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Monoclonal antibody and B-cell epitope mapping of the VP7 protein in bluetongue virus

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Abstract

Bluetongue virus (BTV) VP7 is a group-specific protein that is highly conserved in different serotypes. In this study, BALB/c mice were immunized with purified recombinant BTV-1 VP7 protein expressed in *E. coli*. Then six monoclonal antibodies (mAbs), 2A7, 2B2, 2B3, 2D3, 2D7, and 2H2, against the BTV-1 VP7 protein were produced using hybridoma technology. The reactivity of the mAbs was identified using western blotting, enzyme-linked immunosorbent assay, and immunofluorescence assay. A series of truncated peptides derived from VP7 expressed as glutathione S-transferase fusion proteins were mapped with mAbs by western blotting. The results indicated that 2A7 recognized the epitope ^{71}SAAGINVGPI^{80}, 2B3 recognized ^{110}ARVTGETSTWG^{120}, 2B2 and 2D3 recognized ^{125}PYGFFLETEET^{135}, and 2D7 and 2H2 recognized ^{332}VNPMPGPLTRA^{342}. Amino acid sequence analysis showed that these four epitopes were conserved in 24 typical BTV serotypes. Cross-reaction results showed that mAb 2A7 could recognize the recombinant VP7 protein of BTV-1, African horse sickness virus serotype 1 (AHSV-1), and epidemic hemorrhagic disease virus serotype 1 (EHDV-1). The mAbs 2B2, 2B3, and 2D3 could recognize the recombinant VP7 protein of BTV-1 and EHDV-1, and the mAbs 2D7 and 2H2 specifically recognized the BTV-1 VP7 protein. These specific mAbs and identified B-cell epitopes provided key insights into the structure and function of VP7, while facilitating the development of BTV diagnostics and the design of epitope-based vaccines.

Keywords Bluetongue virus, VP7 protein, Monoclonal antibodies, B-cell epitope mapping

Introduction

Bluetongue (BT) is an infectious, non-contagious, vector-borne viral disease caused by the bluetongue virus (BTV) that affects wild and domestic ruminants such as sheep, goats, cattle, buffaloes, and deer [1]. It is transmitted between vertebrate hosts primarily by *Culicoides* [2]. There are 24 "typical" BTV serotypes and several "atypical" serotypes [3, 4]. To date, up to 36 BTV serotypes have been described [5]. BT is endemic primarily in tropical and subtropical regions between the latitudes of 40°N and 35°S. However, due to climate change, movement of infected animals and vector species, the range of BTV infection is expanding [6–8]. Since the end of the twentieth century, there have been continuous outbreaks of BTV in Europe. Recently, BTV-3 has been epidemic in several European countries such as the United

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Kingdom, the Netherlands, Belgium, France, Germany, Spain, Czech Republic, Switzerland, Denmark and Norway [9–11].

BTV is the type species of the genus *Orbivirus* in the family *Reoviridae* [12], which consists of ten linear dsRNA genome segments encoding seven structural proteins (VP1-VP7) and four nonstructural proteins (NS1, NS2, NS3/NS3 A, NS4) [13, 14]. The VP2 protein binds to surface glycoproteins and promotes clathrin-mediated endocytosis of virions. It is also a major neutralizing antigen and determinant of serotype specificity [15–17]. The VP5 protein penetrates the host cell membrane and delivers the core particle into the host cytosol [18]. The surface layer of the BTV core is formed by VP7, which encloses the VP3 layer, which in turn encloses the viral transcriptase complex (TC), composed of VP1 (RNA-dependent RNA polymerase), VP4 (capping enzyme and transmethylase), and VP6 (RNA dependent ATPase and helicase) [19–23]. Nonstructural proteins are involved in viral replication, assembly, morphogenesis, and release [24–27].

The core surface of BTV is formed of 260 VP7 trimers, each of which consists of two distinct domains. The top domain forms the outer surface of the core and consists of residues 121–249. The bottom domain consists of residues 1–120 and 250–349, which interact with VP3 and also with other trimers of VP7 [20, 28, 29]. In addition to its role in virus assembly, VP7 is a major determinant of serogroup specificity, so it can be used to establish a serological diagnostic method for the detection of BTV antibodies [15]. VP7, as the main component of diagnostic reagents, has been successfully expressed in baculovirus, *E. coli*, yeast, and vaccinia virus [30–33]. Serological diagnosis methods for BTV include enzyme-linked immunosorbent assay (ELISA), virus neutralization, and agar gel immunodiffusion (AGID). Due to cross-reactivity, indirect ELISA and AGID cannot clearly distinguish between BT and EHD serogroups of antibodies [34]. The specificity of mAbs has made competitive-ELISA the primary method for the serological diagnosis of BTV [35].

In this study, the recombinant BTV-1 VP7 protein was expressed in *E. coli* and purified by affinity chromatography. Six mAbs against BTV-1 VP7 protein were prepared and identified. These mAbs recognized four B-cell linear epitopes. Cross-reactivity analysis showed that mAbs 2D7 and 2H2 were able to specifically recognize the VP7 protein of BTV-1, while the mAbs 2B2, 2B3, and 2D3 were able to react with the VP7 protein of BTV-1 and EHDV-1, and the mAbs 2A7 recognized the VP7 protein of BTV-1, AHSV-1, and EHDV-1. This study will lay the foundation for improving serological diagnostics of BTV and the development of epitope-based vaccines.

