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Development of a blocking ELISA to evaluate the neutralizing antibody level against canine distemper virus and identification of novel spatial conformation epitopes

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ABSTRACT

Canine distemper virus (CDV) poses a significant threat to the global pet, fur, and endangered species conservation industries. Vaccines remain the primary means of preventing and controlling CDV, necessitating rapid and accurate detection of neutralizing antibody levels post-vaccination. While the canine distemper virus H protein (CDV-H) serves as the main neutralizing target, key antigenic epitopes and structural information remain unclear. Therefore, this study expressed CDV-H protein using the CHO eukaryotic expression system and developed a neutralizing monoclonal antibody against it using hybridoma technology. The antibody exhibited specificity against prevalent CDV strains and recognized the conformational epitope of CDV-H. Molecular docking identified the amino acids recognizing antigens by this antibody as 120Q, 123 N, 126 N, 127P, 129R, 130E, 133F, 155D, and 418D. And molecular dynamics simulations have confirmed the accuracy of the docking results. Evolutionary tree analysis revealed high conservation of these antigenic sites among all CDV strains. Based on this antibody, a blocking ELISA was established for detecting CDV neutralizing antibodies. A statistically significant positive correlation was observed between blocking ELISA titers and neutralization test titers ($R^2 = 0.6905$, $P < 0.0001$, and after grouping $R^2 = 0.9272$, $P < 0.0085$), indicating that the blocking ELISA can detect neutralizing antibodies in vaccine-immunized dogs and assess vaccine protective immunization effects.

1. Introduction

Canine distemper (CD), caused by canine distemper virus (CDV), is a highly contagious disease affecting a wide range of animals globally [1,2]. CDV infection poses a serious threat to global canine populations and economic animal husbandry, resulting in significant economic losses [3]. CDV is an RNA measles virus, belonging to the *Morbillivirus* genus within the *Paramyxoviridae* family and its genome encodes the CDV-H protein, a type II glycoprotein located in the outer membrane of the CDV vesicle [4]. This protein recognizes host cell-associated receptors and initiates cell fusion, resulting in cellular infection [5,6]. The CDV-H proteins contain numerous neutralizing antigenic epitopes, which are crucial for inducing the production of neutralizing antibodies in the body. While, when individual amino acids in H protein are tated, CDV can recognize non-canine receptors, which in turn causes the expansion of the susceptible host range, and H protein is prone to antigenic drift, which is a key factor leading to the failure of immune protection. Thus CDV-H protein has extensive applications in the

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diagnosis of CD and the development of vaccines [7]. Defining the detailed neutralizing epitopes of CDV-H protein expands the understanding of CDV neutralization mechanisms, which is vital for the advancement of diagnostic techniques and novel vaccines.

Vaccine immunization is crucial for controlling canine distemper (CD), with the level of neutralizing antibodies produced in vaccinated dogs directly correlating with their resistance to CDV infection. Protection is only achieved when the vaccine induces neutralizing antibodies [8,9]. Viral Neutralization Test (VNT) is a reliable method to assess the level of neutralizing antibodies induced by vaccine immunization, but VNT is time-consuming, labor-intensive, expensive, and impractical for large-scale field testing in clinical settings [10]. In contrast, the enzyme-linked immunosorbent assay (ELISA) is a method for detecting CDV antibodies with higher specificity, sensitivity, and practicality [11]. However, commercially available ELISA kits are only suitable for evaluating the level of total antibodies produced by vaccinated animals, and do not reflect the neutralizing antibody titer, thus preventing their direct apply in assessing the protective efficacy of vaccination against CDV infection [12]. Therefore, the development of blocking ELISA methods based on neutralizing antibodies to evaluate the effectiveness of vaccine immunization is essential in controlling CD infection.

In this study, neutralizing monoclonal antibody against CDV-H protein was developed, which target multiple CDV strains to provide a drug reference in animals onset. The key amino acid sites of CDV-H antigenic epitopes were identified, which further enriched the antigenic structural information of CDV-H protein, and could provide reference information for vaccine preparation. Meanwhile, the blocking ELISA method was established. Because the recognized epitope was a neutralizing epitope, the blocking ELISA indicated that the serum contained neutralizing antibodies against the neutralizing epitope, so the antibody effect can be fed back by the blocking rate. This study provided a reliable method for evaluating the level of neutralizing antibodies produced after animal vaccination and to evaluate whether the vaccine can effectively prevent.

2. Materials and methods

2.1. Cell, virus, antibody and serum samples

Vero-SLAM cells, SP2/0, America-1 genotype CDV strain, Asia-1 genotype CDV strain and Asia-4 genotype CDV strain were maintained by this laboratory. DMEM medium (Gibco), Roswell Park Memorial Institute (RPMI) 1640 medium, ABW serum were purchased by this laboratory. Commercially purchased HRP-coupled goat anti-mouse IgG (BF03001, Biodragon) and Alexa Fluor 488 labeled goat anti-mouse IgG (H + L) (A0428, Beyotime). Canine positive serum and negative serum were maintained in this laboratory. Canine immune serum were collected from meat dog farms.

2.2. CDV-H protein expression purification

Amino acids 59–607 of the extracellular domain of the CDV H protein were selected for CHO system optimization and synthesized by Beijing Tsingke Biotech Co., Ltd. (Beijing, China) according to the sequence submitted in GenBank (AHH82065.1, amino acids: 59–607). CHO highly expression cell line constructs against CDV-H protein were performed after gene synthesis. The screened high-expressing cell lines were subjected to bulk expression, and the supernatant of cell cultures was collected for nickel-column purification.

