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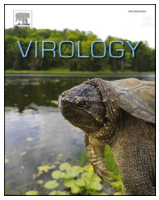

Research on the construction method and characterization of neutralizing mouse-canine chimeric antibody against canine distemper virus

Zhengguo Wang [a,b], Guishan Ye [a,b], Leilei Zhang [a,b], Kuijing He [a,b], Cong Cai [a,b], Yue Wu [a,b], Anding Zhang [a,b,c,d], Long Li [a,b,c,d,*], Li Han [a,b,c,d,**]

[a] National Key Laboratory of Agricultural Microbiology, Hubei Hongshan Laboratory, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei, 430070, China
[b] Key Laboratory of Preventive Veterinary Medicine in Hubei Province, The Cooperative Innovation Center for Sustainable Pig Production, Wuhan, Hubei, 430070, China
[c] Key Laboratory of Development of Veterinary Diagnostic Products, Ministry of Agriculture of the People's Republic of China, Wuhan, Hubei, 430070, China
[d] International Research Center for Animal Disease, Ministry of Science and Technology of the People's Republic of China, Wuhan, Hubei, 430070, China

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ABSTRACT

Canine distemper (CD) is an acute infectious disease that poses significant health risks to canines. Neutralizing monoclonal antibody (mAb) therapy has demonstrated substantial efficacy in prevent CDV infection. However, immune rejection reactions prevent the use of mouse-derived mAbs in the prophylactic protection of CD. Based on the previously developed neutralizing mAb 9-7B targeting the CDV-H protein, this study successfully utilized both single and dual expression vector strategies to integrate the variable regions of mouse-derived mAb with the constant regions of canine-derived mAb, yielding M-C-H/L and M-C-L-H mouse-canine chimeric antibodies through the CHO expression system. The SDS-PAGE and WB results indicate that both chimeric antibodies possess the correct antibody structure. Both chimeric antibodies specifically recognized the CDV-H protein and CDV in indirect ELISA and indirect immunofluorescence experiments and their neutralizing effect on CDV was confirmed in neutralization assays. This study constructed mouse-canine chimeric antibodies against CDV using two methods for the first time, which provides significant guidance for research on prophylactic CD antibody-based drugs.

1. Introduction

Canine distemper (CD) is an infectious disease caused by canine distemper virus (CDV) that seriously jeopardizes the health of canines, and is highly contagious, with a high infection rate and high mortality (Cai et al., 2025; Zhang et al., 2020). Affected dogs initially exhibit symptoms such as fever, anorexia, watery discharge from the eyes and nose, and diarrhea, which progressively worsen in later stages (Li et al., 2024; Rivera-Martínez et al., 2024). Notably, widespread use of live-attenuated CDV vaccines has significantly reduced distemper mortality in domestic dogs, underscoring vaccination as the cornerstone of prevention (Gulliver et al., 2025). However, numerous reports have shown that a certain proportion of vaccinated dogs still become infected with CDV (Gulliver et al., 2025), potentially due to antigenic divergence between vaccine strains and field variants, maternal antibody interference, or inadequate individual immune responses. Therefore, despite the critical role of vaccination in reducing CDV prevalence, therapeutic interventions for breakthrough infections remain a significant challenge in veterinary medicine.

Current CDV prevention strategies remain limited: while pre-exposure or early post-exposure administration of CDV-specific monoclonal antibodies (mAbs) or hyperimmune serum can be effective, these approaches face major drawbacks. Conventional antiserum is costly, difficult to standardize, and carries risks of viral contamination (Zhang et al., 2021), while mouse-derived mAbs trigger immune rejection in heterologous species, reducing therapeutic efficacy (Li et al., 2024). Critically, neither modality efficiently penetrates the blood-brain barrier, leaving neurologic cases untargeted. These limitations underscore

* Corresponding author. National Key Laboratory of Agricultural Microbiology,Hubei Hongshan Laboratory, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei 430070, China.
** Corresponding author. National Key Laboratory of Agricultural Microbiology,Hubei Hongshan Laboratory, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei, 430070, China.
E-mail addresses: lilong@mail.hzau.edu.cn (L. Li), hanli209@mail.hzau.edu.cn (L. Han).