Materials and methods

Cells, virus, serum and proteins

BHK-21 cells were cultured with Dulbecco's modified Eagle's medium containing 5% fetal bovine serum (FBS, Gibco, USA) at 37 °C in a humidified incubator under 5% CO_2. Mouse myeloma cells (SP2/0) were maintained in RPMI 1640 (Gibco, USA) supplemented with 20% FBS. BTV-1 strain (GS/11), rabbit anti-BTV-1 -positive serum, recombinant VP7 of African horse sickness virus serotype 1 (AHSV-1), and epidemic hemorrhagic disease virus serotype 1 (EHDV-1) expressed in *E. coli* were stored in our laboratory.

Expression and purification of recombinant VP7

The full-length gene encoding BTV-1 VP7 (GenBank accession number: AY776331.1) was optimized according to the *E. coli* codon bias and synthesized by GeneCreate Biological Engineering Co., Ltd. (Wuhan, China). The gene was then ligated into a pET-28a plasmid by using restriction endonuclease sites *Bam*HI and *Xho*I and named pET-BTVVP7. The recombinant plasmid pET-BTVVP7 was transformed into *E.* coli BL21 (DE3) competent cells (TransGen Biotech, Beijing, China) for protein expression by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The recombinant protein VP7 (rec-VP7) was expressed at 37 °C, and the expression products were collected at intervals of 2 h after IPTG induction for SDS-PAGE analysis. The expression product was purified at 8 h using Ni–NTA Agarose (QIAGEN, Germany). The purified proteins were analyzed with SDS-PAGE and identified using anti-6 ×His tag monoclonal antibody (Proteintech, Wuhan, China,) and rabbit anti-BTV-1 serum.

Preparation of monoclonal antibody against VP7

Six-week-old female BALB/c mice were immunized using a subcutaneous multi-point injection in the back. The first immunization was purified rec-VP7 at 50 μg/mouse mixed with an equal volume of complete Freund's adjuvant. Booster immunization was performed every 2 weeks using purified rec-VP7 mixed with an equal volume of incomplete Freund's adjuvant. At 14 days after the third immunization, mice blood was collected and antibody titers were determined by indirect ELISA. The mice with the highest antibody titer were intraperitoneally injected with 50 μg rec-VP7. After 3 days, the splenocytes of the mice were isolated and fused with SP2/0 myeloma cells using polyethylene glycol (Sigma, USA). The fused cells were screened by using HAT selection medium in a 96-well plate. Seven days later, the fused cells were cultured with HT medium. When the hybridoma cells had grown to more than one-third of the bottom area of each well, the supernatant of the hybridoma cells was analyzed

with indirect ELISA. The positive hybridoma cells were subcloned 3 ~ 5 times using the limited dilution method. In addition, the isotypes of the mAbs were identified using a mouse monoclonal antibody isotyping kit according to the manufacturer's instructions.

Indirect ELISA

To determine the reactivity of the mAbs, 96-well plates were coated with rec-VP7 in 0.05 M carbonate buffer (pH 9.6) at 4℃ overnight and blocked with 5% skimmed milk for 1 h at 37℃. After washing three times with PBST, 100 µL of hybridoma supernatant was added to the plates and then incubated at 37℃ for 1 h. After three washes, a 1:10 000 dilution of HRP-conjugated goat anti-mouse IgG (Abcam, UK) was added to the plate at 100 µL each well and incubated at 37 °C for 1 h. Following three washes with PBST,100 µL chromogenic substrate solution (TMB) was added and incubated for 10 min. The reaction was stopped with 2 M H_2SO_4, and the optical density was measured at 450 nm using a Multiskan SkyHigh full-wavelength Microplate Reader. An OD_{450} value exceeding 2.1-fold of the negative controls was considered a positive result.

Western blotting

To analyze the reactivity of mAbs with rec-VP7 and natural VP7 in BTV-1-infected BHK-21 cells, VP7 was separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk in PBST at 37℃ for 1 h. Then, the membranes were incubated with hybridoma supernatants in a blocking buffer at 4℃ overnight. After washing three times with PBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:10 000, Abcam, UK) at 37℃ for 1 h. After three washes with PBST, enhanced chemiluminescence (ECL) substrate (ThermoFisher, USA) was added to the membrane and the protein signal was visualized and captured by using a ChemiDoc XRS + Imaging System.

Immunofluorescence assay

BHK-21 cells were infected with BTV-1 at a multiplicity of infection of 0.1 for 24 h. The cells were fixed with precooled 4% paraformaldehyde for 10 min, permeabilized by 0.1% Triton X-100 in PBS for 10 min, and then blocked with 3% BSA for 1 h. The cells were incubated with hybridoma supernatant as primary antibodies for 1 h at room temperature. After washing three times with PBS, cells were incubated with Alexa Fluor488-conjugated goat anti-mouse IgG (1:1 000, Abcam, UK) for 1 h. The cell nuclei were stained with Hoechst 33,342 solution for 10 min. The stained cells were visualized under a Leica TCS SP8 laser scanning confocal microscope.