2.3. Preparation and characterization of monoclonal antibodies against recombinant H protein

Monoclonal antibodies against CDV-H protein were prepared using conventional hybridoma cell methods as reported in the literature [13]. Mice were immunized with the CDV-H protein as an immunogen. Blood was collected from mice via tail vein bleeding, and serum antibody titres were measured by indirect ELISA. Hybridoma cells were screened simultaneously by indirect ELISA, indirect immunofluorescence experiments, and neutralization experiments. After three consecutive subclones, hybridoma monoclonal cells that could stably secrete antibodies were screened. The obtained hybridoma cells 9-7B were used for stable monoclonal antibody secretion. Hybridoma cells were collected into injected mice and ascites was collected and purified. The reactivity and antibody titer of monoclonal antibodies with CDV-H protein were determined by indirect ELISA. Western blot analysis and dot blot assays were employed to assess whether the monoclonal antibodies recognized linear epitopes of CDV-H. Analysis of whether monoclonal antibodies can recognize and neutralize CDV of multiple genotypes by indirect immunofluorescence and neutralization experiments. The mAb isotypes were classified using an IsoStrip kit (Sigma-Aldrich).

2.4. Western blot and dot blot assays

Monoclonal antibodies were analyzed by Western Blot (WB) using purified CDV H protein [14]. The CDV-H protein was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The blocking solution was blocked for 1 h, the primary antibody was incubated for 1 h, the secondary antibody was incubated for 1 h, the color was developed and the results were visualized. The dot blot assays, a nitrocellulose (NC) membrane was cut to the required size. Supernatant from CDV-H-expressing CHO high-expression cell lines 2 μL were spotted onto the NC membrane. The membrane was then dried in a 37 °C oven and subsequently closed in blocking solution for 37 °C for 1 h. The following steps were similar to the WB experiment.

2.5. Indirect immunofluorescence assay

The reactivity of mAbs toward CDV was determined by IFA as described previously [15]. Vero-SLAM cells were infected with America-1 genotype CDV strain, Asia-1 genotype CDV strain and Asia-4 genotype CDV strain in 48-cell well plate. Uninfected cells were used as negative controls. 48 h later, cells were fixed with 4 % paraformaldehyde and then permeabilized with 0.02 % Triton X-100. The cells were blocked with 10 % goat serum at 37 °C for 1 h and incubated with monoclonal antibody 9-7B as primary antibody at 37 °C for 1 h. Alexa Fluor 488 labeled goat anti-mouse IgG(H + L) (A0428, Beyotime) was used as a secondary antibody for incubation at 37 °C for 1 h. he results were observed under the fluorescence microscope (Mshot).

2.6. Neutralization assay

Neutralization experiments were performed to determine the neutralizing activity of monoclonal antibody 9-7B against America-1 genotype CDV strain, Asia-1 genotype CDV strain and Asia-4 genotype CDV strain [14]. Briefly, a twofold dilution of the monoclonal antibody was incubated with 200 $TCID_{50}$ in DMEM for 1 h at 37 °C. The antibody-virus mixture was then added to Vero-SLAM cells at a ratio of 1×10^5 cells/well in a 96-well plate and incubated at 37 °C. The cells were observed every 24 h after inoculation for 3 consecutive days and cytopathic effects were recorded. Neutralization experiments were also performed to test for neutralizing antibodies in serum, which was inactivated in a water bath at 56 °C for 30 min before performing the experiments.

2.7. Indirect ELISA

The titer of monoclonal antibody 9-7B was determined by an established indirect ELISA. CDV-H protein was encapsulated in an ELISA plate overnight at 4 °C, and the blocking solution was blocked at 37 °C for 1 h. Ascites of monoclonal antibody 9-7B was diluted 10-fold in 11

gradients. The diluted ascites was incubated as primary antibody at 37 °C for 1 h. HRP-coupled goat anti-mouse IgG was incubated as secondary antibody at 37 °C for 1 h. The plates were incubated with substrate solution tetramethyl benzidine (TMB) at 37 °C for 10 min and the reaction was stopped with 2 M H_2SO_4 in each well. The OD_{450nm} values were read as the experimental results.

2.8. Sequencing and molecular docking of monoclonal antibodies

The monoclonal antibody 9-7B was sequenced, and the sequencing results were put into NCBI for IgBlast and analyzed to derive the sequence of the variable region of the light chain and the sequence of the variable region of the heavy chain of the monoclonal antibody 9-7B. The sequence of CDV-H protein was molecularly docked with the variable region of the monoclonal antibody using AlphaFold3 [16]. We conducted molecular dynamics (MD) simulations using GROMACS (version 2025.0) to investigate the interactions between monoclonal antibodies and their target molecules. The monoclonal antibody 9-7B prepared in this study targets the CDV-H protein, and 9-7B can specifically bind to the CDV-H protein. CDV belongs to the *Morbillivirus* genus within the *Paramyxoviridae* family, while PDCOV belongs to *Deltacoronavirus* genus within the *Coronaviridae* family. The homology between these two is low, as is the homology between the N proteins. Therefore, PDCOV-N was chosen as the control protein.

Two complex systems, “9-7B-CDV-H" and “9-7B-PDCOV-N", were simulated for 500 ns each. Both the monoclonal antibodies and target protein were parameterized using the AMBER ff14SB force field. Each system was solvated in a cubic box with a minimum solute-to-box edge distance of 1.0 nm, using the TIP3P water model. To neutralize the systems and mimic physiological conditions, Na^+ and Cl^- counterions were added to achieve a final salt concentration of 0.15 M Prior to the production run, all systems underwent energy minimization (EM) and equilibration to relieve any steric clashes. Energy minimization was performed using the steepest descent algorithm for 10,000 steps. This was followed by equilibration in two stages: an NVT ensemble phase, during which the system was gradually heated from 0 to 300 K over 1.0 ps with harmonic restraints; and an NPT ensemble phase, where the system was equilibrated for 2 ns at 300 K and 1 atm under periodic boundary conditions in all directions. Production MD simulations were then carried out under the NPT ensemble for 500 ns. Temperature and pressure were controlled using the V-rescale thermostat and the C-rescale barostat, respectively. Long-range electrostatics were calculated using the Particle Mesh Ewald (PME) method with a real-space cut-off of 1.0 nm. All bonds involving hydrogen atoms were constrained using the LINCS algorithm. Simulations were performed with a 2.0-fs integration time step, and system coordinates were saved every 5000 steps, resulting in a total of 50,000 frames for each system. This provided comprehensive sampling of the mAb-target interactions. Binding free energy calculations were performed using the gmx_MMPBSA tool. Frames were sampled every 10 steps from the full trajectory, yielding 10,000 conformations per system for energy analysis. The improved MM/GBSA method was applied using the GB-Neck2 model. The ionic strength was set to 0.15 M, and the temperature and force field parameters were kept consistent with those used in the MD simulations.

