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Monoclonal antibody against canine distemper virus H protein potently neutralizes the giant panda-derived strain

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Abstract: Canine distemper virus (CDV) is a multi-host pathogen with high morbidity and mortality, posing a severe threat to endangered carnivores, including giant pandas. Current vaccines carry risks of vaccine-induced disease in non-target species, creating an urgent need for safe and effective therapeutic alternatives. The hemagglutinin (H) protein is the primary antigen for stimulating neutralizing antibodies. In this study, we generated three murine monoclonal antibodies (mAbs)—2D1, 6G4, and 6H4—using the H protein from a giant panda-derived CDV strain expressed in a mammalian system to preserve authentic glycosylation and conformation. All three mAbs exhibited potent neutralizing activity against the giant panda-derived CDV strain *in vitro*. Western blot analysis revealed distinct epitope specificities: mAb 2D1 recognized a continuous epitope, as it bound to both eukaryotic and prokaryotically expressed H protein, whereas mAbs 6G4 and 6H4 only reacted with the eukaryotic-expressed protein, suggesting their recognition of non-continuous, conformational epitopes. These data indicate that these mAbs represent promising immunotherapeutic candidates for the control of CDV in giant pandas and other susceptible wildlife.

Keywords:

Canine distemper virus; Monoclonal antibody; Virus neutralizing activity; Giant panda

Introduction

Canine distemper (CD) is an acute, virulent, and highly contagious infectious disease caused by the canine distemper virus (CDV). Its clinical manifestations include anorexia, biphasic fever, conjunctivitis, severe gastroenteritis, and neurological symptoms. The disease exhibits high transmission and mortality rates (von Messling et al., 2003; Beineke et al., 2009). Since the first report of CDV, its host range has continued to expand, making it a worldwide endemic pathogen. It primarily infects carnivores—including canids, felids, bears, raccoons, and otariids—as well as other mammals such as primates, artiodactyls, proboscideans, and rodents (Glisic et al., 2024; Marino et al., 2024; Minamikawa et al., 2024; Rendon-Marín et al., 2019; Walton et al., 2024). In recent years, multiple domestic and international incidents of CDV infection in wildlife have resulted in animal deaths (Liang et al., 2024). For example, between 1999 and mid-2019, successive fatal cases of CDV infection occurred in red pandas in several zoos, cumul affecting approximately 40 individuals (Liu et al., 2024). Between December 2014 and April 2015, five giant pandas at the Qinling Giant Panda Research Center in Shaanxi Province died from CDV infection, causing serious losses (Zhao et al., 2017).

Initially, CDV genotype distribution correlated strongly with geographic location. However, due to wildlife migration and increasing global trade, outbreaks of different CDV genotypes have occurred in multiple countries (Martella et al., 2002). Currently, many species cannot be effectively vaccinated against CDV. This is due to constant viral mutation in wildlife and differences among prevalent strains (Rendon-Marín et al., 2024; Zhao et al., 2010). Furthermore,

genetic recombination between vaccine strains and wild-type viruses has compromised the protective efficacy of existing vaccines, posing a major challenge to wildlife conservation and disease control.

CDV is a single-stranded, negative-sense RNA virus belonging to the genus *Morbillivirus* within the family *Paramyxoviridae*. The viral genome is 15,690 bp in length and encodes six proteins from the 3' to 5' end: nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin (H), and large polymerase protein (L) (von Messling et al., 2001). The H protein is a major glycoprotein that forms surface spikes on the viral envelope. It specifically binds to receptors on target cells, facilitating viral recognition. It also assists the F protein in mediating membrane fusion and viral entry into host cells (Bi et al., 2015; Rendon-Marin et al., 2019).

The H protein contains multiple neutralizing antigenic epitopes that determine CDV host specificity. It is the principal antigen that stimulates the host to produce neutralizing antibodies (Ke et al., 2015). Previous studies indicate that the fusion ability of different CDV strains is primarily determined by the H protein. Therefore, developing specific therapeutic agents targeting the H protein is of great importance for the diagnosis and treatment of canine distemper (von Messling et al., 2001).