Mapping of the linear B-cell epitope of VP7

The VP7 sequence was separated into three fragments for cloning and expression to test reactivity with the mAbs. Then, after two rounds of truncation, the reactive peptides were shortened to 8, 10, or 11 amino acids (Fig. 1). A series of overlapping and truncated fragments were cloned into a pGEX-6p-1 expression vector by using restriction endonuclease sites *EcoR* I and *Xho* I and then expressed as GST-containing fusion proteins in *E. coli* BL21 (DE3). The reactivity of peptides with mAbs was analyzed using western blotting.

Conservation analysis of the identified epitopes

To verify the conservation of the identified VP7 epitope, the VP7 sequences of three orbiviruses including BTV, AHSV, and EHDV were downloaded from GenBank. The amino acid sequence alignment and conservation analysis was implemented by BioEdit software. The spatial distribution and structure of the identified epitope in VP7 (PDB: 1BVP) were visualized by PyMOL. In addition, western blotting was used to analyze the cross-reactivity between the mAbs and the recombinant AHSV and EHDV VP7 proteins expressed in *E. coli*.

Results

Expression and purification of recombinant VP7

The recombinant plasmid pET-BTVVP7 was transformed into *E. coli* BL21 (DE3) competent cells for protein expression. The bands resulting from SDS-PAGE showed that the rec-VP7 protein had a molecular weight of approximately 40 KDa (Fig. 2A). Solubility analysis showed that the rec-VP7 was mainly present in the precipitate after ultra-sonication and centrifugation (Fig. 2B). The rec-VP7 was purified from the precipitate by Ni–NTA Agarose and the results were confirmed by SDS-PAGE (Fig. 2C). Western blotting verified that the purified rec-VP7 was recognized by anti-6 × His tag mAb (Fig. 2D) and anti-BTV-1 rabbit positive serum (Fig. 2E).

Preparation and characterization of mAbs against VP7

Positive clones of hybridoma cells were screened by indirect ELISA based on rec-VP7. After three rounds of screening and subcloning, six hybridoma cell clones, 2A7, 2B2, 2B3, 2D3, 2D7, and 2H2, were generated (Fig. 3A). The reactivity of the six mAbs was tested using western blotting and immunofluorescence assay. The western blot results showed that six mAbs could react with rec-VP7 and natural VP7 in BTV-1-infected BHK-21 cells (Fig. 3B). Immunofluorescence assays showed that six mAbs were also able to react with natural VP7 in BTV-1-infected BHK-21 cells (Fig. 3C). Isotype determination showed that 2A7 and 2D7 were subclass IgG2b, 2B2,