2.9. Bioinformatics analysis

To investigate the homology of epitopes with CDV sequences, representative strains provided from NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/) were compared. H protein sequences of 446 representative CDV strains of different lineages were selected for comparison. The H protein phylogenetic tree was constructed using the neighbor-joining method based on MEGA software, and bootstrap values were calculated based on 1000 replicates. Based on this, the conservation and variability of the predicted key amino acids were analyzed.

2.10. Establishment of the blocking ELISA method

After using the monoclonal antibody 9-7B labeled with HRP, the optimal encapsulation concentration of CDV-H protein and the optimal dilution of HRP-mAb were determined by checkerboard titration to be 0.5 μg/mL and 1:400, respectively. In addition, other important conditions for the blocking ELISA were also optimized. The optimal reaction conditions for the blocking ELISA were antigen encapsulation at 4 °C for 12 h, 2 % BSA as the blocking solution, serum dilution of 1:16, reaction time of 60 min for serum samples, reaction time of 60 min for HRP-MAb, and time for color development of 10 min. Percentage of Inhibition (PI value) for each test sample: PI (%) = [1- OD_{450nm} value of sample/ OD_{450nm} value of negative control] × 100 %.

2.11. Criticality, diagnostic sensitivity and specificity measurements

To calculate the optimal cut-off value as well as the associated diagnostic sensitivity and specificity. Serum from known CDV positive and negative samples were detected by blocking ELISA. Subjects were analyzed for working characteristics ROC analysis and degree of concordance (kappa value) using SPSS software. Receiver operating characteristic (ROC) curve analysis was applied to determine the cut-off value. The Youden index (J = sensitivity+specificity-1) was applied to identify the threshold that maximized the discriminatory power between positive and negative controls. The area under the curve (AUC) was calculated to evaluate the diagnostic accuracy. In order to determine the sensitivity of blocking ELISA, the established blocking ELISA method was used to dilute CDV positive serum 2 times (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024) to determine its sensitivity. In order to determine the specificity of blocking ELISA, the established blocking ELISA method was used to detect the serum positive for four common viruses in dogs, namely, positive serum for canine distemper virus (CDV), positive serum for canine parvovirus (CPV), positive serum for canine parainfluenza virus (CPIV) and positive serum for canine adenovirus 2 (CAV-2) , to determine their specificity. The serum of 52 immunized CDV-positive canines collected from the laboratory was tested by blocking ELISA and neutralization tests in this experiment. Statistical analysis of the degree of agreement (Kappa value) and correlation between different methods was performed by Pearson correlation coefficient analysis using GraphPad Prism software version 6.0. $P < 0.05$ was statistically significant.

3. Result

3.1. Expression purification and immunoreactivity analysis of recombinant CDV-H protein

Since CDV-H protein is a type II glycoprotein with glycosylation modification, the CHO eukaryotic system was applied to express CDV-H protein in this study. SDS-PAGE and WB results revealed that a highly purified recombinant CDV-H protein, with a molecular weight of 62 kDa, was successfully obtained after in vitro expression and purification (Fig. 1A and B). To verify the immunoreactivity of the CDV-H protein, WB verification was performed using canine CDV positive serum and canine CDV negative serum. The results demonstrated that the CDV-H protein specifically reacted with canine CDV positive serum and did not react with CDV negative serum, which demonstrated that the in vitro-expressed CDV-H protein exhibited good immunoreactivity (Fig. 1C and D).

3.2. Development of a neutralizing monoclonal antibody against CDV-H protein

In this study, a neutralizing monoclonal antibody 9-7B was successfully developed against CDV-H protein. Indirect ELISA demonstrated that 9-7B specifically recognize CDV-H protein (Fig. 2A). The WB

