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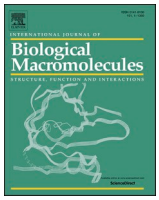

Identification of a novel B cell epitope on the nucleocapsid protein of infectious bronchitis virus

Ran Jing [a,b,d], Jiaru Zhou [a,b], Changcheng Liu [a,b], Mengdi Zhang [a,b], Wenlong Zhu [a,b], Hua Cao [a,b], Bo Hou [c], Hongbo Zhou [a,b], Wentao Li [a,b,d,e,*], Mengjia Zhang [a,b,*]

[a] National Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, China
[b] Key Laboratory of Preventive Veterinary Medicine in Hubei Province, the Cooperative Innovation Center for Sustainable Pig Production, Wuhan, China
[c] Fujian Academy of Agricultural Sciences, Fuzhou, Fujian, China
[d] Frontiers Science Center for Animal Breeding and Sustainable Production, Wuhan, Hubei, 430070, China
[e] Hubei Jiangxia Laboratory, Wuhan, China

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ABSTRACT

Infectious bronchitis (IB) is a highly contagious, acute respiratory disease caused by infectious bronchitis virus (IBV). The coronavirus nucleocapsid protein, essential for viral replication, is highly conserved and strongly antigenic. In the present study, the recombinant IBV N protein expressed via a prokaryotic expression system was employed as an antigen to develop two anti-IBV N monoclonal antibodies (mAbs), 6D1 and 6D2. The mAbs exhibited high specificity for IBV and strongly bound to the IBV N protein, with EC_{50} values of 7.385 ng/mL and 9.994 ng/mL, respectively. Both antibodies are IgG1 subtypes with kappa light chains. In addition, these two mAbs recognized IBV N protein in *pcDNA3*-V5-IBV-N-transfected cells and IBV-infected cells. Further investigation identified the motif ^{218}KADEMAHRR^{226} as the novel minimal linear B-cell epitope recognized by mAbs. Homology analysis showed that this linear epitope is highly conserved among different genotypes, and Western blot and IFA experiments confirmed that these two mAbs can detect the N protein in CEF cells infected with different IBV genotypes. Identifying novel linear epitopes of the IBV N protein is crucial for the development of specific and sensitive diagnostic tools, vaccine development and immune mechanism research, offering new strategies and tools for preventing and controlling IBV infection.

1. Introduction

Avian infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), affects the avian respiratory and urogenital systems and is characterized by tracheal rales, sneezing and coughing [1]. IBV is highly contagious and can lead to high mortality in chicks, reduced feed conversion, a lower growth rate in broilers, and decreased egg production and quality in laying hens, resulting in considerable economic losses globally. First identified in North Dakota, USA, in 1930, IB was reported by Shalk and Hawn in 1931 and soon spread to all major poultry-producing countries. Beach and Schal conclusively determined the viral etiology of the disease until 1936. In 1937, Beaudette and Hudson successfully isolated IBV for the first time by inoculating homogenized tracheal tissue from infected chickens into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs [2,3].

IBV is a representative member of the Gamma Coronavirus family within the *Coronaviridae* family [4]. It possesses a 27 kb single-stranded positive-sense RNA genome, featuring a 5'cap and a 3'poly(A) tail [5]. The genome consists of 5'UTR-1ab-S-3a/3b/*E*-M-5a/5b-N-3'UTR, encoding four structural proteins, namely, spike (S), envelope (E), membrane (M), and nucleocapsid (N), and fifteen nonstructural proteins derived from the precursor polyproteins encoded by the ORF1a and ORF1ab polyproteins [6]. The N gene in the viral genome encodes a phosphorylated protein that has remained relatively conserved throughout evolution [7,8]. The genomic RNA of coronaviruses is replicated within the host cell via a replicase complex and transcribed into multiple subgenomic mRNAs through a discontinuous transcription mechanism. Among these transcripts, the mRNA encoding the N protein is the most abundant [9]. Given the distinct replication and transcription modalities of coronaviruses, the production level of the IBV N protein

* Corresponding authors at: National Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, China.
E-mail addresses: wentao@mail.hzau.edu.cn (W. Li), zhangmengjia0210@mail.hzau.edu.cn (M. Zhang).

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markedly exceeds that of the S protein, with the N protein being approximately six times more abundant than the S protein during viral infection. This disparity in protein yield highlights the pivotal role of the N protein in both viral replication and assembly processes [10,11]. The S protein of IBV contains hypervariable regions, rendering it highly susceptible to mutations [12]. In contrast, the N protein is highly conserved among different IBV genotypes. Antibodies targeting the IBV N protein can recognize multiple genotypes of the virus [13]. In addition to its conservation, the N protein of IBV exhibits robust immunogenicity and is capable of eliciting both specific antibody responses and cytotoxic T-cell-mediated reactions [14,15]. Following live vaccination, S1, S2 and N proteins evoked equivalent, robust antibody titers, whereas the M glycoprotein elicited a markedly attenuated response. S1-, S2- and N-specific antibodies were first detectable at 2 weeks post-vaccination, attained significantly higher titers in chicks vaccinated at 14 days of age than in those vaccinated at 1 or 7 days, and peaked 4 weeks after the booster dose [16]. The IBV N protein has garnered significant attention as a diagnostic target because its antigenic properties are suitable for the development of diagnostic methods capable of detecting IBV infection.

Identifying B-cell epitopes is vital for the development of epitope vaccines, diagnostic tools, and therapeutic antibodies [17–20]. B-cell epitopes are specific regions on the surface of an antigen that are recognized by B-cell receptors or antibodies, initiating immune responses [21]. While numerous epitopes may be present on antigens, only those that adhere to the structural and positional criteria of B-cell epitopes are recognized by B cells [22,23]. The high mutation rate of the IBV S1 gene has led to new genotypes and serotypes, posing significant challenges for the prevention and control of IB due to the limited cross-protection. Recent studies have focused on identifying B-cell epitopes within the conserved N protein. Several linear B-cell epitopes have been reported, including the sequence “FGPRTK” (positions 242–247) on the IBV N protein, as well as the highly conserved epitope “IPLNRGRGGRST” (positions 158–169) and the epitope “DSPAPIIKLGG” (positions 11–21) [24–27].