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the urgent need for next-generation biologics such as chimeric antibodies, which could offer enhanced neutralization potency, species compatibility, and tissue penetration to bridge the gap between prevention and therapy (Bergmann et al., 2021; Zhou et al., 2022).

Based on the previously prepared mouse-derived mAb 9-7B (Wang et al., 2025b), which targets the CDV-H protein and possesses potent CDV-neutralizing activity, this study constructed mouse-canine chimeric antibodies using two strategies. Expression of these two chimeric antibodies was achieved using the CHO cell system, and two mouse-canine chimeric antibodies exhibited favorable biological properties, laying the groundwork for further clinical development in the prophylactic protection of CDV disease.

2. Materials and methods

2.1. Cells, monoclonal antibodies and strains

CDV-H protein, Neutralizing mAb 9-7B, CHO cell line, and Vero-slam cells were stored in our laboratory (Wang et al., 2025b); CDV (Asia-1) isolates were isolated, purified, and kept by our laboratory (Cai et al., 2019). The HRP-conjugated goat anti-mouse IgG and HRP-conjugated rabbit anti-canine IgG were purchased from Biodragon (Suzhou, China), and Alexa Fluor 488-labeled goat anti-mouse IgG was purchased from Beyotime (Shanghai, China). Sequences of the canine heavy chain constant region were obtained from NCBI GenBank: AF354266.1 and sequences of the canine light chain constant region were obtained from NCBI GenBank: CP050603.1.

2.2. Sequencing of heavy chain variable region (VH) and light chain variable region (VL)

Total RNA of mAb 9-7B cell line was extracted by TRIzol reagent and reverse transcribed to cDNA as template. The heavy and light chains (VH and VL) gene fragments were amplified by PCR using universal primers (Table S1) set for mouse-derived antibodies (PCR conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s, with a final extension at 72 °C for 5 min). The correct gels of the target fragments were cut off and sent to sequencing. The VH and VL sequences of the mAb, CH and CL of the canine-derived antibody were CHO optimized to synthesize the sequences. Optimization parameters included: maximization of codon usage frequency based on CHO preferences; elimination of rare codons with <10 % frequency, adjustment of GC content to 40–60 %, and avoidance of extreme GC regions and extended mononucleotide/dinucleotide repeats. By homologous recombination, the fragments and vectors were ligated, fused, transformed, picked bacteria identified and sent for sequencing to construct the chimeric antibody plasmid.

2.3. Construction of highly expressed CHO cell lines stably expressing chimeric antibodies

The chimeric antibodies were designed as IgG class, subclass IgG-C, with canine constant regions derived from GenBank: AF354266.1 and CP050603.1 to maintain Fc-mediated effector functions while minimizing immunogenicity. As shown in Fig. 1, the variable regions of the mouse-derived antibody heavy and light chains (VH and VL) were respectively fused with the constant regions of canine-derived antibody heavy and light chains (CH and CL) to generate chimeric antibodies. In this study, two strategies were employed to construct mouse-canine chimeric antibody plasmids. The first strategy employed a polycistronic vector system, wherein the mouse-derived VL and VH regions were individually fused with canine-derived CL and CH regions, respectively, and subsequently linked via a T2A peptide (Fig. 1). This construct was then cloned into the PXC17.4 vector for chimeric antibody expression in CHO cells. The second strategy utilized a dual-plasmid system for separate expression of antibody light and heavy chains. In this approach, the mouse-derived VL and VH regions were separately fused with canine-derived CL and CH regions and cloned into two distinct PXC17.4 plasmids. These two plasmids were then co-transfected at a 1:1 ratio into the same CHO cells via electroporation to facilitate chimeric antibody expression (Fig. 1). After 48h post-transfection, high-expressing CHO cell lines were selected using 15 μmol methionine sulphoximine (MSX). The vector construction was primarily performed using PCR amplification and homologous recombination techniques, with the corresponding primer information detailed in Table S2. The chimeric antibody for the first scheme was named M-C-L-H, and the chimeric antibody for the second scheme was named M-C-H/L.