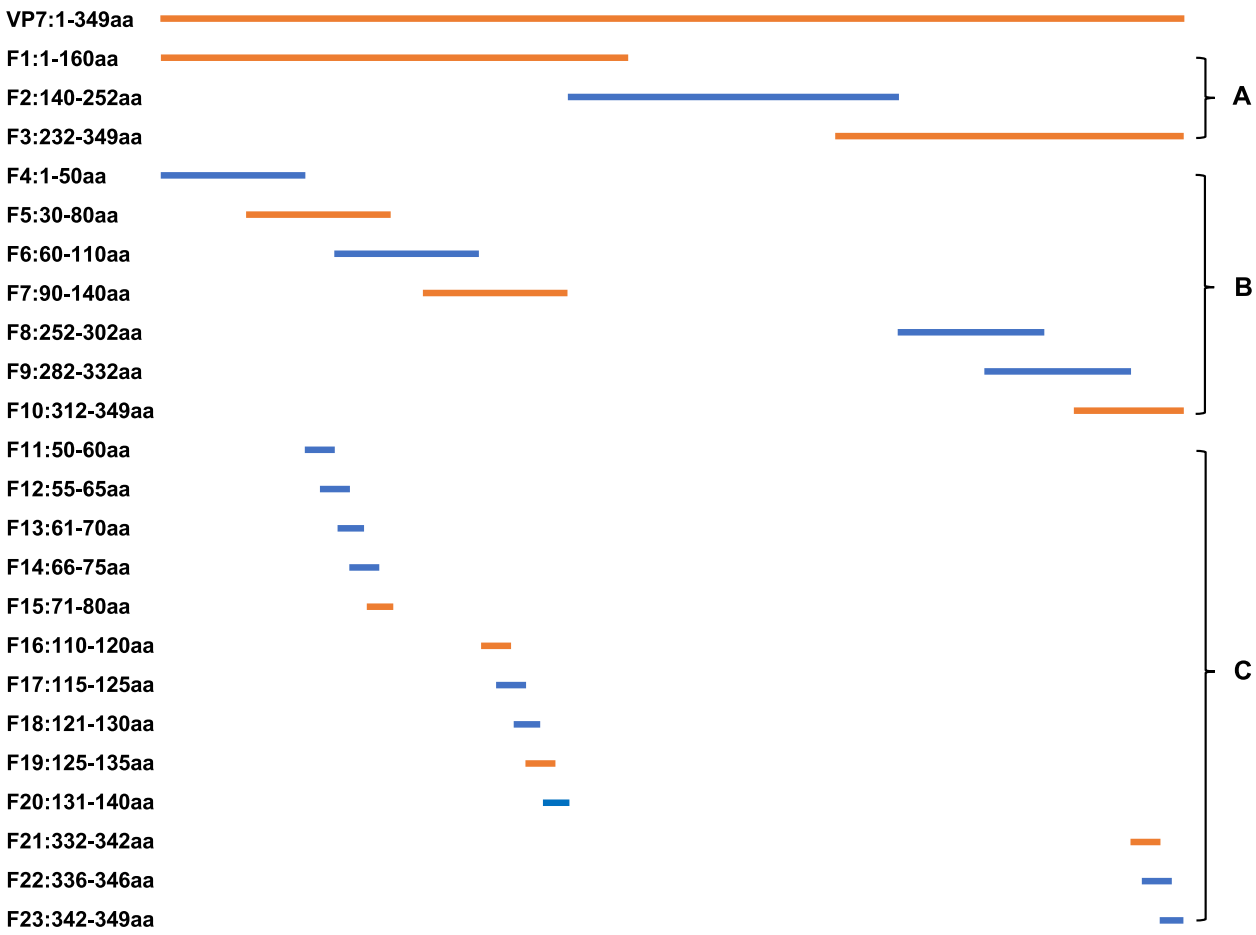

Fig. 1 Schematic diagram of VP7 epitope mapping strategy. The fragments recognized by mAbs 2A7, 2B2, 2B3, 2D3, 2D7, and 2H2 are represented by orange lines, and the fragments that are not recognized by any mAb are represented by blue lines. **A**, **B**, and **C** represent three rounds of truncation

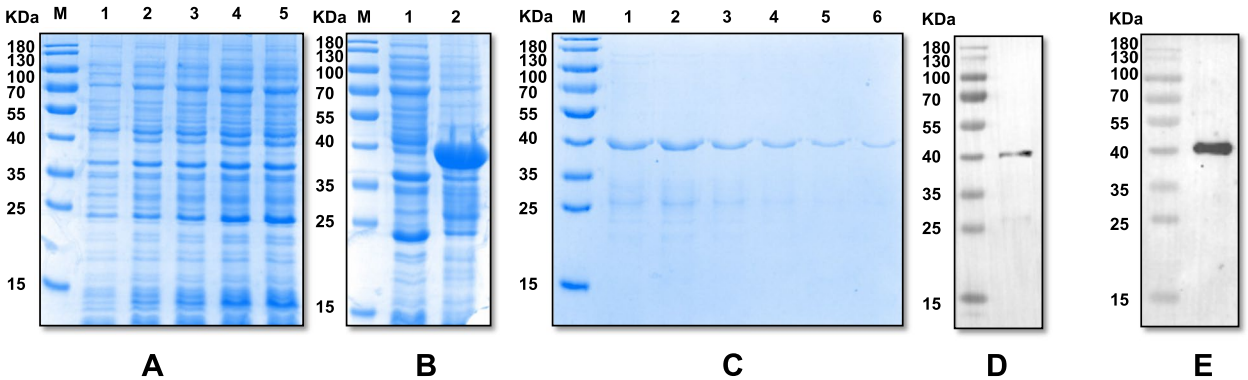

Fig. 2 Expression, purification, and identification of recombinant BTV VP7. **A** SDS-PAGE identification of the expression of VP7. Lane 1: Uninduced bacteria; lanes 2–5: Bacteria induced for 2, 4, 6, and 8 h. **B** Solubility analysis of recombinant VP7. Lane 1: VP7 in supernatant after ultra-sonic treatment; lane 2: VP7 in precipitate after ultra-sonic treatment. **C** Purified VP7 verified by SDS-PAGE. Recombinant VP7 was identified by anti-6 × His tag mAb (**D**) and rabbit anti-BTV-1 positive serum (**E**)