According to recent statistics, the managed giant panda population in China exceeds 700 individuals. Increasing population density in managed environments has accelerated viral transmission and mutation rates, posing a severe threat to population health and stability. However, there are currently no effective drugs available domestically or internationally for preventing or treating CDV in giant pandas—a high-risk viral disease endangering this species (Hu et al., 2016).

Most vaccines used for immunoprophylaxis in giant pandas are canine vaccines. Due to species differences, these vaccines often fail to induce effective antibody production or provide long-term immune protection in giant pandas. Some even carry the potential risk of inducing infection (Wang et al., 2008). Current treatment of CDV infections is primarily symptomatic and generally ineffective. For animals in early stages of infection, administering monoclonal antibodies or hyperimmune serum to neutralize the virus can be beneficial, supplemented by symptomatic treatment of associated complications (Cai and Jin, 2007).

Given the scarcity of virus-specific antibodies for giant pandas and the species' conservation status, this study prepared and characterized three hybridoma cell lines secreting monoclonal antibodies (mAbs) against CDV H protein. All three mAbs were capable of neutralizing the giant panda-derived CDV strain (CDV-giant panda/SX/2014), with the highest neutralizing titer reaching 1:128. These mAbs can be used for further development of genetically engineered antibodies with low immunogenicity and high specificity. They also lay a foundation for research on therapeutic antibodies against CDV in giant pandas.

Materials and Methods

Cells, Virus, and Mice

SP2/0 cells, Vero cells, and GPSLAM-Vero cells were preserved by the Changchun Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The giant panda-derived CDV strain, designated CDV-giant panda/SX/2014, was also maintained in the same laboratory. Female

BALB/c mice were purchased from Si Pei Fu (Beijing) Biotechnology Co., Ltd. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Chengdu Research Base of Giant Panda Breeding (Approval No.: 2022005).

Cloning and Eukaryotic Expression of CDV H Protein

The H protein gene from the canine distemper virus (CDV H) was codon-optimized and artificially synthesized. The gene was cloned into the pATX1 expression vector, incorporating an N-terminal signal peptide for proper secretion and a C-terminal Strep-tag for purification. The recombinant protein was expressed in XtenCHO cells and purified sequentially using Strep-Tactin affinity chromatography and gel filtration chromatography. Protein concentration was determined using a BCA protein quantification kit (Thermo Fisher Scientific), yielding a final concentration of 0.46 mg/mL. The purified protein was stored at -80°C for subsequent use.

Immunization of Mice

Female BALB/c mice (6–8 weeks old) were subcutaneously immunized with 50 μg of the eukaryotic-expressed CDV H protein emulsified in an equal volume of Quick Antibody-Mouse 3W aqueous adjuvant (Suzhou Biodragon Technology Co., Ltd., China). Booster immunizations were administered every two weeks for a total of three boosts. Serum antibody titers and neutralizing antibody levels were monitored by enzyme-linked immunosorbent assay (ELISA) and neutralization tests, respectively. Mice showing high antibody titers were selected and given a final intravenous boost with 100 μg of CDV H protein (without adjuvant) three days prior to cell fusion.

Preparation of Monoclonal Antibodies

Splenocytes from immunized mice were fused with SP2/0 myeloma cells at a 5:1 ratio using ClonaCell™-HY PEG (STEMCELL Technologies, Canada). The fused cells were cultured in ClonaCell™-HY Medium D semi-solid medium (STEMCELL Technologies, Canada) for selection. Supernatants from surviving hybridoma cells were screened by indirect ELISA using purified CDV H protein as the coating antigen. Positive clones were subcloned by limiting dilution, and their supernatants were reassayed by indirect ELISA. Selected hybridoma lines were expanded, and monoclonal antibodies (mAbs) were produced via the ascites method in liquid paraffin-primed BALB/c mice. The mAbs were subsequently purified, and their subclasses were identified.

Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect ELISA was used to determine the anti-CDV H antibody titers in mouse sera and hybridoma supernatants. A 96-well polystyrene microtiter plate (CORNING, USA) was coated with 0.2 μg per well of purified recombinant CDV H protein in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C . After washing three times with PBST (PBS containing 0.05% Tween-20), the plate was blocked with 3% skim milk (Beijing Solarbio Science & Technology Co., Ltd., China) at 37°C for 2 h. Following another wash, diluted serum or hybridoma supernatant was added and incubated at 37°C for 1 h. The plate was washed again, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (BIO-RAD, USA) diluted 1:20,000 was added, followed by incubation at 37°C for 1 h. After five washes with PBST, tetramethylbenzidine (TMB) substrate (Beijing Solarbio Science & Technology Co., Ltd., China) was added and incubated in the dark for 15 minutes at room temperature. The reaction was

stopped with stop solution (Beijing Solarbio Science & Technology Co., Ltd., China), and the absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, USA). The antibody titer was defined as the reciprocal of the highest dilution that yielded an OD₄₅₀ value ≥ 2.1 times that of the negative control.

Indirect Immunofluorescence Assay (IFA)

GPSLAM-Vero cells were infected with the CDV-giant panda/SX/2014 strain for 48 hours. The cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and blocked with 2% bovine serum albumin (BSA) at 37°C for 1 h. The cells were then incubated with the monoclonal antibodies (diluted 1:500) at 4°C for 1 h. After three washes, Alexa Fluor 488-conjugated goat anti-mouse IgG (Beyotime Biotechnology, China) diluted 1:500 was added and incubated at 37°C for 1 h. The stained cells were visualized using an inverted fluorescence microscope (Olympus Corporation, Japan).

Neutralization Assay

The neutralizing titers of the three mAbs (2D1, 6G4, and 6H4) against the CDV-giant panda/SX/2014 strain were determined as follows. The purified mAbs were serially diluted twofold in incomplete DMEM and mixed with an equal volume of virus solution (100 TCID₅₀/mL) in 96-well plates. The plates were incubated at 37°C with 5% CO₂ for 1 h with shaking every 15 minutes. Then, Vero cells were added and cultured for 3–5 days. Cytopathic effect (CPE) was observed daily under a microscope (Olympus Corporation, Japan). The neutralization titer was defined as the highest antibody dilution that completely inhibited CPE.

Antibody Purification and Subclass Identification

Hybridoma cells (8×10^5 cells/mouse) were injected intraperitoneally into female BALB/c mice (8–10 weeks old) that had been pre-treated with liquid paraffin. Ascites fluid was collected 7–10 days later and purified by the octanoic acid-ammonium sulfate method and protein A affinity chromatography. The titer of the purified antibodies was determined by ELISA. The mAb isotypes were identified using a Mouse Monoclonal Antibody Isotype ELISA Kit (BF16001, Suzhou Biodragon Technology Co., Ltd., China).

Western Blot (WB)

Western blot was performed to assess the reactivity of the mAbs with the recombinant H protein. The protein samples were mixed with loading buffer, boiled for 10 minutes, and separated by 12% SDS-PAGE. The proteins were then transferred to a 0.22 μ m nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBST for 2 h at room temperature and washed three times with TBST. The membrane was then incubated with the prepared anti-CDV H protein monoclonal antibodies (diluted in TBST) at 4°C for 1 h with gentle shaking. After three washes with TBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (H+L) (1:20,000 dilution) at room temperature for 1 h. Finally, the membrane was washed again, and protein bands were visualized using a Western Blot chromogenic solution (Thermo Fisher Scientific, USA).

Results

Expression and Purification of the CDV H Protein

The recombinant plasmid pATX1-CDV H, containing a C-terminal Strep-tag, was successfully

expressed in the XtenCHO mammalian expression system. The recombinant protein was purified using Strep-Tactin affinity chromatography followed by gel filtration chromatography. The results showed an immunoreactive band at an apparent molecular weight of >70 kDa (Fig. 1a), which was consistent with the predicted molecular weight of the glycosylated CDV H protein.