Expressed cell lines were detected by dot blot crosses with rabbit anti-canine IgG-HRP against the chimeric antibody. Neutralization experiments were also performed to verify whether the neutralizing effect of the chimeric antibodies. Chimeric antibodies were heavily expressed and purified using Protein A + G. The binding buffer and elution buffer used during the purification process are 20 mM PBS and 0.1 M glycine-HCl (PH2.7) respectively, and neutralized with 1 M Tris-HCl (pH9.0). The chimeric antibodies were characterized by reducing and non-reducing SDS-PAGE and Western blot (WB) to determine the

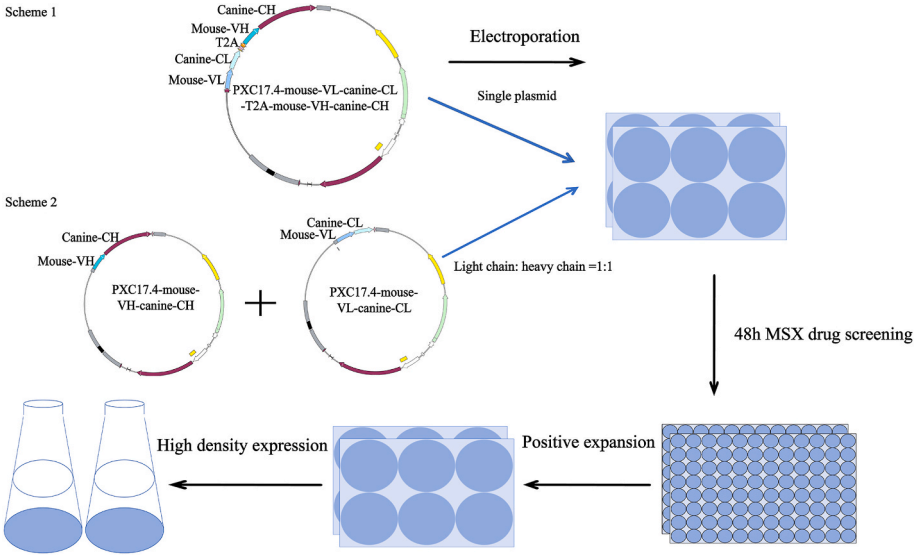

Fig. 1. Schematic design of the chimeric antibody construction.

purification effect and reactivity of the chimeric antibodies expressed by CHO cells.

2.4. Indirect ELISA

The CDV-H protein was expressed and purified in our laboratory as previously described (Wang et al., 2025b) and was coated onto ELISA plates and incubated overnight at 4 °C. The blocking solution was incubated at 37 °C for 1 h. The initial concentration of the chimeric antibody was adjusted to 1 mg/mL, and the chimeric antibody was diluted with 1:100 as the starting point, 2-fold ratio dilution. CHO cell supernatant without electrotransferred plasmid was used as negative control. Incubation was performed at 37 °C for 1h. HRP-conjugated rabbit anti-canine IgG was diluted 1:10000 as secondary antibody. Incubation was performed at 37 °C for 1h. TMB was added to 37 °C dark incubation for 10min, terminated with 2M H_2SO_4, and read the $OD450_{nm}$ reads. The ELISA titer was determined as the maximal dilution that yielding an OD450 value $\geq$ 2.1-fold higher than the negative control. All ELISA assays were performed with $\geq$3 independent biological replicates, with data presented as mean ± SD.

2.5. Neutralization experiment

The chimeric antibodies were diluted to an initial concentration of 1 mg/mL, followed by two-fold serial dilutions. Each diluted antibody preparation was mixed with viral solution at a 1:1 vol ratio (50 μL antibody + 50 μL virus), followed by incubation at 37 °C for 1h. Then, the mixture (100 μl, with 4 replicates) was added to 96-well plates in which Vero-slam cells (2×10^4 cells/100 μl) had been previously added. The 96-well plates were placed in 5 % carbon dioxide and were incubated at 37 °C. The results were observed daily and counted after 5 days.