This study expressed the recombinant IBV N protein via a prokaryotic system and generated two mAbs, 6D1 and 6D2, specific to the IBV N protein. These two mAbs demonstrated strong reactivity with the recombinant IBV N protein, with EC_{50} values of 7.385 ng/mL and 9.994 ng/mL, respectively. Both antibodies were identified as the IgG1 subtype with kappa light chains. Western blot and IFA confirmed that these two mAbs recognized the IBV-N protein both in *pcDNA3*-V5-IBV-N plasmid-transfected cells and in IBV-infected cells. Competition binding assays via biolayer interferometry revealed that the two mAbs share identical epitopes, and ^{218}KADEMAHRR^{226} was identified as the linear B-cell epitope by a series of truncated IBV N proteins. Notably, homology analysis revealed that the linear epitope is highly conserved among different genotypes. Western blotting and IFA revealed that these two mAbs can detect the N protein in CEF cells infected with different IBV genotypes. These findings offer valuable tools for clinical detection, etiological studies, and epidemiological research on IBV and suggest new avenues for developing epitope vaccines and antiviral strategies.

2. Materials and methods

2.1. Cells, viruses and animals

Vero CCL81 (ATCC CRL-1587) cells and chicken embryo fibroblast (CEF) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, 11965092, Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5 % CO_2. The myeloma cell line (SP2/0, ATCC CRL-1581) and the hybrid cells producing mAbs were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI 1640, 11875093, Gibco, USA) supplemented with 20 % FBS. The IBV strain Beaudette CK (GenBank accession number: AJ311317.1) was preserved in our laboratory. The virus was propagated in Vero CCL81 cells in DMEM supplemented with 1 % (*v*/v) FBS. Six-week-old female BALB/c mice were purchased from the Laboratory Animal Center, Huazhong Agricultural University, with approval number 202502170001, and housed in SPF isolators ventilated under negative pressure. All animal care and procedures were conducted in accordance with animal ethics guidelines and approved protocols.

2.2. Expression and purification of the recombinant IBV N protein

To obtain the recombinant IBV N protein in a prokaryotic expression system, the entire ORF of the IBV N gene was amplified via the primers N-F: 5′- GAATTCGAGCTCCGTCGAATGGCAAGCGGTAAAGCA −3′ (containing an EcoR I digestible site) and N-R: 5′-CTCGAGTGCGGCCGCAAGAAGTTCATTCTCTCCTAGAG-3′ (containing a *Xho* I site). After digestion, the PCR product was cloned and inserted into the *Eco*RI and *Xho*I sites of the pET-28a (+) vector. The recombinant plasmid was subsequently successfully identified via Sanger sequencing and transformed into *Escherichia coli* Rosetta (DE3) cells. Recombinant His-IBV-N protein was expressed in Luria–Bertani (LB) medium upon induction with 0.7 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 h at 37 °C. The purified protein was then subjected to SDS–PAGE and Western blotting with an anti-His tag antibody (AE086, ABclonal, China).

2.3. Preparation of monoclonal antibodies against the IBV-N protein

Monoclonal antibodies against the IBV N protein were generated by immunizing four six-week-old female BALB/c mice with 200 μg of purified His-IBV-N protein emulsified in complete Freund’s adjuvant (F5881; Sigma, USA). After the initial immunization, the mice received booster immunization with the same dose every two weeks via incomplete Freund’s adjuvant (F5506; Sigma, USA). Serum samples were collected for monitoring antibody levels via indirect enzyme-linked immunosorbent assay (ELISA). Two weeks after the last boost, the mice were injected with 50 μg of recombinant protein for three consecutive days. The mice were then euthanized, and the splenocyte single-cell suspension was fused with SP2/0 myeloma cells in 96-well plates at a 10:1 ratio via polyethylene glycol (PEG 4000, 95904; Sigma, USA). Hybridomas were selected in RPMI-1640 with Hypoxanthine-Aminopterin-Thymidin (HAT) media supplement (H0262, Sigma, USA) supplemented with 20 % FBS for one week. Hybridomas were screened by Western blot analysis and indirect ELISA. Positive hybridomas were cultured with hypoxanthine-thymidin (HT) media supplement (H0137, Sigma, USA) supplemented with 20 % FBS and subcloned by limiting dilution three times. The mice were intraperitoneally injected with liquid paraffin to stimulate an immune response in the peritoneal cavity. Following sensitization, hybridoma cells were resuspended in serum-free medium to achieve an optimal cell density. After 10 days, ascites was collected via paracentesis. The subtype of the monoclonal antibody was determined via an isotyping ELISA kit (BF06002X, Biodragon, China).

2.4. Indirect enzyme-linked immunosorbent assay (ELISA)

One hundred microliters of purified His-IBV-N protein at 2 μg/mL was coated onto the wells overnight in coating buffer at 4 °C. The plate was blocked with 5 % skim milk for 2 h at 37 °C. Then, 100 μL of the supernatant from hybridoma cells was added as a primary antibody to each well for 1 h at 37 °C. After washing with phosphate-buffered saline (PBS) buffer containing 0.05 % Tween-20, 100 μL of HRP-conjugated goat anti-mouse IgG (H + L) antibody (A5003, ABclonal, China) was added, and the mixture was incubated at room temperature (RT) for 1 h. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB, 501129758, Thermo Fisher Scientific, USA) substrate solution was added, followed by incubation with H_2SO_4 (2 M) to stop the reaction. The absorbance was measured at $OD_{450\ nm}$ via a Model 680 Microplate Reader (Bio-Rad, USA).

Table 1
Sequences of the primers used in this study.