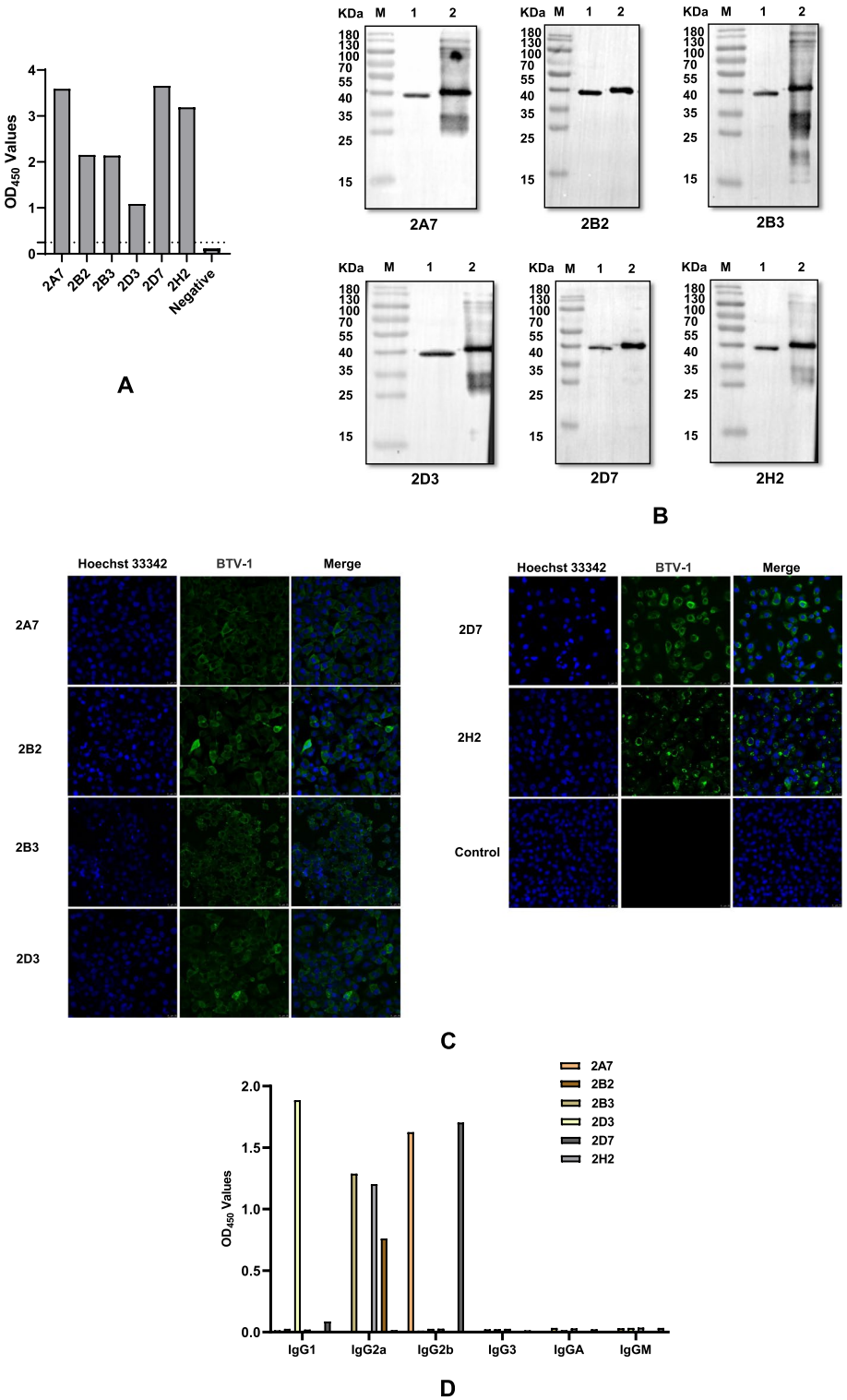

Fig. 3 Characterization of the mAbs. **A** The reactivity of mAbs was analyzed with indirect ELISA. The dotted line is 2.1-fold that of the negative control. **B** The reactivity of monoclonal antibodies was analyzed using western blotting. Lane1: BHK-21 cells infected with BTV-1; Lane 2: Recombinant VP7. **C** Immunofluorescence analysis of the mAbs. BHK-21 cells infected with BTV-1 were subjected to immunofluorescence assay at 24 h. Alexa Fluor488-conjugated goat anti-mouse IgG was used as a secondary antibody. **D** Isotype identification of mAbs