2.6. Indirect immunofluorescence (IFA)

Neutralization experiments were performed first and observed for 48 h. The cell plates were subjected to indirect immunofluorescence assay and the cells were fixed with 4 % paraformaldehyde. The cell plates were permeabilized and closed. The primary antibody was mouse CDV-H protein mAb 9-7B, and the secondary antibody was goat anti-mouse FITC fluorescent secondary antibody. The results were observed under fluorescence microscope.

3. Results

3.1. Construction of chimeric antibody expression plasmid

The heavy chain variable region (VH) and light chain variable region (VL) of the mouse-derived mAb 9-7B were amplified (Fig. 2A). Subsequent to sequencing, CHO codon optimization synthesis was performed, and the expression plasmids were constructed in accordance with the methodology outlined in Fig. 1, named PXC17.4-M-C-H, PXC17.4-M-C-L, and PXC17.4-M-C-L-H, respectively. Ultimately, all three plasmids were successfully constructed (Fig. 2B).

3.2. Expression and purification of mouse-canine chimeric antibodies

The constructed cell lines were expressed and purified, and the chimeric antibodies were characterized. SDS-PAGE analysis under reducing and non-reducing conditions revealed typical light and heavy chain sizes for the chimeric antibodies, indicating that they could form complete antibody molecules through covalent disulfide bond formation (Fig. 3A and B). In the M-C-L-H construct, the light chain is expressed as a VL + CL + T2A fusion, where the T2A peptide undergoes cotranslational cleavage between its C-terminal glycine (G) and proline (P). This results in a light chain product retaining the T2A peptide (calculated MW: ~29.33 kDa), compared to the standalone VL + CL (26.95 kDa) in the M-C-H/L construct. The observed higher apparent MW (~35 kDa) in SDS-PAGE may arise from partial glycosylation or altered electrophoretic mobility due to the T2A sequence. Notably, the size difference confirms successful T2A-mediated processing. WB results under reducing and non-reducing conditions showed that the corresponding regions of the chimeric antibody had been successfully replaced with those of the canine (Fig. 3A and B). Both M-C-H/L and M-C-L-H chimeric antibodies achieved a concentration of 300 mg/L.

3.3. Indirect ELISA for the determination of chimeric antibody potency

To determine whether the chimeric antibody had antibody activity, indirect ELISA was performed. The results showed that the ELISA titer of M-C-H/L was 1×10^{-5} mg/mL, and the ELISA titer of M-C-L-H was 4.88×10^{-6} mg/mL. The chimeric antibody can react specifically with the CDV-H protein (Fig. 4).

3.4. Specific identification of chimeric antibodies

Due to the suboptimal performance of HRP-conjugated canine

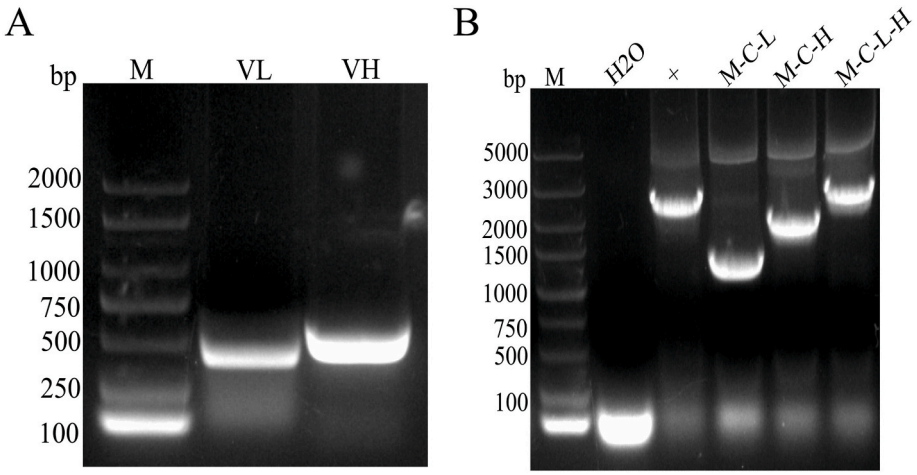

Fig. 2. Plasmid construction of chimeric antibodies. (A) The VH and VL of mAb 9-7B were amplified by PCR. The target fragments of VL and VH are approximately 500 bp. (B) The PCR identification of the plasmid. H_2O was used as the negative control. The fragment sizes of the positive controls, M-C-L, M-C-H, and M-C-L-H, were approximately 2300 bp, 1300 bp, 2000 bp, and 2800 bp, respectively.

