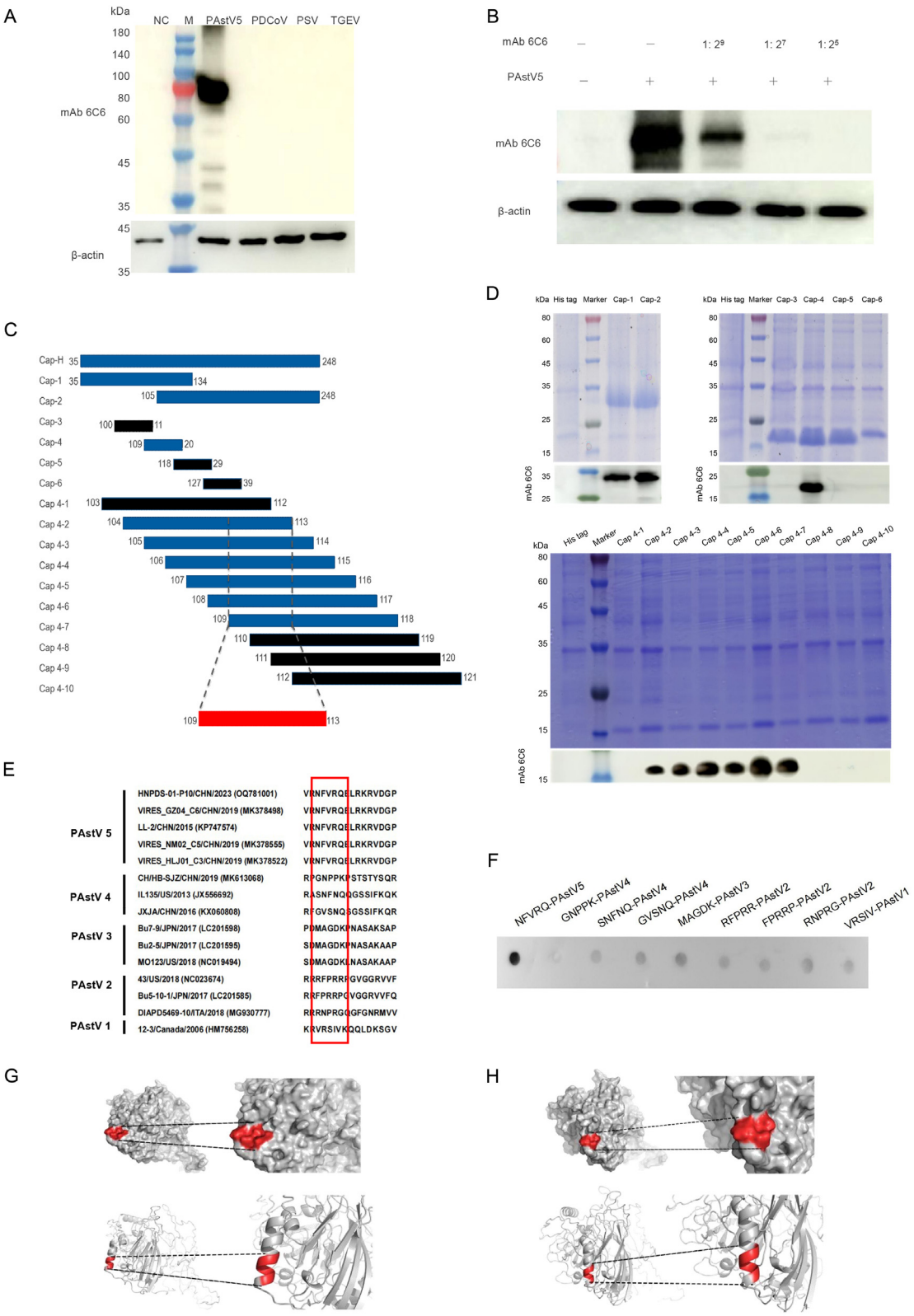

Fig. 1. Mapping and analyzing of the epitope recognized by the mAb 6C6. **A** Identification of mAb 6C6 specificity by WB. **B** LLC-PK1 cells were infected with mixture (100 $TCID_{50}$ PAstV5 and 2-fold dilutions mAb 6C6) and then the PAstV5 Cap amounts were identified with WB method. **C** Schematic diagram of the epitope mapping. The segments that could be recognized by the mAb 6C6 are highlighted in blue. The segments in black are those that could not be recognized by the mAb 6C6. The red area is the smallest epitope region recognized by the mAb 6C6. **D** Expression of the truncated proteins was detected by SDS-PAGE. Binding of the mAb 6C6 with the truncated proteins was determined by WB. His tag as negative controls. **E** Comparison of the 6C6 epitope amino acid sequence among different PAstV strains. Amino acid differences were marked with red boxes. **F** Peptide-Dot Blot was used to detect the reactivity between peptides and mAb 6C6. **G, H** In the 3D structure predicted by the PAstV5 Cap, the relative localization of the identified the mAb 6C6 is highlighted in red on the surface.

Virus neutralization assay was used to detect the neutralizing activity of the mAb 6C6 against PAstV5 by IFA (Supplementary Fig. S2E) and WB (Fig. 1B). The 100 μL of 2-fold serial diluted the mAb 6C6 samples were incubated with 100 μL 100 $TCID_{50}$ of PAstV5 for 1.5 h at 37 °C. Then, the 200 μL PAstV5-mAb 6C6 mixture was inoculation into LLC-PK1 cells. Neutralizing assay revealed that the mAb 6C6 showed potent neutralization against PAstV5.