Preparation and Identification of CDV H Antibodies

To generate monoclonal antibodies (mAbs) against CDV H protein, five female BALB/c mice were immunized with the purified recombinant antigen. After four rounds of immunization, all five immunized mice developed high-titer antibody responses, with serum titers exceeding 1:640,000 (Fig. 1b). Splenocytes from mouse No. 414, which showed a strong immune response, were fused with SP2/0 myeloma cells. Through indirect ELISA screening, three hybridoma cell lines stably secreting anti-CDV H mAbs were obtained and designated as 2D1, 6G4, and 6H4. The ELISA binding titer of the hybridoma supernatants was determined to be 1:256, 1:4096, and 1:1024, respectively.

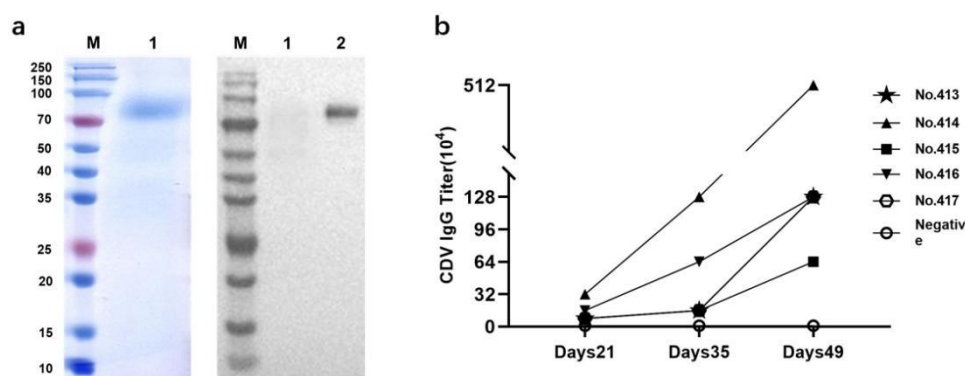


Figure 1. Preparation of CDV H protein antibodies. a. SDS-PAGE and Western Blot analysis of purified CDV H protein. b. Detection of serum titers in immunized mice by ELISA.

Characterization of Purified Monoclonal Antibodies

The three mAbs were purified and analyzed by SDS-PAGE, which revealed heavy and light chains with approximate molecular weights of 55 kDa and 20 kDa, respectively (Fig. 2a). Isotype analysis indicated that all three mAbs belonged to the IgG1 κ subclass (Fig. 2b). The binding titers of the purified mAbs, as measured by ELISA, were 1:10⁷, 1:10⁸, and 1:10⁸, respectively. Western blot analysis further demonstrated that all three mAbs recognized the eukaryotic-expressed H protein, while only mAb 2D1 also reacted with the prokaryotically expressed H protein (Fig. 2c), indicating that 2D1 recognizes a continuous epitope.