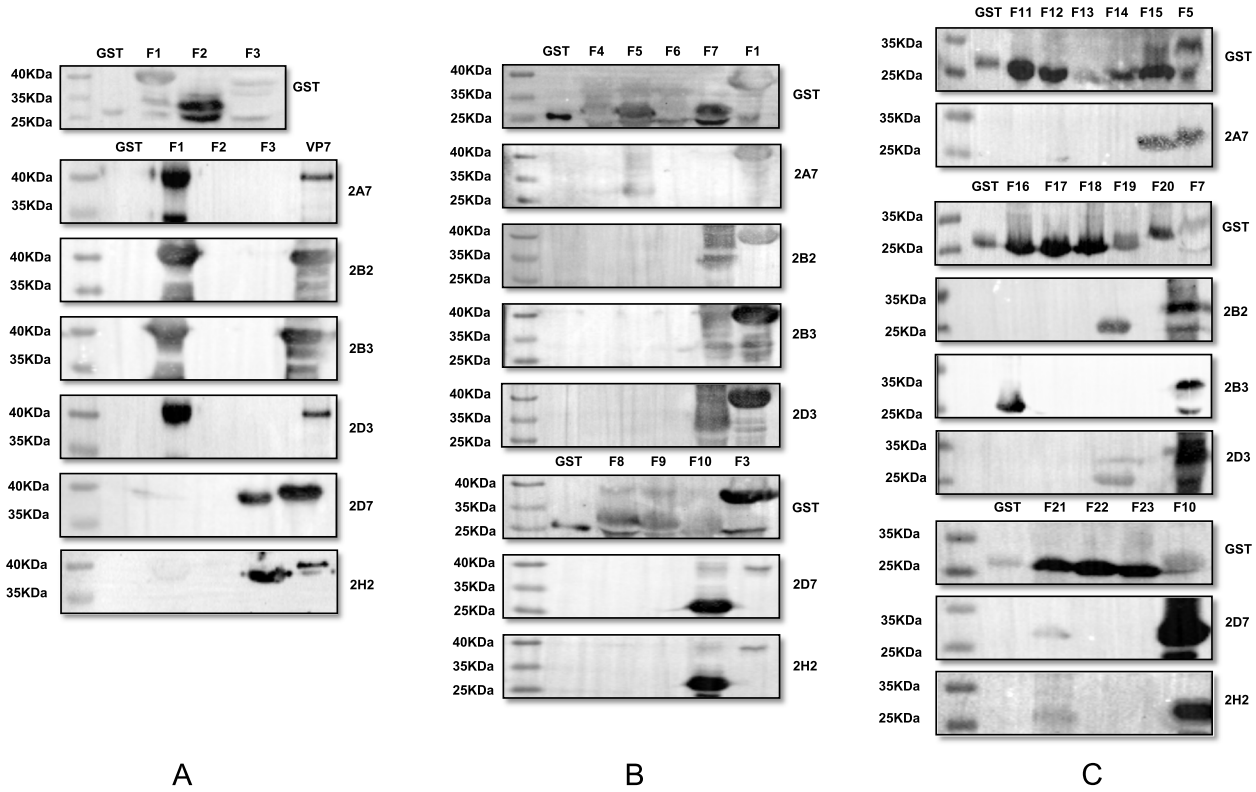

Fig. 4 Fine-mapping of the epitopes with mAbs. Primary (**A**), secondary (**B**), and tertiary (**C**) VP7 fusion proteins truncated by the prokaryotic expression were screened to show the antigenic epitopes of the six mAbs by western blotting. The empty vector pGEX-6p-1-GST and full-length VP7 were used as the negative and positive controls, respectively

2B3 and 2H2 were subclass IgG2a, and 2D3 was subclass IgG1 (Fig. 3D).

Mapping of the epitopes recognized by the 6 mAbs

To identify the epitopes recognized by mAbs, VP7 was first truncated into three fragments and named F1 (1–160 aa), F2 (140–252 aa), and F3 (232–349 aa). Western blotting results showed that 2A7, 2B2, 2B3, and 2D3 recognized the F1 fragment, 2D7 and 2H2 recognized the F3 fragment (Fig. 4A). To further determine the epitopes recognized by these six mAbs, two rounds of truncation were performed based on the F1 and F3 fragments and validated with western blotting. The results showed that the epitope recognized by 2A7 was located in the 71–80 aa region, the epitope recognized by 2B3 was located in the 110–120 aa region, the epitope recognized by 2B2 and 2D3 was located in the 125–135 aa region, the epitope recognized by 2D7 and 2H2 was located in the 332–349 aa region (Fig. 4B and C).

Homology and cross-reactivity analysis

To evaluate the conservation of epitopes in orbiviruses, the VP7 amino acid sequences of BTV, AHSV, and EHDV were downloaded from GenBank and analyzed using BioEdit software. The alignment results showed that the epitopes ^{71}SAAGINVGPI^{80}, ^{110}ARVTGETSTWG^{120}, and ^{332}VNPMPGPLTRA^{342} were conserved in the typical serotypes of BTV (except for BTV-15 and BTV-19), and the amino acid "T" at the position 135 of epitope ^{125}PYGFFLETEET^{135} was replaced by a "V" in individual serotypes (Fig. 5). The four epitopes were not conserved in AHSV and EHDV. However, cross-reactivity analysis showed that the mAb 2A7 was able to react with the rec-VP7 protein of BTV, AHSV, and EHDV. The mAbs 2B2, 2B3, and 2D3 were able to react with the rec-VP7 protein of BTV and EHDV. The mAbs 2D7 and 2H2 only recognized BTV VP7 specifically (Fig. 6).

Bioinformatic analysis of the BTV VP7

The 3D structural models of BTV-1 VP7, AHSV-1 VP7, and EHDV-1 VP7 were predicted using SWISS-MODEL (https://swissmodel.expasy.org/) with 1BVP templates. Structure analysis showed that the antigenic epitopes of 2B2 and 2D3 were mapped onto the core of the top domain, and those of 2A7, 2B3, 2D7, and 2H2 were mapped onto the bottom domain. The epitope ^{71}SAAGINVGPI^{80} was located at the N-terminus, and the epitope ^{332}VNPMPGPLTRA^{342} was located at the