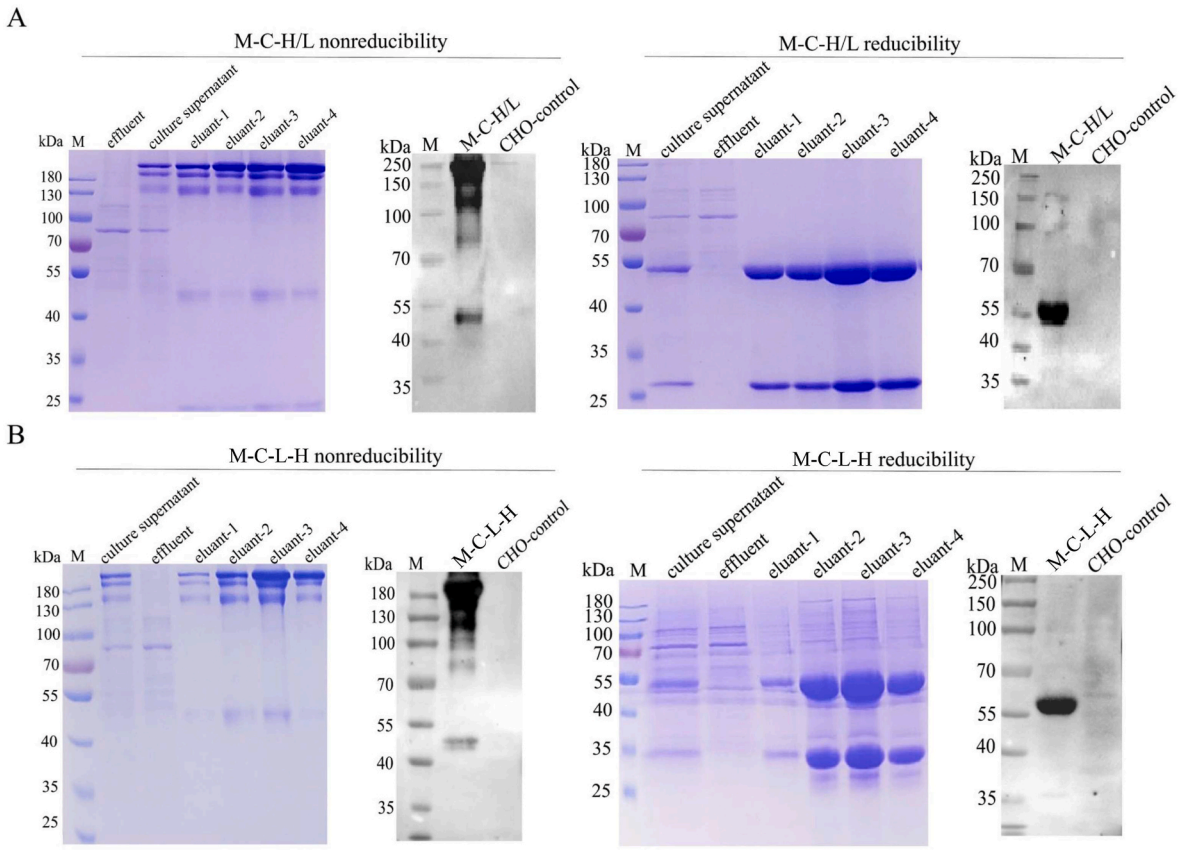

Fig. 3. Identification of mouse-canine chimeric antibody by SDS-PAGE and WB. (A) The structural integrity of M-C-H/L was verified by reduced/non-reduced SDS-PAGE and WB analysis. M:protein marker. Culture supernatant: The culture supernatant was harvested from high-density CHO cells expressing a chimeric antibody. Effluent: the eluate containing impurity proteins was washed with 20 mM PBS (pH7.0). Eluant-1-4: The chimeric antibody was eluted with 0.1 M Gly-HCl (pH2.7) and neutralized with 1 M Tris-HCl (pH9.0). (B)The structural integrity of M-C-L-H was verified by reduced/non-reduced SDS-PAGE and WB analysis. The legend information of Figure A and Figure B is the same.