Primer name	Primer sequences (5′-3′)	Position in N gene	Fragment size (bp)
IBV N-F	GAATTCGAGCTCCGTCGAATGGCAAGCGGTAAAGCA	1–1227	1227
IBV N-R	CTCGAGTGCGGCCGCAAGAAGTTCATTCTCTCCTAGAG		
IBV NA-F	GATCCGAATTCGAGCTCCGTCGAATGGCAAGCGGTAAAGCAGCTGGAAA	1–333	333
IBV NA-R	TGCTCGAGTGCGGCCGCAAGATCTTGAGTATCACCCCAGTTCAGGTCA		
IBV NB-F	GAATTCGAGCTCCGTCGAATGGGAACAGGACCTGCCGCTGACCTG	289–666	378
IBV NB-R	TGCTCGAGTGCGGCCGCAAGCATTTCATCTGCCTTTGCCTTGGTAATGCG		
IBV NC-F	GAATTCGAGCTCCGTCGAATGCAGAAAAAGGGCTCTCGCATTACCAAG	622–942	321
IBV NC-R	TGCTCGAGTGCGGCCGCAAGATCAAACTGCGGGTCATCACATGG		
IBV ND-F	GAATTCGAGCTCCGTCGAATGTTTGAATTTACTACTGTGGTCCCATGTG	898–1227	330
IBV ND-R	GTGCTCGAGTGCGGCCGCAAGAAGTTCATTCTCTCCTAGAGCTGCATC		
IBV NC1-F	GCCCCTGGGATCCCAGAAAAAGGGCTCTCGCAT	622–720	99
IBV NC1-R	CCCGGGAATTCCGGTTGATCAACCCTATAATTAGGTGGGATAGTG		
IBV NC2-F	GGGGCCCCTGGGATCCCCACCTAATTATAGGGTTGATCAAGTGT	697–792	96
IBV NC2-R	CCCGGGAATTCCGGCTTAATACCTTCCTCATTCATCTTGTCATCA		
IBV NC3-F	GGCCCCTGGGATCCATGAATGAGGAAGGTATTAAGGATGGG	772–870	99
IBV NC3-R	CCCGGGAATTCCGGTTTGGGTGTCACTCTACTTCCAAAA		
IBV NC4-F	GGCCCCTGGGATCCTTTGGAAGTAGAGTGACACCCAAAC	847–942	96
IBV NC4-R	CGACCCGGGAATTCCGGATCAAACTGCGGGTCATCACAT		
IBV NC1A-F	CCCCTGGGATCCCAGAAAAAGGGCTCTCGCATT	622–681	60
IBV NC1A-R	GACCCGGGAATTCCGGATACCGGCGATGAGCCATT		
IBV NC1B-F	CAGGGGCCCCTGGGATCCACCAAGGCAAAGGCAGATG	643–705	63
IBV NC1B-R	TCGACCCGGGAATTCCGGATTAGGTGGGATAGTGCGCT		
IBV NC1C-F	GGCCCCTGGGATCCGAAATGGCTCATCGCCGGTA	661–720	60
IBV NC1C-R	CCCGGGAATTCCGGTTGATCAACCCTATAATTAGGTGGGATAG		
IBV 1B-13-F	GATCCAAGGCAAAGGCAGATGAAATGGCTCATCGCCGGTATTGCC	646–684	39
IBV 1B-13-R	TCGAGGCAATACCGGCGATGAGCCATTTCATCTGCCTTTGCCTTG		
IBV 1B-11-F	GATCCGCAAAGGCAGATGAAATGGCTCATCGCCGGTATC	649–681	33
IBV 1B-11-R	TCGAGATACCGGCGATGAGCCATTTCATCTGCCTTTGCG		
IBV 1B-9-F	GATCCAAGGCAGATGAAATGGCTCATCGCCGGC	652–678	27
IBV 1B-9-R	TCGAGCCGGCGATGAGCCATTTCATCTGCCTTG		

2.5. SDS–PAGE and western blotting

SDS–PAGE was performed using a 12 % polyacrylamide gel. The gels were subsequently stained with Coomassie Blue R-250 (161–0400, Bio-Rad, USA). IBV-infected Vero CCL81 cells or Vero CCL81 cells transfected with *pcDNA3*-V5-IBV-N were washed with cold PBS and lysed with RIPA lysis buffer (P0013B, Beyotime, China). The lysates were subjected to 12 % polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (IPVH00010, Millipore, USA). After being blocked with 5 % skim milk in PBST (PBS supplemented with 0.05 % Tween 20, pH 7.4) for 1 h, the membranes were incubated with the primary antibody overnight at 4 °C, followed by washing and incubation with an HRP-conjugated secondary antibody for 1 h at RT (AS002/AS003, ABclonal, China). After washing with PBST buffer, the proteins were visualized via Western Lightning Plus-ECL reagent (NEL104001EA, PerkinElmer, USA) and an enhanced chemiluminescence (ECL) imager (Tanon, China).

2.6. Immunofluorescence assay (IFA)

IBV-infected Vero CCL81 cells or Vero CCL81 cells transfected with *pcDNA3*-V5-IBV-N were washed once with PBS and fixed with 4 % paraformaldehyde (P0099, Beyotime, China) for 15 min. The cells were permeabilized with 0.1 % Triton X-100 (108,643, Merck, Germany) for 15 min and blocked in PBS with 5 % BSA (BS114, Biosharp, China) for 2 h at RT. Finally, the cells were washed with PBST three times and incubated with 4′,6-diamidino-2-phenylindole (DAPI) (C0065, Solarbio, China) for 7 min. The cells were incubated with hybridoma supernatants or primary antibodies for 1 h at 37 °C. After being washed three times with PBST, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (615–004-214, Jackson, USA) diluted 1:500 in PBS for 45 min at RT. After being stained with 4′,6-diamidino-2-phenylindole (DAPI, BL105A, Biosharp, China), fluorescence images were acquired via a fluorescence microscope (SOPTOP ICX41, China).

2.7. Determination of the half maximal effective concentration (EC_{50})

The purified IBV N protein was coated onto the plate at a concentration of 1 μg/mL (100 μL per well). These two mAbs were initially diluted to 1 μg/mL and then serially diluted fourfold across twelve gradients. Detection was performed via the use of an HRP-conjugated anti-mouse secondary antibody at a dilution of 1:5000. After 15 min of substrate development, the reaction was terminated with H_2SO_4 (2 M), and the absorbance at OD $_{450\ nm}$ was measured, followed by determination of the EC_{50}.

2.8. Competitive binding assay

A competitive binding assay was performed via BLI on the ForteBio Data Analysis system. Specifically, the protein A sensor (18–5010, Sartorius, Germany) was first immobilized with the monoclonal mAb 6D1, followed by saturation with the purified IBV N protein. After blocking with mouse IgG, the sensor was exposed to the mAb 6D2, and the resulting interference spectral shift (in nanometers) was measured as an indicator of the binding capacity of the 6D1 antibody. Finally, the sensor was regenerated with 10 mM glycine solution (pH 3.0).

2.9. Overlapping truncated IBV N design and B-cell epitope identification

The fragment of the IBV N protein was first divided into four segments, namely, NA (amino acids 1–111), NB (amino acids 97–222), NC (amino acids 207–314), and ND (amino acids 299–409), to identify the B-cell epitope in the IBV N protein. Truncated IBV N proteins were expressed and subjected to western blotting with the mAb 6D1 and an anti-His tag antibody. Furthermore, overlapping sequences of the IBV N protein, specifically NC1 (amino acids 207–240), NC2 (amino acids 233–264), NC3 (amino acids 258–290) and NC4 (amino acids 282–314), were cloned and inserted into pGEX-6P-1 and expressed as GST-tagged proteins. Three polypeptides spanning the NC1 region (NC1A: amino acids 207–227; NC1B: amino acids 215–235; NC1C: amino acids 221–240) were subsequently expressed and identified via western

Table 2
IBV strains used in this study for comparison.