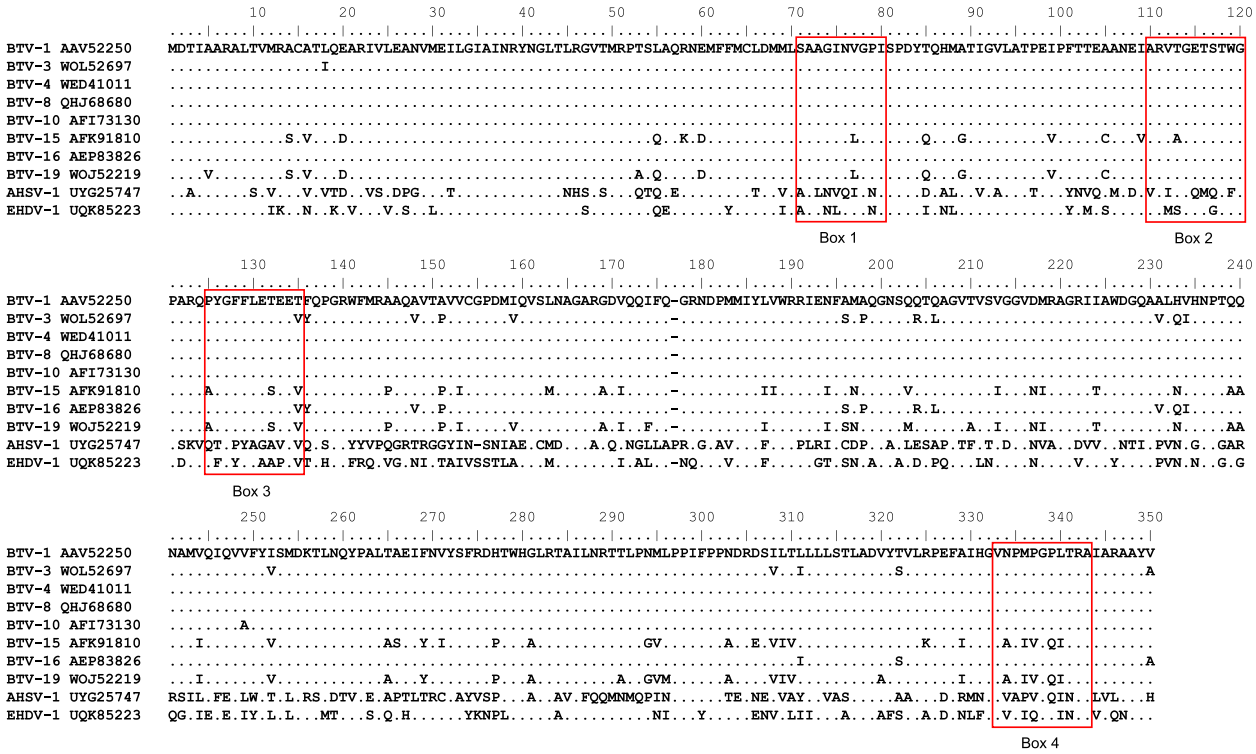

Fig. 5 Sequence alignment of VP7 of three orbiviruses. The epitope [71]SAAGINVGPI[80] (Box 1) identified by 2A7, the epitope [110]ARVTGETSTWG[120] (Box 2) identified by 2B3, the epitope [125]PYGFFLETEET[135] (Box 3) identified by 2B2 and 2D3, and the epitope [332]VNPMPGPLTRA[342] (Box 4) identified by 2D7 and 2H2 are circled with red. Black dots indicate the same amino acids, and the dotted lines indicate the gap regions

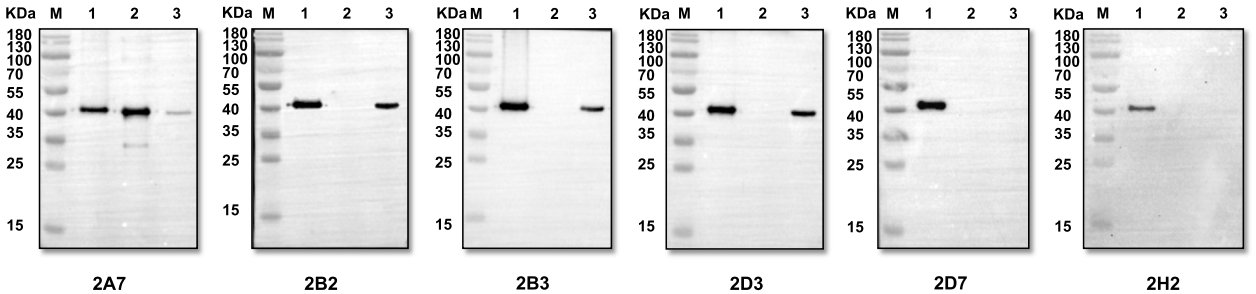

Fig. 6 Cross-reactivity assays between mAb and AHSV and EHDV VP7 proteins. Reaction of mAbs 2A7, 2B2, 2B3, 2D3, 2D7, and 2H2 with the VP7 of three orbiviruses. Lane 1: rec-BTV VP7, lane 2: rec-AHSV VP7, lane 3: rec-EHDV VP7

C-terminus, and they were fully exposed to the surface of the trimer (Fig. 7A). Models of BTV-1 VP7, AHSV-1 VP7, and EHDV-1 VP7 were superimposed on each other, and the results showed that their structures were highly similar (Fig. 7B).

Discussion

VP7 is a highly conserved immunodominant protein within the genus Orbivirus of the family *Reoviridae*, which is the target of group-specific diagnosis [36]. BTV and EHDV are closely related orbiviruses with structural, antigenic, and molecular similarities, both are transmitted by the bite of hematophagous insects (*Culicoides*) and often co-transmitted geographically, causing similar clinical manifestations in susceptible animals [37]. The cross-reactivity between members of the BT and EHD serogroups raises the possibility that an isolate of EHDV could be mistaken for BTV [38]. Due to the similarity of these two viral antigens, the diagnosis of infection is often confounded [36, 37]. The competitive ELISA method based on specific monoclonal antibodies can be used for the detection of BTV and EHDV serogroup-specific