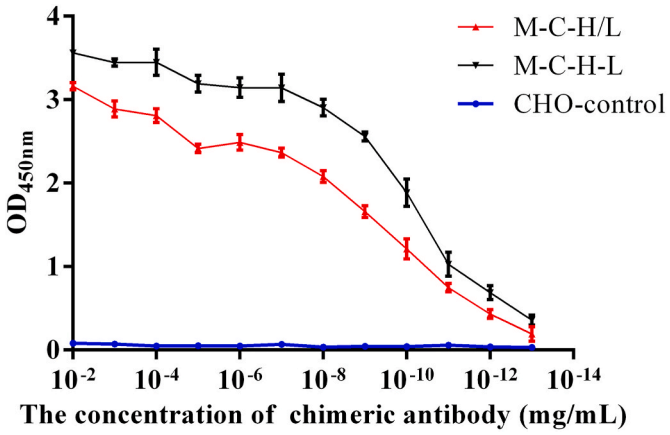

Fig. 4. Reactivity analysis of mouse-canine chimeric antibody against CDV-H protein.

antibodies, the reactivity of the chimeric antibodies with CDV was determined by immunofluorescence experiments. A neutralization assay was performed by incubating the mouse-canine chimeric antibody with CDV, followed by detection using HRP-conjugated mouse-derived mAb 9-7B to quantify CDV. The result indicated that the mouse-canine chimeric antibody has neutralized CDV, resulting in no cellular lesions and therefore no specific reaction with the antibody (Fig. 5). These results proves that the mouse-canine chimeric antibody can be correctly folded and assembled into an active antibody protein.

3.5. Neutralization experiments with chimeric antibodies

The neutralizing activity of chimeric antibodies against CDV was determined using a cellular neutralization assay. The results showed that the neutralizing potency of the M-C-H/L chimeric antibody reached 4×10^{-3} mg/mL and that of the M-C-L-H chimeric antibody reached 1.56×10^{-2} mg/mL (Fig. 6). It indicated that these chimeric antibodies were neutralizing antibodies that could effectively bind to CDV and block its infection of cells. It suggested that mammalian systemically expressed chimeric antibodies retained the neutralizing activity of the original mAb against CDV. These chimeric antibodies demonstrate potential for early post-exposure prophylaxis when administered within the critical 48–72 h window following CDV exposure, and may be developed into biologics for pre-exposure prevention of infection.

4. Discussion

CD is a febrile, highly contagious infectious disease that affects mammals across more than 20 families, including canids and felids (Chen et al., 2023; Dong et al., 2021). While live-attenuated CDV vaccines are the cornerstone of distemper prevention, emerging genotypes may reduce vaccine efficacy in specific cases, highlighting the need for adjunct therapies (Gulliver et al., 2025; Khosravi et al., 2025; Shi et al., 2025). Therefore, active and effective prophylactic protection is essential once CD occurs in pet dogs. Traditional mouse-derived mAbs are limited in their early post-exposure intervention efficacy due to their inability to bind canine Fc receptors, often triggering xenogeneic immune responses (Li et al., 2024; Zhou et al., 2022). Consequently, the development of canine-derived neutralizing antibodies targeting CDV