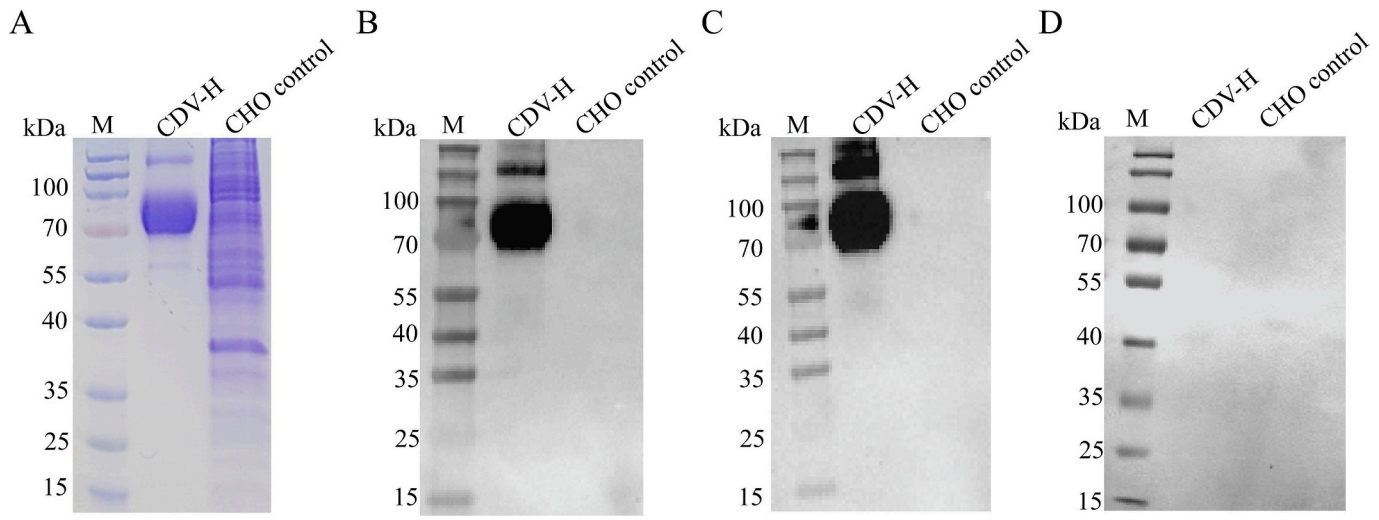

Fig. 1. Expression, purification, and validation of the CDV-H protein. (A) SDS-PAGE analysis of recombinant CDV-H protein obtained by expression and purification in CHO system. (B) Western blot analysis of the reactivity of the recombinant CDV-H protein and HIS-tag. (C) Immunoreactivity analysis of recombinant CDV-H protein with canine CDV positive serum. (D) Immunoreactivity analysis of recombinant CDV-H protein with canine CDV negative serum. The above upper band information was consistent. Lane 1, Protein marker. Lane 2, Purified recombinant CDV-H protein. Lane 3, Supernatant of CHO Cells without electroporated plasmid as negative control.

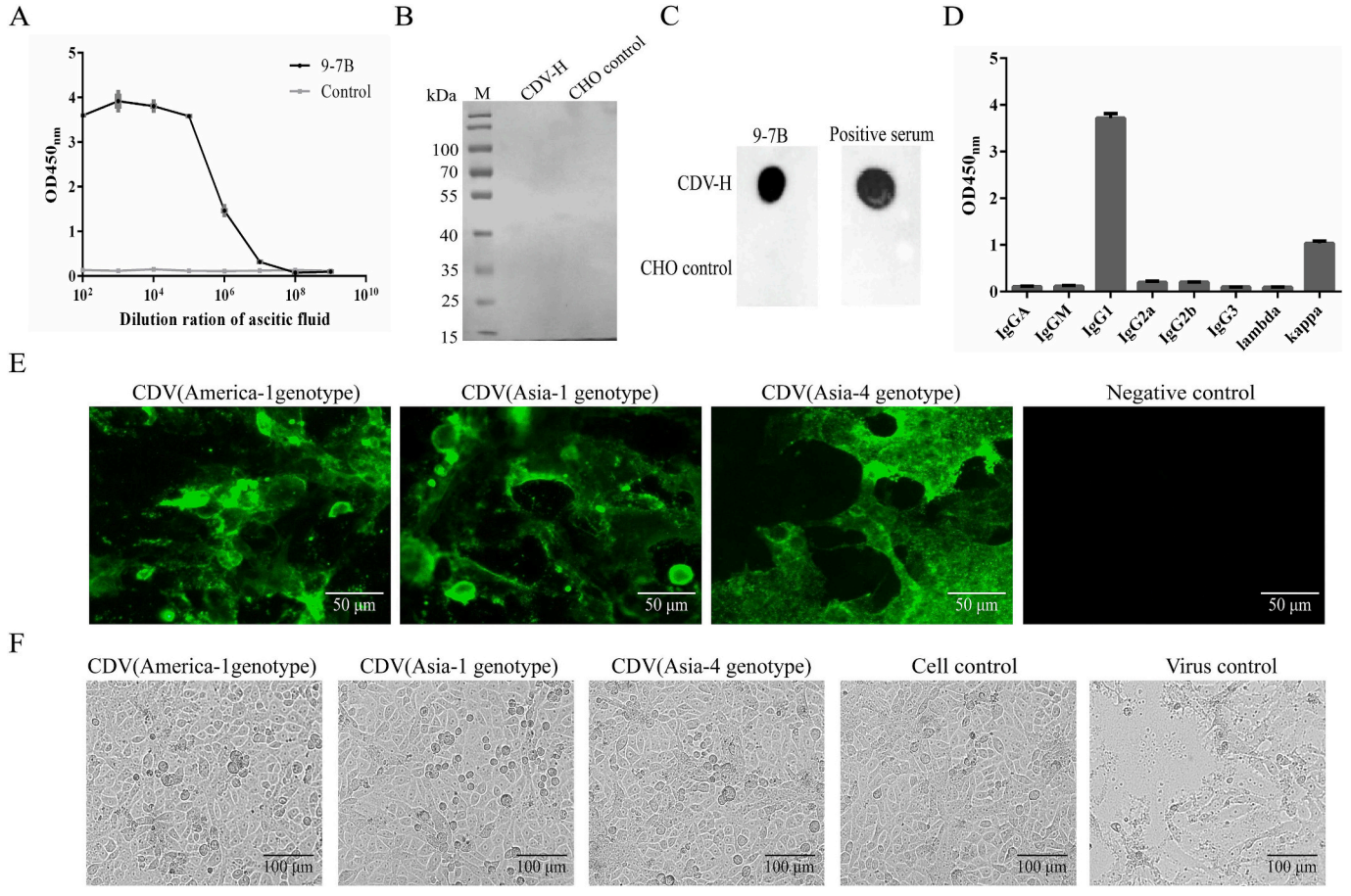

Fig. 2. Validation of monoclonal antibody 9-7B. (A) The ascites titer of the monoclonal antibody 9-7B was determined by indirect ELISA. Means±S.D. of data from three repeated experiments performed. (B) The reaction of the monoclonal antibody 9-7B with the CDV-H protein was verified by WB. Lane 1, Protein marker. Lane 2, Purified recombinant CDV-H protein. Lane 3, Supernatant of CHO Cells without electroporated plasmid as negative control. (C) The reaction of the monoclonal antibody 9-7B with the CDV-H protein was verified by dot blot assays. CDV-H ，Supernatant from untreated CHO high-expressing cell lines expressing CDV-H protein. CHO control, Supernatant of CHO Cells without electroporated plasmid as negative control. (D) Subclass identification of monoclonal antibody 9-7B. Means±S.D. of data from three repeated experiments performed. (E) Validation of monoclonal antibody 9-7B by indirect immunofluorescence. (F) Validation of monoclonal antibody 9-7B by neutralization assay.