IBV strain	GenBank accession number	Country
QX	MN548289.1	China
ck/CH/HB/20	MZ456995.1	China
ck/CH/JS/TAHY/2018	ON260865.1	China
Korea/415/2010	OR050557.1	Korea
3595-20LM/23	OR397128.1	Mexico
India/ck/03/23	OR824987.1	India
H52	EU817497.1	Vaccine
H120	ON350836.1	Vaccine
M41	DQ834384.1	America
ck/CH/LJL/111054	KC506155.1	China
JP/Shimane/98 RNA	LC716901.1	Japan
Beaudette CK	AJ311317.1	America

blotting. The specific epitope was ultimately identified via three truncated polypeptides, which were subsequently cloned and inserted into pGEX-6P-1. An empty vector or pGEX-6P-1 vector linked with an unrelated protein was selected as the control for the experiment. The sequences of primers used for amplification are shown in Table 1.

2.10. Epitope conservation and visual analysis

To verify the conservation of the identified IBV-N epitope, the IBV-N sequences of different genotypes were downloaded from GenBank. The IBV-N protein sequences used in the present study are shown in Table 2. The amino acid sequence alignment and conservation analysis were implemented via the Protein Data Bank (PDB, https://www.rcsb.org). Different genotypes of IBV-infected CEF cells were harvested after a 48-h incubation for IFA and western blot analysis. The spatial location of epitope recognized by mAb 6D1 was predicted based on the crystal structure of IBV N protein (PDB ID: 2CA1), as deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank. The epitope position was mapped onto the monomer.

3. Results

3.1. Expression, purification and identification of the recombinant His-IBV-N protein

The entire ORF of the IBV N gene of the IBV strain Beaudette CK (GenBank accession no. AJ311317.1) was cloned and inserted into the pET-28a (+) vector with a His-tag for expression in *Escherichia coli Rosetta* (DE3) cells. Since the majority of the recombinant protein was expressed in the soluble fraction, the IBV N protein was purified under native conditions using a Ni-NTA affinity column. SDS–PAGE and western blot analysis were performed to detect protein expression. As shown in Fig. 1A, the induced protein mainly presented in the supernatant, indicating successful soluble expression. The purified protein had a molecular weight of approximately 47 kDa. These findings demonstrate that the recombinant His-IBV-N protein was successfully expressed, making it suitable for use as an immunogen to produce mAbs in mice.

3.2. Generation and characterization of monoclonal antibodies targeting the IBV N protein

Following the fourth immunization of BALB/c mice with the recombinant IBV N protein, the serum was collected. Indirect ELISA demonstrated that antibody titers targeting the IBV N protein in all immunized mice exceeded 1/25,600 (data not shown). The mouse with the highest specific antibody titer was selected for cell fusion. The cells were subsequently cultured in selective medium. The supernatants were harvested to screen hybridomas that secreted mAbs via indirect ELISA and IFA. After subcloning and screening, two positive hybridomas secreting anti-IBV N mAbs (6D1 and 6D2) were obtained. Purified mAbs were prepared from ascites via protein A agarose beads according to the manufacturer's instructions. SDS–PAGE analysis revealed that the purified mAbs represented heavy chains (HCs, ~55 kDa) and light chains (LCs, ~25 kDa) (Fig. 2A). As shown in Fig. 2B, the heavy chains of these two mAbs are both IgG1, and the light chains were both identified as kappa type (Fig. 2B). Indirect ELISA was performed to detect the binding of these two mAbs to the purified IBV N protein, and the EC_{50} was calculated via nonlinear regression analysis. As shown in Fig. 2C, these two mAbs could bind to the purified IBV N protein, with EC_{50} values of 7.385 ng/mL and 9.994 ng/mL, respectively.

3.3. Specificity of monoclonal antibodies targeting the IBV N protein

To confirm the specificity of the two mAbs, the eukaryotic expression plasmid *pcDNA3*-V5-IBV-N was constructed and transfected into Vero CCL81 cells. IFA and western blotting were performed using the two mAbs. As shown in Fig. 3A, specific bans of approximately 45 kDa were observed when the anti-V5 tag antibody and the two mAbs were used, which is identical to the size of the predicted IBV N protein. IFA analysis revealed specific fluorescence in Vero CCL81-transfected cells but no fluorescence in empty vector-transfected cells (Fig. 3B).

Furthermore, the ability of the two mAbs to detect IBV- infected cells

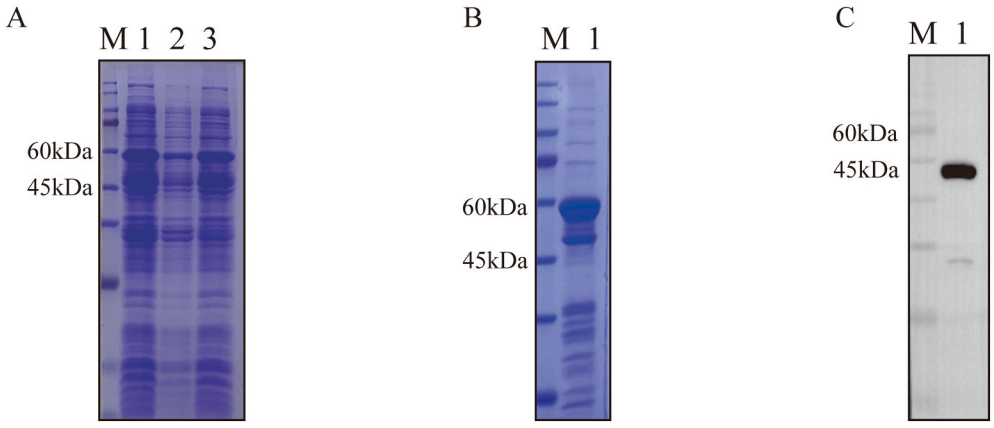

Fig. 1. The expression and purification of the recombinant His-IBV-N protein.
(A) Cell lysates of *Escherichia coli Rosetta* (DE3) carrying the pET28a-His-IBV-N identified by SDS-PAGE. Lane M: protein marker; Lane 1: total lysate of cells induced to express recombinant His-tagged IBV N protein; Lane 2: pellet fraction (insoluble fraction) of the lysate; Lane 3: supernatant (soluble fraction) of the lysate. (B) The purification of the recombinant His-IBV-N protein identified by SDS-PAGE. Lane M: protein marker; Lane 1: purified IBV-N-His protein (C) Western blot analysis of the recombinant His-IBV-N protein using an anti-His mAb. Lane M: protein marker; Lane 1: purified IBV-N-His protein.