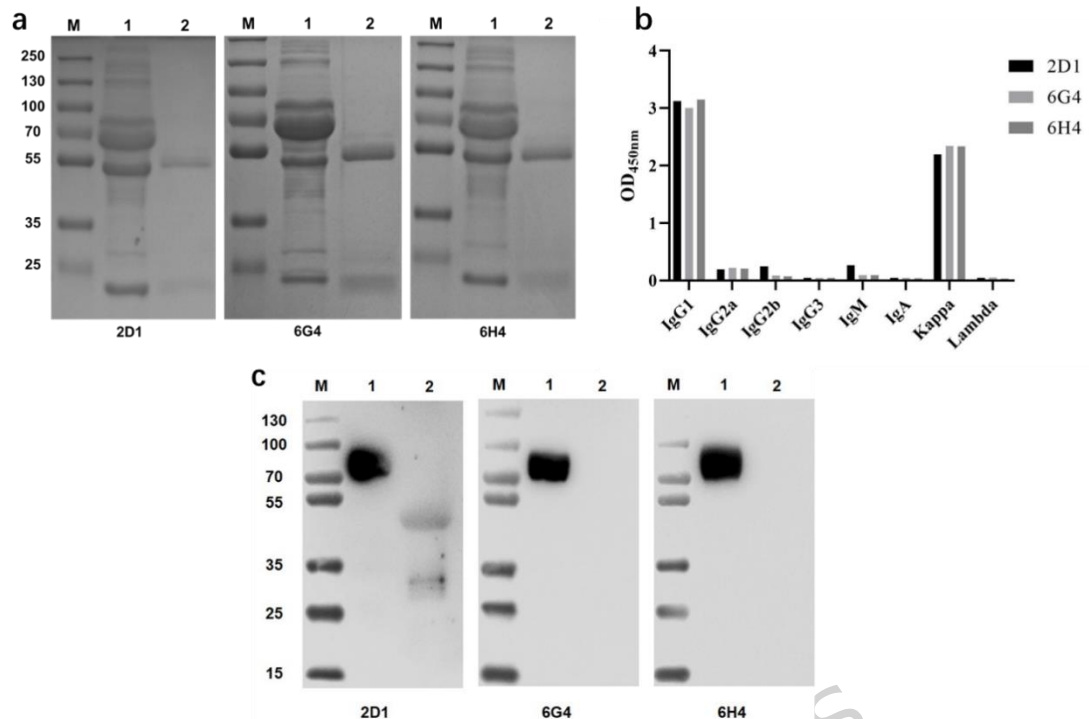


Figure 2. SDS-PAGE analysis, subclass identification and Western Blot identification of purified mAbs against the CDV H protein. a. SDS-PAGE analysis M: protein marker; 1: unpurified ascites; 2: purified ascites. b. subclass identification. c. Western Blot identification. M: protein marker; 1: eukaryotic expressed H protein; 2: prokaryotic expressed H protein.

Immunofluorescence Reactivity of mAbs with CDV-infected Cells

The specificity of the mAbs was evaluated by indirect immunofluorescence assay (IFA) in GPLSLAM-Vero cells infected with the CDV-giant panda/SX/2014 strain. All three mAbs, along with positive mouse serum, specifically bound to the virus-infected cells, exhibiting clear green fluorescence (Fig. 3). The fluorescence signals of mAbs 6G4 and 6H4 were notably stronger than that of 2D1, suggesting a higher binding capacity to the native viral antigen. No fluorescence was detected in uninfected cells, confirming the specificity of the mAbs for CDV.

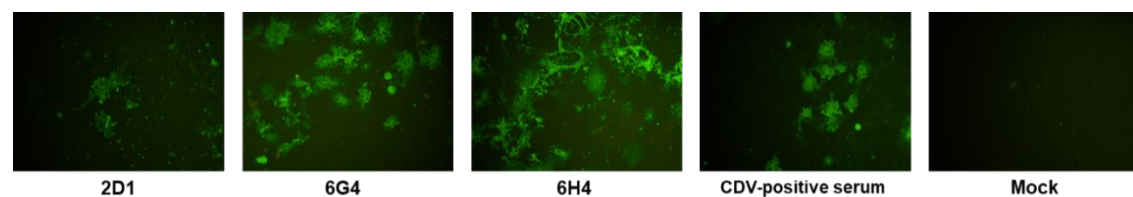


Figure 3. Immunofluorescence reactivity of mAbs 2D1, 6G4 and 6H4 with the CDV-giant panda/SX/2014 strain in GPLSLAM-Vero cells. CDV positive serum was used as the positive control; uninfected GPLSLAM-Vero cells were used as the negative control.

Neutralizing Activity of mAbs Against CDV-giant panda/SX/2014

The neutralizing potency of the mAbs was assessed using a cell-based protection assay. All three mAbs—2D1, 6G4, and 6H4—effectively protected GPLSLAM-Vero cell monolayers from virus-induced cytopathic effect (CPE) (Fig. 4). The neutralization titers, defined as the highest dilution that completely inhibited CPE, were determined to be 1:64 for 2D1, 1:128 for 6G4, and 1:32 for

6H4. No CPE was observed in the uninfected control group, confirming the specificity of the neutralization assay.