results revealed that 9-7B does not recognize the linearized CDV-H protein (Fig. 2B). However, the dot blot assays demonstrated that 9-7B exhibited a specific reaction with the non-denatured CDV-H protein supernatant, which proved that the CDV-H antigen epitope recognized by 9-7B was a spatial conformational epitope (Fig. 2C). Subclass identification confirmed that 9-7B belongs to the IgG1/Kappa subtype (Fig. 2D). To verify the reactivity and broad-spectrum of 9-7B against CDV, indirect immunofluorescence experiments were performed with America-1 genotype CDV strain, Asia-1 genotype CDV strain and Asia-4 genotype CDV strain stored in our laboratory. The results showed that 9-7B reacted specifically against all three strains, which proved its broad-spectrum reactivity (Fig. 2E). The results of the neutralization test showed that the virus control had membrane fusion, which was a typical lesion of CDV, while the cell morphology of the cell control and the experimental group was normal without obvious cellular lesions. The results indicated that 9-7B has a neutralizing effect on all three strains, confirming its good neutralizing activity (Fig. 2F).

3.3. Antigenic epitope identification and conservation analysis

The sequences of the heavy and light chain variable regions of antibody 9-7B were obtained through PCR amplification technology. Since 9-7B recognizes the conformational epitope of CDV-H antigen, molecular docking was performed using AlphaFold3 (Fig. 3A). The docking results indicated that the key antigenic sites recognized by 9-7B were 120Q, 123 N, 126 N, 127P, 129R, 130E, 133F, 155D, 418D (Table S1). The root mean square deviation (RMSD) value can be used to evaluate the stability of dynamic equilibrium. The results of the molecular dynamics showed that 9-7B-CDV-H complex had no significant conformational changes were observed in the RMSD and spatial conformation. This indicated good overall structural stability. In contrast, the control 9-7B-PDCOV-N complex showed larger fluctuations in RMSD throughout the simulation, and exhibited interface drift and even partial dissociation in later stages, supporting its instability as a nonspecific complex (Fig. 3B). The total energy of the 9-7B-CDV-H