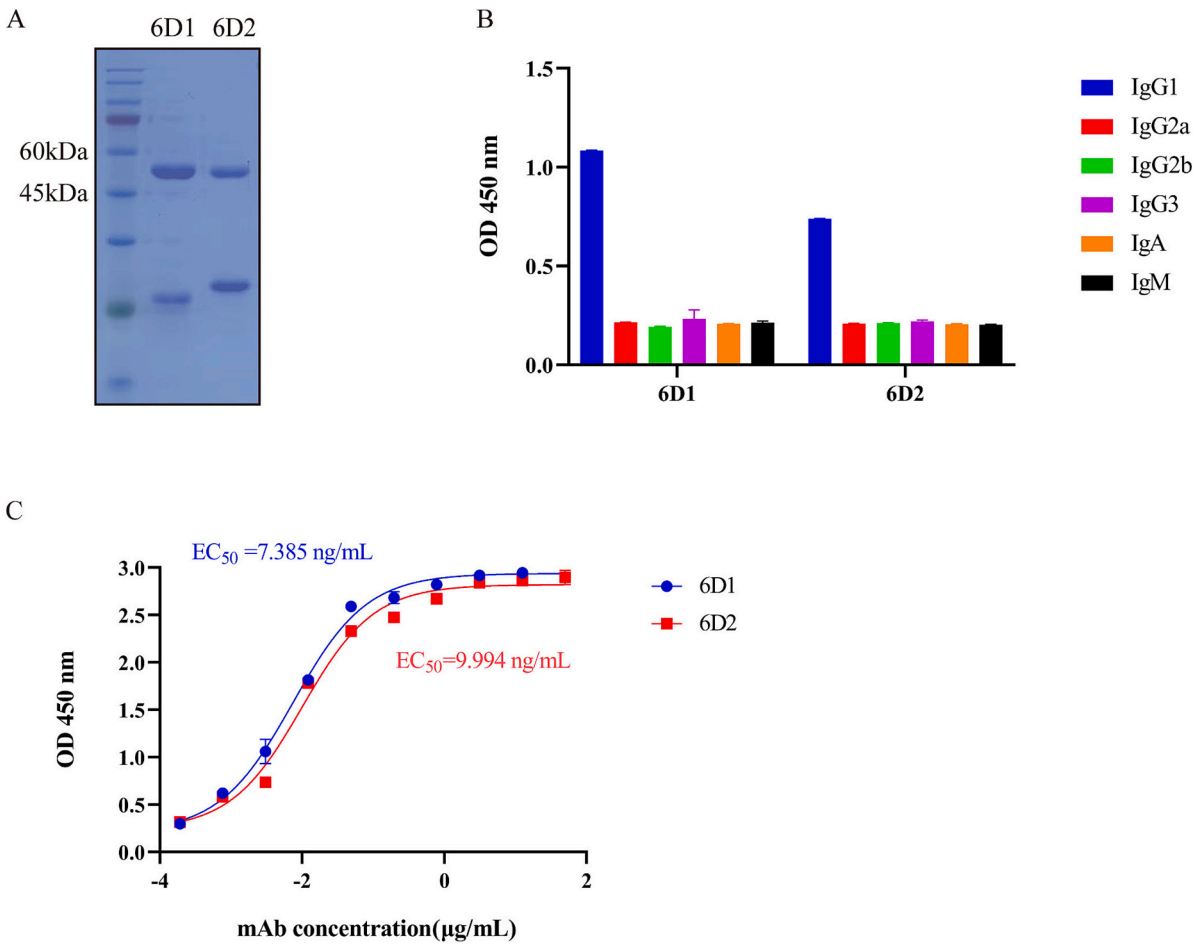

Fig. 2. The generation and characterization of the mAbs 6D1 and 6D2.
(A)The identification of the purified mAbs 6D1 and 6D2 by SDS-PAGE. (B)Subtype analysis of these two mAbs heavy and light chains. (C)The binding of these two mAbs to the recombinant His-IBV-N protein was assessed using ELISA. The EC_{50} values were determined by ELISA of purified these two mAbs to His-IBV-N protein. The values calculated using nonlinear regression analysis.

was assessed. Vero CCL81 cells were inoculated with the IBV Beaudette strain at an MOI of 0.1. Following a 20-h incubation period, the IBV-infected Vero CCL81 cells were harvested for IFA and western blot analysis. Both mAbs detected protein bands of similar size to those observed in *pcDNA3*-V5-IBV-N-transfected cells in lysates of IBV-infected Vero CCL81 cells (Fig. 3C). Additionally, specific green fluorescence was observed in IBV-infected Vero CCL81 cells, whereas no fluorescence was detected in mock-infected cells (Fig. 3D).

3.4. mAb 6D1 and mAb 6D2 share identical epitopes

The competitive binding interactions between the mAbs 6D1 and 6D2 were investigated via BLI. When the sensor immobilized with the monoclonal mAb 6D1, followed by saturation with the purified IBV-N protein, was immersed in the mAb 6D2, no change in the biolayer thickness was observed. The interference spectrum curve did not shift, and the fitting curve after data processing did not exhibit a second peak. The results revealed that the mAb 6D2 was unable to bind to the protein A sensor coated with the mAb 6D1 and the IBV-N protein. The mAbs 6D1 and 6D2 compete for binding to the same epitope region within the IBV-N protein antigen, as depicted in Fig. 4A.

3.5. Mapping the epitopes of the 6D1 and 6D2 mAbs

In accordance with the results of the BLI assay, the mAb 6D1 was selected for subsequent epitope identification. On the basis of the predicted B-cell epitopes, the 491 amino acids of the N protein were divided into four overlapping peptide fragments (Fig. 4B). These truncated N proteins fused with a 6 × His-tag were expressed in *Escherichia coli Rosetta* (DE3). Western blot analysis revealed that only the NC (aa 207–314) could be recognized by the mAb 6D1 (Fig. 4C). To narrow down the region, the NC (aa 207–314) was further divided into four overlapping peptide fragments: NC1: aa 207–240, NC2: aa 233–264, NC3: aa 258–290, and NC4: aa 282–314. Western blot analysis revealed that only NC1 (aa 207–240) could be recognized by the mAb 6D1 (Fig. 4D). Further truncation analysis of three overlapping peptide fragments of NC1 (NC1A: aa 207–227, NC1B: aa 215–235, NC1C: aa 221–240) revealed that the mAbs recognized NC1B (Fig. 4E). Sequential truncation and expression analysis finally mapped the epitope recognized by mAb 6D1 on the IBV N protein to the peptide sequence ^{218}KADEMAHRR^{226} (Fig. 4F).