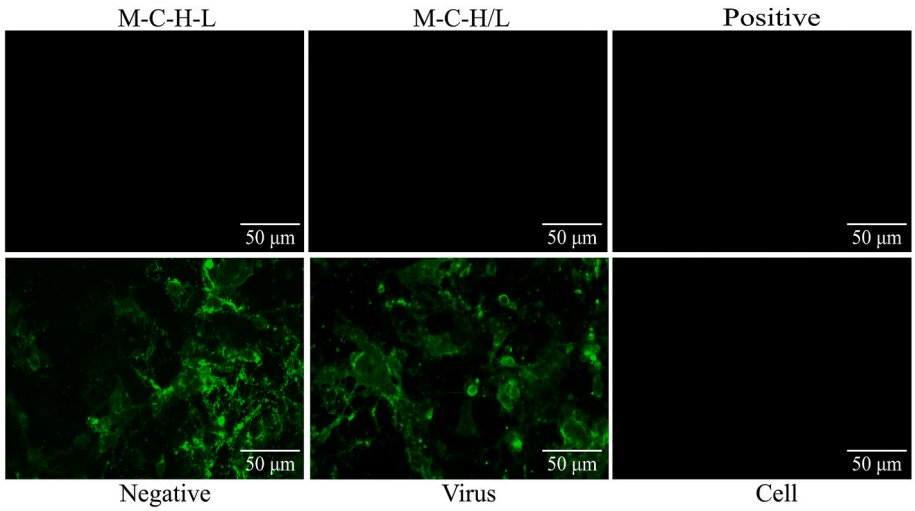

Fig. 5. Analysis of the reactivity of mouse-canine chimeric antibodies with CDV. M-C-L-H and M-C-H/L group: chimeric antibody + CDV pre-incubation group; Positive group: mAb 9-7B + CDV pre-incubation group; Negative group: PBS + CDV pre-incubation group; Virus group: CDV-infected cells; Cell group: uninfected cells.

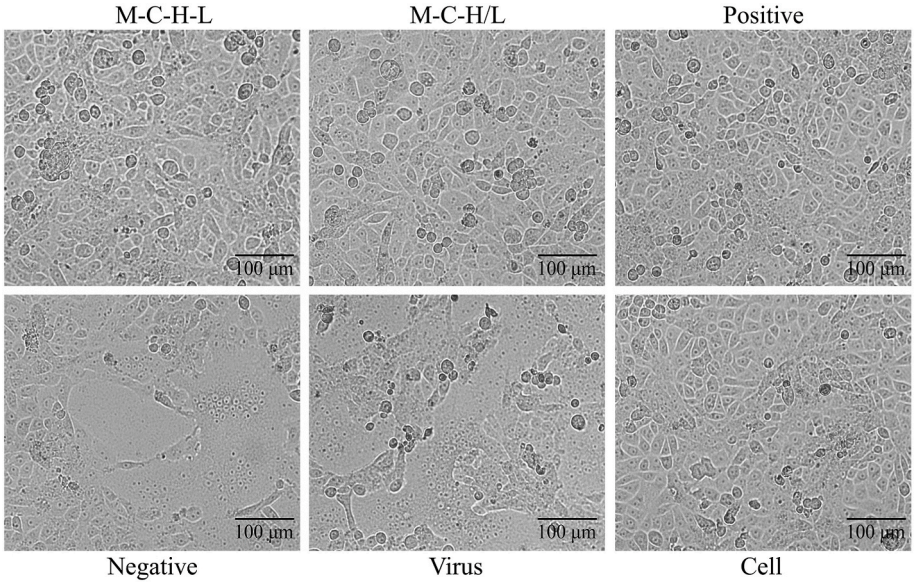

Fig. 6. Analysis of neutralizing activity of mouse-canine chimeric antibody. M-C-L-H and M-C-H/L group: chimeric antibody + CDV pre-incubation group; Positive group: mAb 9-7B + CDV pre-incubation group; Negative group: PBS + CDV pre-incubation group; Virus group: CDV-infected cells; Cell group: uninfected cells.

infection is of paramount importance. In antibody engineering, chimeric antibodies were constructed by fusing the variable regions of mouse heavy and light chains with the constant regions of canine heavy and light chains. These chimeric antibodies retain the antigen-binding specificity of the parental mouse antibodies while reducing their immunogenicity, thereby enhancing stability in vivo(DeLuca et al., 2021). Compared to other expression systems, mammalian cell expression systems, such as CHO cells, produce recombinant antibody molecules with structural and glycosylation patterns more closely resembling those of natural antibodies. This ensures correct antibody conformation, preserves biological activity, and facilitates large-scale suspension or serum-free culture(Kunert and Reinhart, 2016; Wang et al., 2025a; Zhang et al., 2022). Leveraging these advantages, this study constructed mouse-canine chimeric antibodies and expressed them using the CHO expression system.