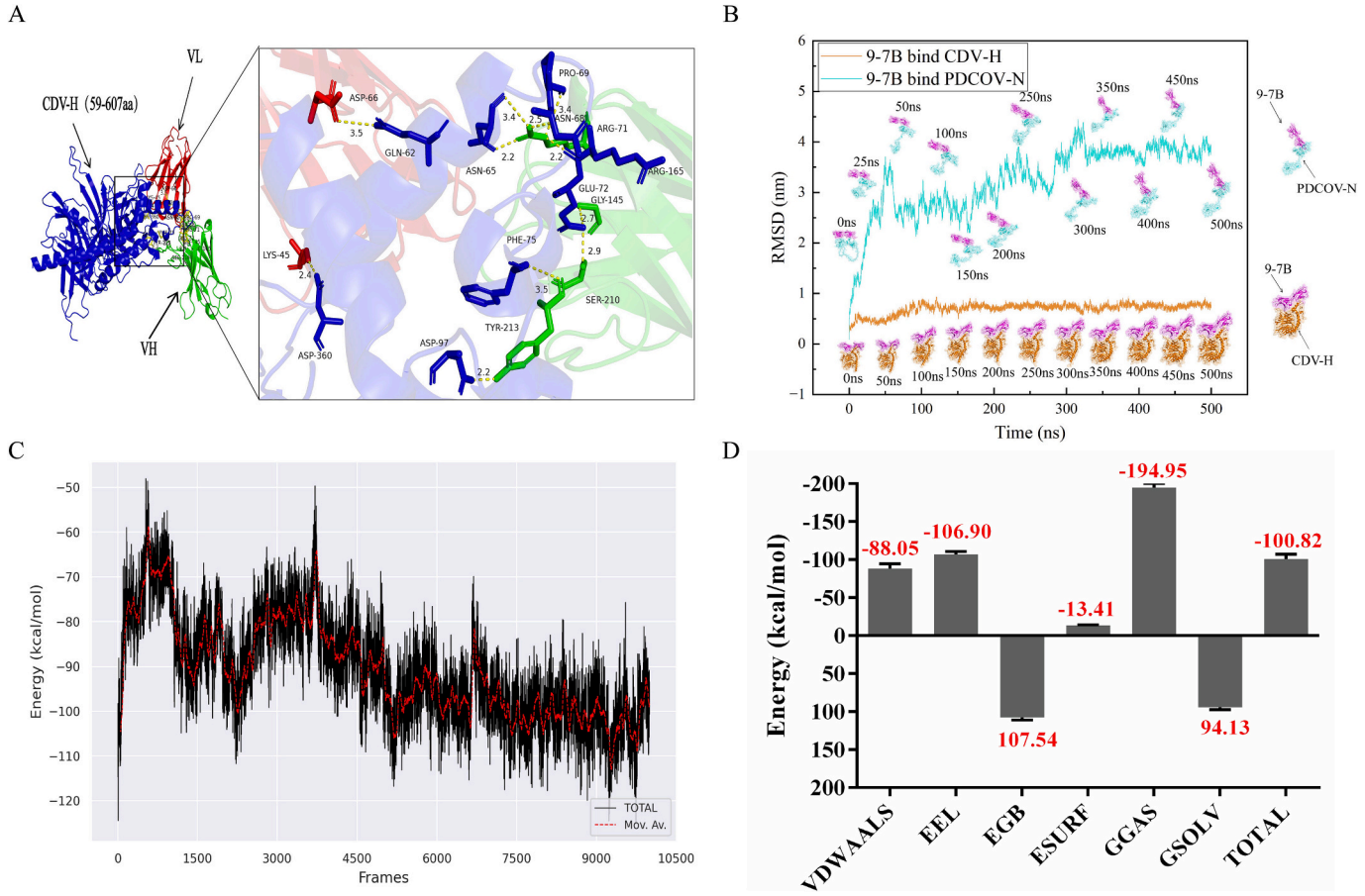

Fig. 3. Sequence analysis and molecular docking of 9-7B. (A) Antigen-antibody molecular docking results. Blue: antigen; red: light chain; blue: heavy chain. The yellow dashed line is the hydrogen bond for antigen-antibody binding. (B) Visualization of RMSD variation curve and conformational change during 500 ns molecular dynamics simulation of 9-7B-CDV-H protein complex and 9-7B-PDCOV-N protein complex. (C) Total energy fluctuation analysis of 9-7B-CDV-H complex. The x-axis was the frame number (representing the interval of 0–500 ns), and the y-axis was the binding free energy value calculated based on different frames. The red curve was a smoothed version of the black curve, obtained by averaging every 50 frames of binding free energy. (D) Interaction energy analysis of 9-7B-CDV-H complex (Calculated from frame 7000 to frame 10,000 in Fig. 3C). VDWAALS (Van der Waals): Van der Waals interaction energy, which describes the dispersive and repulsive forces between hydrophobic molecules. Based on the Lennard-Jones potential function, it includes the induced dipole interactions between atoms. EEL (Electrostatic Energy): Electrostatic interaction energy, which describes the Coulombian attraction and repulsion between charged groups or polar groups, is based on Coulomb's law and is affected by charge distribution and distance. EGB (Generalized Born Solvation Energy): The generalized Born solvation energy describes the electrostatic solvation effect of a molecule in a solution and estimates the polarization in the solvent based on the generalized Born approximation. ESURF (Non-Polar Solvation Energy):The generalized Born solvation energy describes the electrostatic solvation effect of a molecule in a solution and estimates the polarization in the solvent based on the generalized Born approximation. GGAS (Gas Phase Energy):The total energy of the gas phase, usually the sum of VDWAALS and EEL, represents the interaction energy in a vacuum or gas phase, GGAS = VDWAALS + EEL. GSOLV (Solvation Free Energy): Solventization free energy, which is the sum of EGB and ESURF, represents the contribution of solvent effect to binding free energy, GSOLV = EGB + ESURF. TOTAL (Total Binding Free Energy):The total binding free energy, which represents the net energy change of ligand binding to receptor, TOTAL = GGAS + GSOLV = (VDWAALS + EEL) + (EGB + ESURF).

complex fluctuated very little in the later part of the whole and remained stable (Fig. 3C). Interactions exhibit strong electrostatic attraction (EEL) and good van der Waals interactions (VDAWWLS). However, we also found that polar solvation energy (EGB) was unfavorable for the binding of 9-7B-CDV-H, which was offset by electrostatic interactions. This indicated that the binding between 9-7B-CDV-H was primarily dominated by van der Waals interactions. These results supported the rationality of the AlphaFold3 prediction model from an energetic perspective (Fig. 3D). The results of molecular dynamics simulation showed that the predicted molecular docking model had a stable structure. To evaluate the conservation of these key antigenic sites across different CDV-H proteins, a phylogenetic tree of CDV-H proteins was constructed using the adjoining method (Fig. S1). The conservation of the nine key antigenic sites was highly consistent among all CDV strains, ranging from 92.83 % to 100 % (Table S2). Notably, the 155D residue mutated to glutamate in the America-1 vaccine strain, yet monoclonal antibody 9-7B could still neutralize it (Fig. 2F), indicating that the mutation of this antigenic locus did not impair the recognition of America-1 virulent strain (vaccine strain) by 9-7B.

3.4. Determination of the cut-off, specificity, and sensitivity of the blocking ELISA

After optimizing the blocking ELISA method, this study used 30 canine serum, and all serum samples were analyzed by serum neutralization experiment to confirm the antibody status. Later, the canine serum was tested with the blocking ELISA, and the test results were analyzed by the receiver operating characteristics (ROC) curves to determine the cut-off value of the blocking ELISA method. The ROC analysis showed that the area under the curve (AUC) of the established test reached 0.898 with a 95 % confidence interval of 0.761–1.000, indicating a maximum diagnostic sensitivity of 86.70 % when the cut-off value reached 41.23 % (Fig. 4A and B). Therefore, the cut-off value for this blocking ELISA method was 41.23 %, indicating that serum samples were determined to be positive when the blocking ELISA measured a PI value >41.23 %, and negative otherwise. To determine the specificity of the blocking ELISA, Common canine virus (CPV, CPIV, CAV-2, and CDV) positive sera were tested. The results indicated that all sera except CDV-positive sera were negative, indicating the good specificity of the established blocking ELISA (Fig. 4C). Sensitivity analysis revealed that blocking ELISA had a sensitivity of 1:1024 (Fig. 4D).