3.6. Cross-reactivity and conserved epitope mapping of mAbs 6D1 and 6D2 on the IBV N protein

IBV is characterized by extensive antigenic and genetic diversity, which leads to the continuous emergence of new genotypes, lineages, serotypes, and variants [28–30]. The N protein is a highly conserved structural protein. To evaluate the conservation of epitopes in the IBV-N protein, the IBV-N amino acid sequences of different genotypes were downloaded from GenBank. The alignment results revealed that the epitope ^{218}KADEMAHRR^{226} was conserved in the different IBV

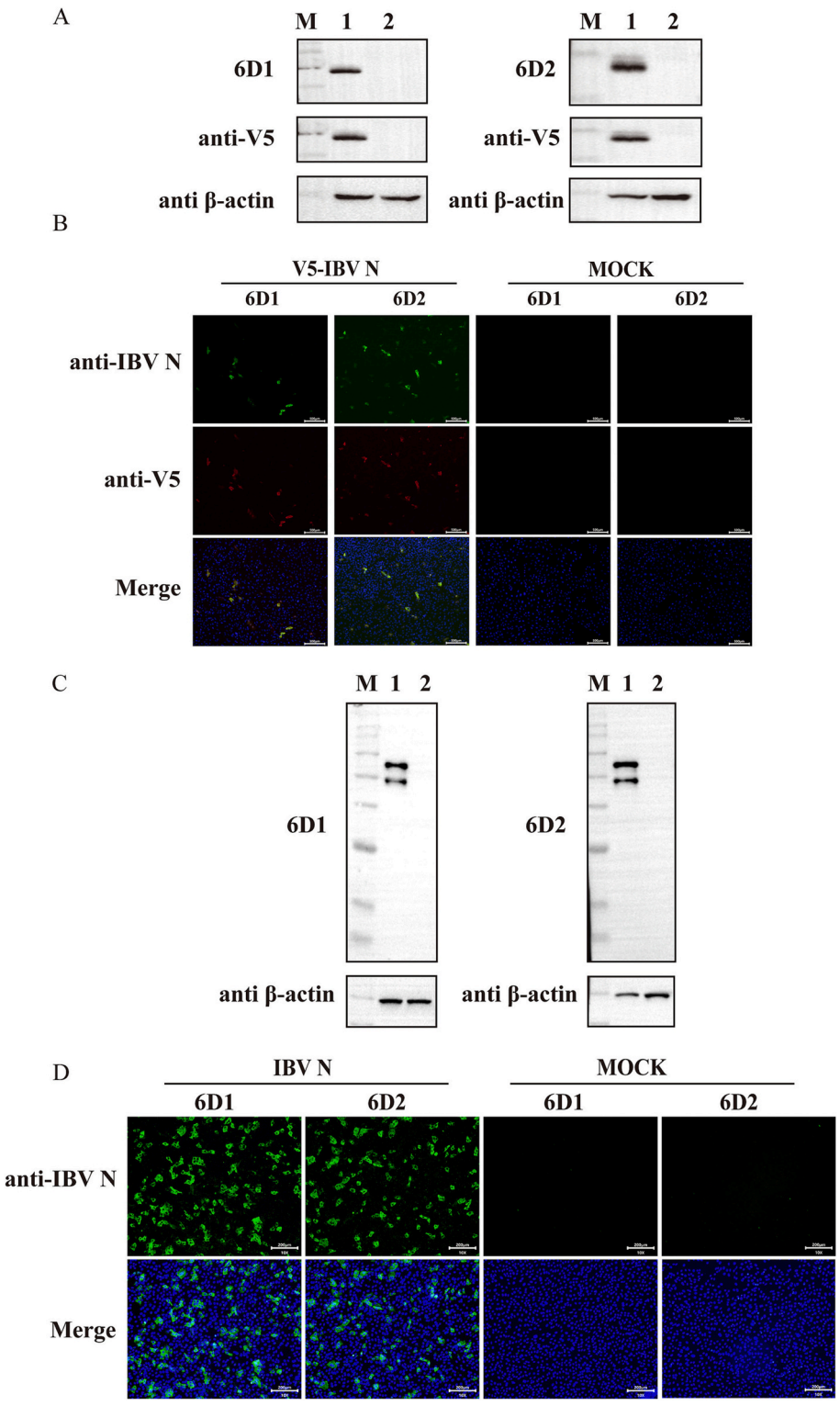

Fig. 3. Specificity of mAbs 6D1 and 6D2 targeting IBV N protein were determined by western blot and IFA.
(A) Western blot detection of the IBV N protein in transfected Vero-CCL81 cells using the mAb 6D1, mAb 6D2 and anti-V5 mAb. Lane M: protein marker; Lane 1: transfected Vero-CCL81 cells; Lane 2: Vero-CCL81 cells mock. (B) IFA detection of the IBV N protein in transfected Vero-CCL81 cells using these two mAbs and anti-V5 mAb. (C) Western blot detection of the IBV N protein in IBV-infected cells using these two mAbs. Lane M: protein marker; Lane 1: IBV-infected cells; Lane 2: uninfected cells mock. (D) IFA detection of the IBV N protein in IBV-infected cells using the mAbs 6D1and 6D2.

genotypes. Furthermore, different genotypes of IBV were infected with CEF cells, including M41 of GI-1 (mass type), CK/CH/HB/2018/09, CK/CH/JS/2018/16, CK/CH/HB/2018/25, CK/CH/JS/2018/38 of GI-13 (4/91 type), and CK/CH/JS/2018/21 of GI-19 (QX type). IFA and western blot analyses were performed 48 h postinfection. The results demonstrated that these two mAbs can detect the N protein in CEF cells infected with different IBV genotypes (Fig. 5B and C), indicating its strong cross-reactivity against IBV of different genotypes. To determine