In order to identify the epitopes on the PAstV5 Cap that mAb 6C6 recognized, the PAstV5 Cap-H gene was truncated into a series of fragments for three rounds (Fig. 1C) using a series of specific primers (Supplementary Table S1). Firstly, the Cap-H was truncated into two overlapped fragments, aa 35–134 and aa 105–248. The result showed the mAb 6C6 recognized both Cap-1 and Cap-2 in WB analysis (Fig. 1D), and we deduced that the epitope binding for the mAb 6C6 located in the region of aa 105–134 of the PAstV5 Cap. In order to ensure the accuracy of the epitope, we supplemented 5 aa before and after aa 105–134 for the secondary truncation. The result showed the mAb 6C6 recognized Cap-4 in WB analysis (Fig. 1D) and the epitope binding for the mAb 6C6 was located in the region of aa 109–120 (Cap-4 fragment) of the PAstV5 Cap. At the last round, the mAb 6C6 recognized Cap4-2 to Cap4-7 in WB analysis (Fig. 1D). The results indicated the minimal antigenic epitope recognized by the mAb 6C6 was amino acids residues ^{109}NFVRQ^{113} of the PAstV5 Cap (Fig. 1C).

In order to assess the conservation of the epitope among different PAstV genotypes, representative PAstV ORF2 sequences were obtained from GenBank and the corresponding parts were aligned by MegAlign software. Notably, this identified epitope was relatively conserved among the PAstV5 strains from East Asia (Fig. 1E). The phylogenetic tree was constructed for PAstV5 ORF2 gene and showed that PAstV1 had the closest evolutionary relationship to PAstV5 and PAstV3 had the most distant evolutionary relationship to PAstV5 (Supplementary Fig. S2F). The artificial synthesized peptides containing the corresponding regions of other genotypes of PAstV1–5 were tested by peptide-ELISA (Supplementary Fig. S2G) and peptide-Dot blot method (Fig. 1F). The results showed that mAb 6C6 specifically target a PAstV5 conserved epitope^{109}NFVRQ^{113}.

To investigate the conformation of the antigenic epitope recognized by mAb 6C6 in detail, the three-dimensional structure of the PAstV5 Cap was predicted using I-TASSER software and edited using PyMOL software, and spatialized the epitope recognized by 6C6 (Fig. 1G).Then, the three-dimensional structure of the protein was rotated horizontally by 90° to better understand its morphology (Fig. 1H). The results showed that the identified epitope recognized by the mAb 6C6 formed an alpha spiral structure and located outside the three-dimensional structure of the protein.

Astrovirus is a type of virus that infects mammals and poultry and has a wide host range. Astrovirus infection in humans can cause gastrointestinal diseases, and there have been a few cases of neurological symptoms. A list of animal species, including humans, cattle, sheep, cats, dogs, deer, chickens, turkeys, and ducks, had been found susceptible to astrovirus infection. The rich genetic diversity among mammalian astroviruses may be caused by the cross-species transmission in wild animals, domestic animals, or humans. Recently, there has been a dramatic increase in astroviruses infected in animal species. Astrovirus infections have been associated with neurological diseases in cattle and pigs (Ykema and Tao, 2021), interstitial nephritis in young chickens (Jin et al., 2021), and hepatitis in ducks (Zhang et al., 2023).

Epidemiological surveys indicate that PAstV is spread worldwide, infecting mainly piglet. PAstV infection is often observed in combination with other viruses and could cause mild diarrhea, growth retardation, small intestinal mucosa damage (Lee et al., 2021). Therefore, effective PAstV prevention strategies are needed to address this major public health issue and burden.

The therapeutic mAb engineering field represents the most promising potential in medicine. As an effective antiviral strategy and diagnostic agent, the mAbs have been used in antibody therapy development, genotype or serotype diagnosis. Although monoclonal antibodies (mAbs) targeting PAstV have been developed in previous studies, research on monoclonal antibodies and antigenic epitope regions specific to PAstV5 remains limited. For example, four monoclonal antibodies targeting the PAstV1 capsid protein have been reported, and two novel B-cell epitopes (^{120}GNNTFG^{125} and ^{485}RISDPIWFSA^{494}) were identified; however, the neutralization capacities of these antibodies have not been fully explored. In contrast, this study developed a monoclonal antibody, mAb 6C6, targeting the PAstV5 Cap, which, after preliminary validation, demonstrated certain neutralization potential. Moreover, this study identified a novel B-cell epitope, ^{109}NFVRQ^{113}, on PAstV5 for the first time.

Currently, there are no commercial vaccines and serum diagnostic products to control PAstV infection. In this study, the mAb 6C6 against the PAstV5 Cap with neutralizing activity was prepared. Further, the minimal unit of B cell epitope recognized was identified. Overall, the development and characterization of the mAb 6C6 against the PAstV5 Cap lays a solid foundation for the study of the biological properties of the Cap, including its antigenicity and regulatory mechanisms, as well as the development of immunodiagnostic reagents and vaccines against PAstV5.

FOOTNOTES

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