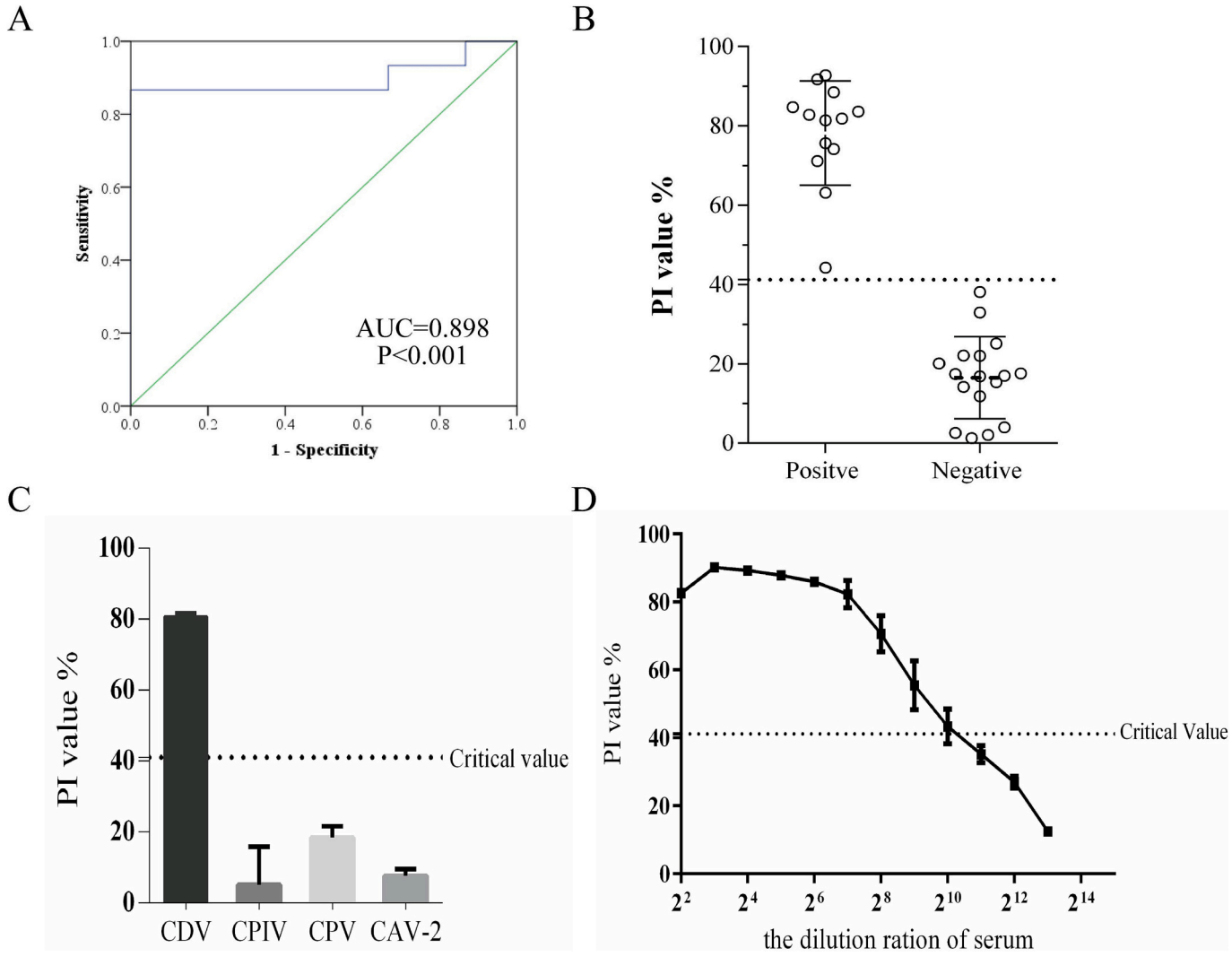

Fig. 4. Cut-off value, sensitivity, and specificity analysis for blocking ELISA. (A) ROC curve analysis of blocking ELISA. ROC analysis was calculated for 13 known positive serum samples and 17 known negative serum samples collected. Data from three repeated experiments performed. (B) Interactive point analysis of PI value of serum samples. Data from three repeated experiments performed. (C) Specificity analysis of blocking ELISA. Means±S.D. of data from three repeated experiments performed. (D) Sensitivity analysis of blocking ELISA. Means±S.D. of data from three repeated experiments performed.

3.5. Correlation analysis of blocking ELISA methods with neutralization tests

By comparing the PI values of the blocking ELISA to the neutralization experimental titers, the results indicated a statistical correlation between two, with a correlation coefficient of 0.6905 (Fig. 5A). According to the antibody PI values, clinical sera were divided into 5 groups: 45 %–55 %, 55 %–65 %, 65 %–75 %, 75 %–85 %, and 85 %–95 %. The mean value of serum neutralization potency within each group was calculated, the correlation analysis was performed again, and the correlation coefficient was as high as 0.9272 (Fig. 5B). Overall, there was a strong positive correlation between the PI values in blocking ELISA and the neutralizing titer in neutralization tests, with the neutralizing titer in serum increasing as the blocking rate increases. It demonstrated that the blocking ELISA method established in this study could preliminarily assess the neutralization potency of the serum from vaccine-immunized dogs. Specifically, when the PI value was greater than the critical threshold of 41.23 %, the neutralization potency was >1:16, and a higher PI value corresponded to a higher neutralization potency, which provided a convenient and reliable method for evaluating vaccine immunization effect.

4. Discussion and conclusion

CDV induced CD is a highly infectious, multi-host, morbidity and mortality viral disease that causes serious economic losses to the pet market and to farms of dogs, foxes and minks [17,18]. Currently, no approved drugs exist for the treatment of CD, making vaccination is the primary means of controlling CD. Therefore, the design of effective vaccines and evaluation of their immunization effects are crucial [19].

Accurate resolution of antigenic epitopes and structural information can facilitate the development of vaccine design [20]. The CDV-H protein is essential for CDV to infect the host and can elicit the production of neutralizing antibodies [21]. Various studies have reported linear non-neutralizing epitopes of CDV-H, such as ^{87}QVIDVLTPLFK^{97}, ^{120}QKTNFFNPNREFDFR^{134}, and ^{178}ARGDIFPPY^{186}, as well as linear neutralizing epitopes such as ^{126}FNPNREFD^{133}, ^{193}TSVGRFFPL^{201}, ^{238}DIEREFD^{244}, and ^{238}DIEREFDT^{245} [22–25]. However, no spatial conformational epitopes targeting CDV-H proteins have been reported to date. In this study, a monoclonal antibody 9-7B that recognizes a spatial conformational epitope of the CDV-H protein was developed. Molecular docking revealed the key antigenic sites for CDV-H recognition by 9-7B to be 120Q, 123 N, 126 N, 127P, 129R, 130E, 133F, 155D, and 418D. Taken together, the structural and energetic analyses from MD simulations indicate that the 9-7B-CDV-H complex exhibits superior conformational and thermodynamic stability compared to the non-specific control. These results validate the credibility of the AlphaFold3-predicted binding model and provide a solid structural basis for further mechanistic investigation and antibody engineering efforts. These nine key antigenic sites are highly conserved across all CDV strains, with a conservation rate ranging from 92.83 % to 100 %, which not only provides precise antigenic site information for vaccine design, but also elucidates the broad-spectrum recognition effect of the antibody 9-7B developed in this study. Meanwhile, blocking ELISA indicated that the epitope recognized by the neutralizing antibody could be simultaneously recognized by the antibodies immunized by multiple genotypes of vaccines. Therefore, the epitope recognized by this antibody could serve as a target site for the evaluation of neutralization effect.