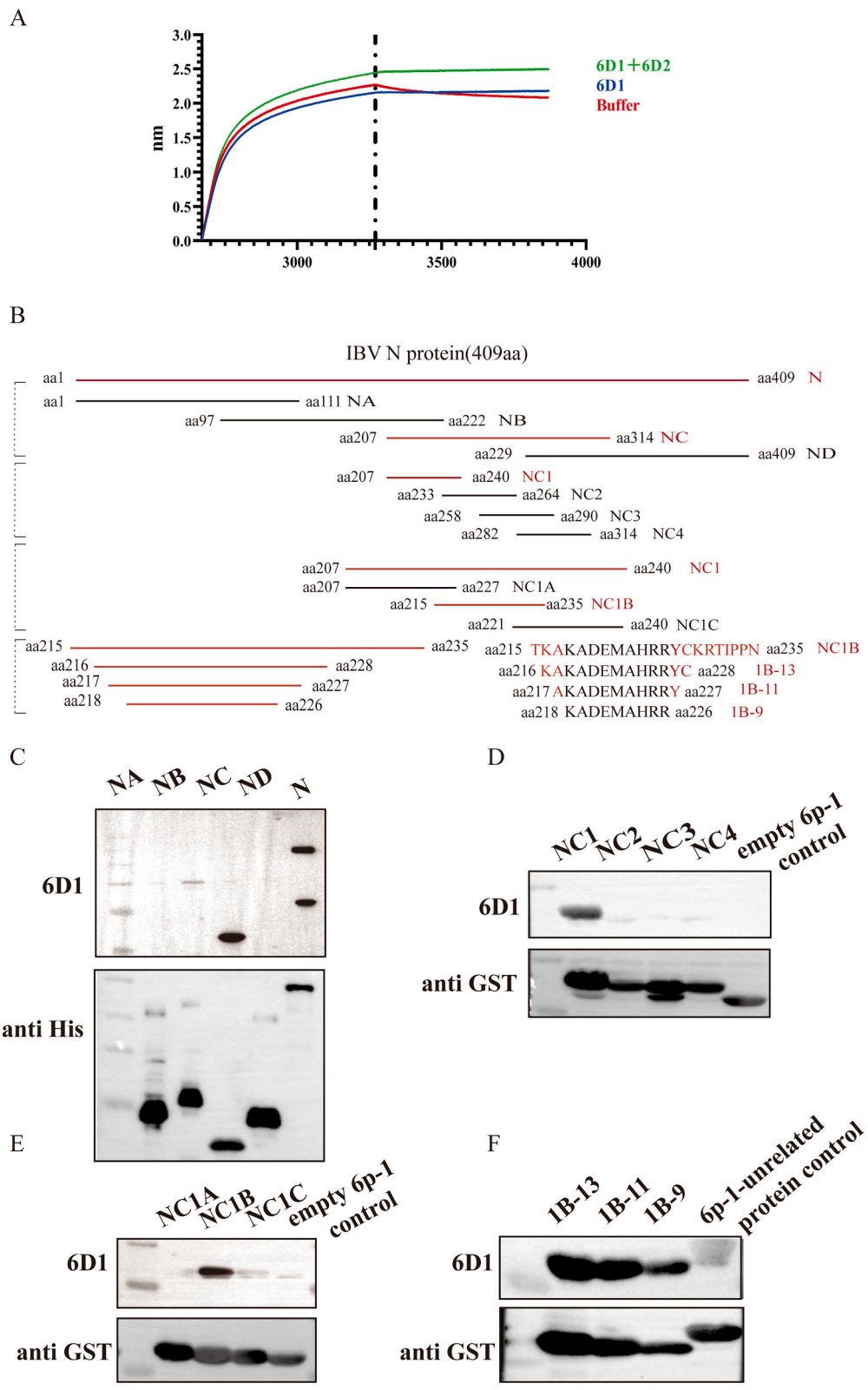

Fig. 4. Epitopes mapping by mAbs 6D1 and 6D2 against IBV N protein.
(A)Competitive binding assay. Biofilm interferometry (BLI) assay was used to determine whether the binding between purified mAbs and IBV N proteins depended on the same epitope. (B) Schematic representation of IBV N fragments used for B cell epitope mapping. The segments that could be recognized by mAb 6D1 is highlighted in red.(C) Western blot analysis of IBV N protein (namely N, NA, NB, NC, ND) using mAb 6D1 and anti-His mAb.(D) Western blot analysis of IBV N protein (namely NC1,NC2, NC3, NC4) and pEGX-6p-1 empty vector using mAb 6D1 and anti-GST mAb.(E) Western blot analysis of IBV N protein (namely NC1A,NC1B, NC1C) and pEGX-6p-1 empty vector using mAb 6D1 and anti-GST mAb.(F) Western blot analysis of IBV N protein (namely 1B-13,1B-11,1B-9) and an unrelated protein expressed from pEGX-6p-1, using mAb 6D1 and anti-GST mAb.