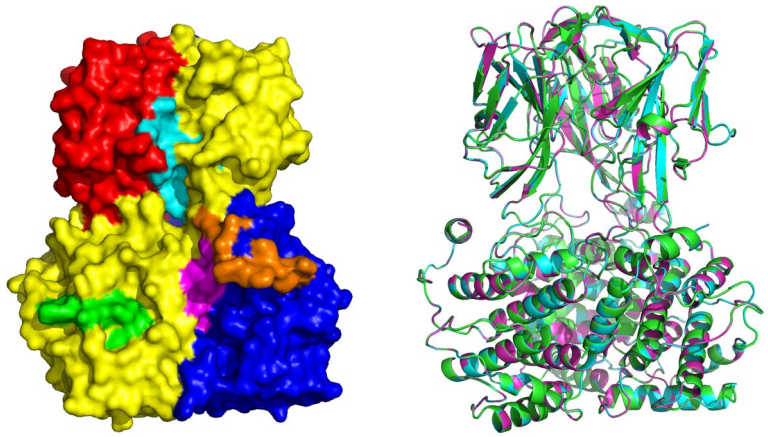

Fig. 7 Structural analyses of VP7. **A** VP7 structure and four epitopes recognized by mAbs. Red, yellow, and blue represent monomer structures of VP7. The epitopes ^{71}SAAGINVGPI^{80}, ^{110}ARVTGETSTWG^{120}, ^{125}PYGFFLETEET^{135}, and ^{332}VNPMPGPLTRA^{342} are colored in green, magenta, cyan, and orange, respectively. **B** The VP7 models of BTV (blue), AHSV (green), and EHDV (magenta) have been superimposed on to each other to show structural similarity

antibodies, respectively, to distinguish between BTV and EHDV infection [39].

Previous studies have shown that some mAbs binding to conformational or linear epitopes were able to be used as a blocking antibody for BTV diagnosis [40–43]. In this study, we inserted the BTV VP7 gene into the pET28a vector and expressed the rec-VP7 protein in *E. coli*. The rec-VP7 protein was purified and inoculated into BALB/c mice to produce mAbs against the BTV VP7 protein. ELISA, western blot, and immunofluorescence assay were used to screen and identify the hybridoma cells secreting antibodies. Six mAbs with excellent reactivity were obtained. These mAbs will provide alternative biomaterials for further research on developing BTV diagnosis methods.

To further investigate the corresponding antigenic epitopes of these monoclonal antibodies on the BTV VP7 protein, the epitopes of mAbs were mapped using western blot analysis. The BTV VP7 gene was artificially divided into 24 different overlapping truncated segments and expressed in *E. coli* as a GST-tagged peptide. Four linear epitopes ^{71}SAAGINVGPI^{80}, ^{110}ARVTGETSTWG^{120}, ^{125}PYGFFLETEET^{135}, and ^{332}VNPMPGPLTRA^{342} were identified on the VP7 protein. The 3D structure showed that the epitopes ^{71}SAAGINVGPI^{80} and ^{332}VNPMPGPLTRA^{342} were accessible at the surface of the VP7 trimer, and the epitopes ^{110}ARVTGETSTWG^{120} and ^{125}PYGFFLETEET^{135} were located in the center of the top and bottom domains, respectively. In other studies, mAbs have been used to identify the binding epitopes of BTV VP7. These epitopes have been identified according to the prepared mAbs, which recognize both conformational and linear epitopes [41, 44]. This study has identified four antigenic epitopes on BTV VP7 using monoclonal antibodies, which will contribute to the design of peptide-based and subunit vaccines against BTV.

Analysis of the amino acid sequences of 24 typical bluetongue virus serotypes revealed that the amino acid sequences of epitopes ^{71}SAAGINVGPI^{80}, ^{110}ARVTGETSTWG^{120}, and ^{332}VNPMPGPLTRA^{342} were almost conserved. The results of amino acid sequence analysis showed that the four epitopes identified by mAbs were not completely conserved in VP7 of AHSV and EHDV compared with BTV VP7. Cross-reactivity results showed that 2A7 reacted with VP7 of BTV, AHSV, and EHDV. 2B2, 2B3, and 2D3 reacted with VP7 of BTV and EHDV, whereas 2D7 and 2H2 reacted only with VP7 of BTV. Based on these results, it has been inferred that mAbs 2D7 and 2H2 can be used as candidate antibodies for serological differential diagnostic methods of BTV and EHDV.

Conclusion

In summary, this study generated six monoclonal antibodies against the BTV VP7 protein and mapped their corresponding B-cell antigen epitopes. Further studies have shown that 2A7 reacted with VP7 of BTV, AHSV, and EHDV. 2B2, 2B3, and 2D3 reacted with VP7 of BTV and EHDV, whereas 2D7 and 2H2 reacted only with VP7 of BTV. This will not only provide a basis for further understanding the structure and function of BTV VP7 but will also provide data for the establishment of diagnostic methods and the development of epitope-based peptide vaccines for orbiviruses.

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Authors' contributions
X-BH performed experiments, collected and analyzed the date, and drafted the manuscript. JX, YZ and F-HM participated in the experiments. Z-CT and J-XL provided resources and supervised the experiments. G-GQ and HY revised the manuscript and acquired funding. J-ZD designed the study, revised the manuscript, and acquired funding. All authors read and approved the final manuscript.

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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 were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the study was approved by The Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Permit No. LVRIAEC-2022–081).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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