The immunizing effect of a vaccine is assessed by the level of neutralizing antibodies in the animal. Viral neutralization tests are considered the “gold standard” for assessing the level of neutralizing antibodies, but they are time-consuming, labor-intensive, and exhibit variable sensitivity [26,27]. Alternatively, the ELISA method, which is simple, rapid, sensitive and low cost, has been proposed as a viable alternative to VNTs [28]. However, in the field of CD antibody detection, most ELISA methods developed thus far target antigens or whole antibodies, failing to reflect the immune protective ability of the antibody toward the organism. Canine vaccination typically induces high-titer neutralizing antibody responses that confer lifelong immunity. The neutralization titer of 1:32 is conventionally considered the protective threshold against CDV infection [29,30]. Therefore, in this study, a CDV neutralizing antibody strain 9-7B was developed, and established a blocking ELISA method. The cut-off value in the blocking ELISA method was 41.23 %, which the serum neutralizing antibody titer was 1:16. The serum neutralizing antibody titer was 1:32–1:64 when the PI value was between 45 % and 75 %, the serum neutralizing antibody titer was 1:128–1:256 when the PI value was between 75 % and 85 %, the serum neutralizing antibody titer was 1:256–1:512 when the PI value was between 85 % and 95 %. These can be determined whether the animal's neutralizing antibody levels can provide lifelong protection and vaccine efficacy.

The R^2 before grouping was 0.6905 and the R^2 after grouping was as high as 0.9272. It can be seen that the antibody levels shown by blocking ELISA were positively correlated with neutralizing antibody titers, but not precisely, but the overall trend was consistent through the group analysis. The reason for the low correlation coefficient may be due to differences in samples, variations between ELISA and neutralization tests, and insufficient test samples. Future research will focus on enhancing sample quality control to ensure consistency in conditions across groups and expanding sample sizes to optimize data. Of course, this study also had some limitations. When the PI value was >85 %, the neutralization valence growth may slow down because of antibody binding saturation, and it was difficult to accurately distinguish high antibody level samples. The H protein used in this study was the Asia-1 type popular in China, and if the antibody level of other genotypes was tested, the H protein of the corresponding genotype strain was required

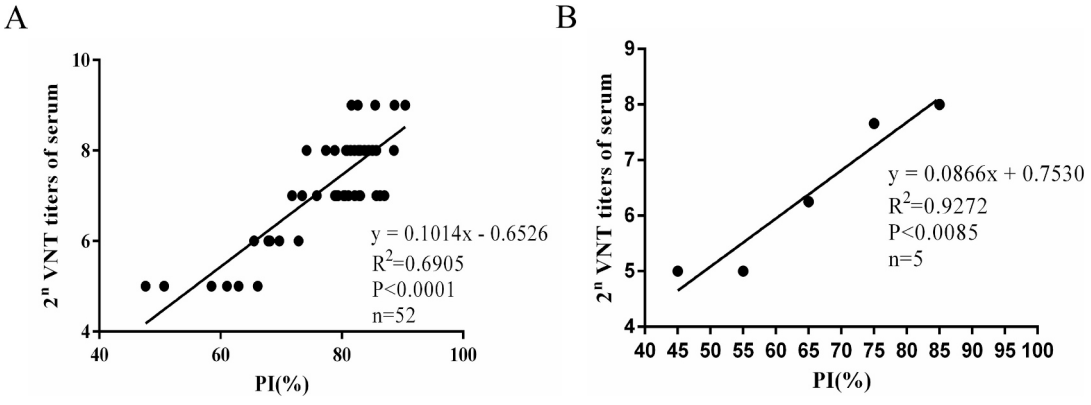

Fig. 5. Correlation between the antibody titers determined by VNT and the blocking ELISA. (A) Correlation analysis of PI value and neutralization antibody titers. Means±S.D. of data from three repeated experiments performed. (B) PI value and neutralization antibody titers analysis after grouping.

as the envelope.

In conclusion, this study developed a neutralizing monoclonal antibody 9-7B against CDV-H protein and identified highly conserved key amino acids within this protein, thereby enriching the understanding of immune epitopes in CDV-H protein. Additionally, a blocking ELISA was successfully developed for the detection of neutralizing antibodies against CDV in vaccine-immunized dogs. This blocking ELISA exhibits high sensitivity and specificity, serving as a valuable tool for evaluating vaccination effectiveness.

CRediT authorship contribution statement

Zhengguo Wang: Investigation. **Kuijing He:** Methodology. **Guishan Ye:** Methodology. **Shuang Jiang:** Methodology. **Linlin Mu:** Methodology. **Cong Cai:** Methodology. **Yue zeng:** Investigation. **Anding Zhang:** Data curation, Conceptualization. **Long Li:** Writing – review & editing, Funding acquisition, Methodology. **Li Han:** Writing – review & editing, Data curation, Funding acquisition.

Statement

During the preparation of this work, the authors used Ernie Bot in order to improve the readability and language of the manuscript. After using Ernie Bot, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Ethics approvals

All animal experiments in this study followed the ethical guidelines for animal experiments of the Laboratory Animal Center of Huazhong Agricultural University and were approved by the Animal Ethics Committee of the Laboratory Animal Center of Huazhong Agricultural University (approval number:HZAUMO-2025-0018).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.145622.

Data availability

Data will be made available on request.

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