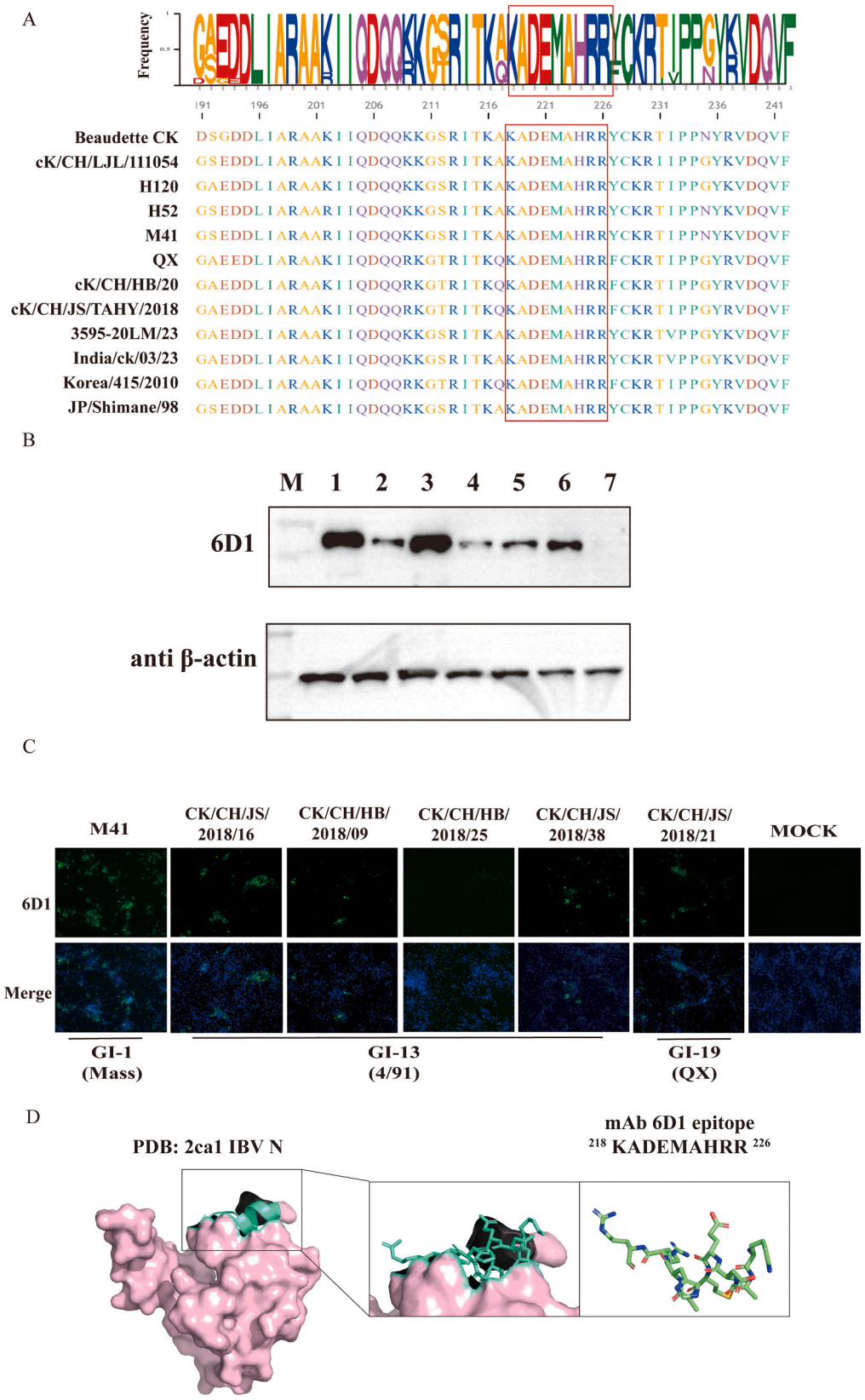

Fig. 5. Homology analysis of the epitope and cross-reactivity analysis of the mAbs 6D1 and 6D2. (A) Comparison of the amino acid sequence of these two mAbs epitope among IBV strains of different genotypes. (B) Reactivity analysis of mAb 6D1 with different IBV genotypes-infected cells determined by western blot. Lane M: protein marker; Lane 1:M41-infected cells; Lane 2: CK/CH/JS/2018/09 –infected cells; Lane 3: CK/CH/HB/2018/16-infected cells; Lane 4:CK/CH/HB/2018/25-infected cells; Lane 5:CK/CH/JS/2018/38-infected cells; Lane 6:CK/CH/JS/2018/21 -infected cells; Lane 7: CEF cells mock. (C)Analysis of reactivity of mAb 6D1 with different IBV genotypes-infected cells determined by IFA. (D) Localization of the epitope recognized by mAb 6D1 on the IBV N protein crystal structure (PDB ID: 2CA1), visualized using PyMOL.

the spatial location of the epitope recognized by mAb 6D1, the published crystal structure of the IBV N protein (PDB ID: 2CA1) was visualized using PyMOL. The analysis revealed that the epitope is exposed on the surface of the IBV N protein (Fig. 5D). Combined with experimental data, it has been confirmed that the epitope ^{218} KADEMAHRR ^{226} is likely to be a linear epitope targeting the IBV N protein. These results suggest that the mAb 6D1 can serve as a universal tool for the detection of IBV antigens from different genotype.

4. Discussion

The genetic diversity and rapid evolution of IBV pose significant challenges for the diagnosis and prevention of IBV infection [31]. Recent advancements in the identification of viral epitopes have become increasingly important for virus detection and therapeutic interventions [32]. In this study, we generated two monoclonal antibodies targeting the N protein of IBV and identified a highly conserved epitope. Notably, the antibodies we developed can detect infection by various IBV genotypes, underscoring the potential of this conserved epitope as a robust target for diagnostic and therapeutic applications.

The N protein is the most conserved structural protein among coronaviruses and is also the most abundant owing to the unique discontinuous viral transcription process [33]. Studies have reported that the majority of IgG antibodies in serum following coronavirus infection specifically target the N protein and that these IgG antibodies are present even in the early stages of infection [34,35]. Therefore, the N protein of coronavirus is crucial for developing diagnostic methods as an immunogen. In this study, the IBV N protein was successfully expressed, and two monoclonal antibodies, 6D1 and 6D2, were generated after BALB/c mice were immunized with the recombinant IBV N protein. Furthermore, these two mAbs exhibited high binding affinities for the expressed IBV N protein, with EC_{50} values of 7.385 ng/mL and 9.994 ng/mL, respectively. Although the recombinant IBV N protein was expressed via an *E. coli* prokaryotic expression system, the generated mAbs efficiently detected the N protein in both transfected cells and IBV-infected cells.

The upper respiratory tract-related symptoms induced by IBV are similar to those caused by other avian viruses, such as Newcastle disease (NDV) and avian influenza (AIV) [36], complicating the clinical diagnosis of IBV. Epitope-based serological diagnostic approaches have demonstrated high sensitivity and specificity [31]. Given the complexity of the IBV epidemic, the identification of highly conserved epitopes is critical for developing diagnostic approaches. To date, limited B-cell linear epitopes have been characterized on the IBV N protein, with most being located at the N-terminus. A notable exception is the ^{242}FGPRTK^{247} epitope reported by Han et al. at the C-terminus of the N protein. Here, we identified a novel epitope, ^{218}KADEMAHRR^{226}, located at the C-terminus of the IBV N protein. Multiple sequence alignment and homology analysis revealed that the epitope is conserved across various IBV genotypes. These findings provide valuable resources for the establishment of improved IBV diagnostic methods.

Since the epitope located on the N protein of IBV is highly conserved, we infected CEF cells with different IBV strains of various genotypes. Western blot and IFA analyses revealed that these two mAbs can detect the N protein in CEF cells infected with different IBV genotypes, suggesting that the mAbs can be used as powerful tools to detect IBV. However, given that the linear epitope is also conserved in the N protein of vaccine strains, these two mAbs cannot distinguish between field strains and vaccine strains. Future investigations will combine phage display technology with clinical IBV infection-positive serum to identify additional antigenic epitopes targeting the N protein.

In conclusion, two monoclonal antibodies targeting the N protein of IBV were developed, and a novel linear epitope, ^{218} KADEMAHRR ^{226}, located at the C-terminus of the N protein of IBV, was identified. Notably, this epitope is highly conserved among different genotypes of IBV. Our findings elucidate the antigenic structure of the IBV N protein and provide a basis for the establishment of IBV detection and diagnostic methods.

CRediT authorship contribution statement

Ran Jing: Writing – original draft, Visualization, Project administration, Formal analysis, Data curation, Conceptualization. **Jiaru Zhou:** Writing – original draft, Project administration, Formal analysis, Conceptualization. **Changcheng Liu:** Software, Investigation, Formal analysis. **Mengdi Zhang:** Investigation, Formal analysis. **Wenlong Zhu:** Writing – review & editing, Supervision. **Hua Cao:** Writing – review & editing. **Bo Hou:** Resources. **Hongbo Zhou:** Resources. **Wentao Li:** Writing – review & editing, Funding acquisition. **Mengjia Zhang:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

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Data availability

Data will be made available on request.

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