In this study, The IgG-C subclass was chosen due to its Fc domain properties that optimally balance Fc-mediated ADCC activity with extended serum persistence, characteristics essential for effective CDV prevention requiring sustained protection. Currently, various vector design strategies have been developed for mAb expression in CHO cells. These include co-transfecting separate vectors encoding light chain (LC) and heavy chain (HC) genes or employing a single vector with a polycistronic vector system to drive LC and HC expression (Yang et al., 2024). This study utilized both vector systems to transfect CHO-K1 cells. Previous studies have suggested that overexpression of LC relative to HC in polycistronic vector system can enhance mAb expression (Ebadat et al., 2017). Therefore, an LC-T2A-HC arrangement was adopted in this study. Interestingly, the results revealed comparable antibody expression levels between the two vector systems, contrary to reports indicating significantly higher expression in polycistronic vector system (Li et al., 2018). This discrepancy may be attributed to the use of limiting dilution cloning during screening, where only high-expressing clones were selected, potentially masking differences in expression levels between the systems. Thus, both systems proved viable for obtaining

high-expressing CHO cell lines.

It has been reported that the Asia-1 strain is the predominant epidemic lineage of CDV in China (Doan et al., 2025). Furthermore, we previously developed *MAVS*-inactivated MDCK cell lines, which significantly enhance the propagation efficiency of CDV (Cai et al., 2019). Notably, all CDV strains successfully isolated from veterinary hospitals in our study were identified as Asia-1. Therefore, this study primarily employed the Asia-1 strain to evaluate the neutralizing activity of the chimeric antibodies. In terms of antibody activity, chimeric antibodies from both systems specifically recognized CDV-H protein and CDV, demonstrating neutralizing effects in neutralization assays. The ELISA assay measures the binding capacity of the chimeric antibody to the CDV-H protein, whereas the VN assay detects the chimeric antibody's ability to specifically block CDV entry. The high ELISA titer indicates strong binding affinity between the chimeric antibody and the target protein (Fig. 4). However, the low VN titer demonstrates that the local antibody concentration required for effective neutralization is substantially higher than that needed for simple antigen binding (Figs. 5 and 6). Similar discrepancies are well-documented for other viruses. For example, anti-PRRSV Mabs showed 4900-fold higher ELISA than VN titers (Sun et al., 2025), as neutralization demands precise epitope targeting and steric hindrance. The dual-vector system exhibited a fourfold higher neutralizing activity compared to the polycistronic vector system. This difference may be due to the incomplete "self-cleaving" efficiency of T2A, leading to the production of fusion or truncated proteins that compromise antibody functionality (Chng et al., 2015).

In summary, this study successfully constructed mouse-canine chimeric antibodies using two distinct strategies and established high-expressing CHO cell lines for their production. The chimeric antibodies exhibited correct structural integrity and biological activity, specifically recognizing and neutralizing CDV. These findings provide valuable insights for the development of prophylactic CDV antibody drugs targeting early-stage infection.

CRediT authorship contribution statement

Zhengguo Wang: Writing – original draft, Supervision, Methodology, Data curation, Conceptualization. **Guishan Ye:** Methodology. **Leilei Zhang:** Validation, Writing – review & editing. **Kuijing He:** Methodology. **Cong Cai:** Methodology. **Yue Wu:** Methodology. **Anding Zhang:** Methodology, Data curation, Conceptualization. **Long Li:** Writing – review & editing, Methodology, Funding acquisition. **Li Han:** Writing – review & editing, Funding acquisition, Data curation.

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.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence 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.virol.2025.110667.

Data availability

Data will be made available on request.

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