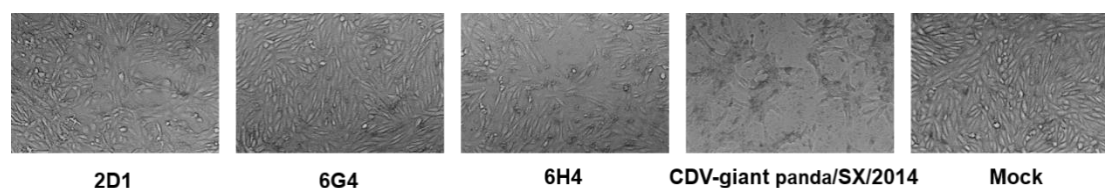


Figure 4. Detection of neutralization activities of mAbs 2D1, 6G4 and 6H4 against the CDV-giant panda/SX/2014 strain in GPSLAM-Vero cells. GPSLAM-Vero cells infected with the CDV-giant panda/SX/2014 strain were set as negative control; GPSLAM-Vero cells without infection were set as untreated control.

Discussion

Canine distemper virus (CDV) remains one of the most significant viral threats to giant pandas and other endangered carnivores, as its outbreaks can cause high mortality rates (Seimon et al., 2013). There are no effective vaccines or therapeutic drugs for canine distemper in giant pandas, either at home or abroad (Geng et al., 2020; Hu et al., 2016; Wang et al., 2008). Current vaccination strategies, which are primarily developed for domestic dogs, present a dilemma in wildlife conservation. While essential for population protection, their use in non-target species—including giant pandas, red pandas, and mustelids—carries risks of inducing vaccine-associated diseases (Greenacre 2003; Xiu 2000; Zhao et al., 2014). This safety concern has led to justified caution in giant panda breeding institutions, creating an urgent need for alternative immunoprophylactic approaches, such as passive immunotherapy with neutralizing monoclonal antibodies (mAbs).

Against this backdrop, we developed a panel of three murine mAbs (2D1, 6G4, 6H4) targeting the CDV H protein—the primary target for neutralizing antibodies. A critical aspect of our approach was the use of a eukaryotic expression system (XtenCHO cells) to produce the H protein immunogen. This strategy was designed to preserve the complex higher-order structure and post-translational modifications of the native viral protein, thereby increasing the likelihood of generating antibodies against functionally relevant, conformation-dependent epitopes (Cai et al., 2024). The success of this strategy is evidenced by the fact that all three mAbs recognized the eukaryotically expressed H protein and exhibited potent neutralizing activity against the giant panda-derived CDV-giant panda/SX/2014 strain, confirming their targeting of epitopes critical for viral entry.

The distinct immunoreactivity patterns of the mAbs revealed their recognition of different epitopes. Notably, mAb 2D1 bound both eukaryotic and prokaryotically expressed H protein, a finding that is consistent with the recognition of a continuous epitope that remains accessible even on a denatured antigen. In contrast, the exclusive reactivity of 6G4 and 6H4 with the eukaryotic-expressed protein suggests their binding is dependent on non-continuous, conformation-sensitive epitopes. This diversity in epitope recognition is particularly advantageous. It not only provides a valuable set of tools for epitope mapping and diagnostic assay development but also support the future investigation of a cocktail therapy. Such a strategy, utilizing antibodies against distinct epitopes, could enhance neutralization breadth and mitigate the risk of viral escape mutants, a

critical consideration given the genetic and antigenic variability of the CDV H protein, including the previously identified Y549H substitution in our target strain (Feng et al., 2016).

While our data confirm the potent neutralizing capacity of these mAbs, the precise molecular mechanism of neutralization remains to be fully elucidated. For the conformation-specific mAbs 6G4 and 6H4, neutralization likely occurs through blocking receptor engagement or interfering with the H-protein's interaction with the fusion (F) protein. It is noteworthy that emerging evidence from related morbilliviruses indicates that some potent neutralizing antibodies target the F protein, stabilizing it in a pre-fusion state and blocking the conformational changes required for membrane fusion (Imhoff et al., 2007; Zyla et al., 2024). Future work should investigate whether any of our mAbs employ a similar mechanism, which would represent a significant finding for CDV immunology.

In conclusion, the mAbs generated in this study provide a promising foundation for combating CDV in giant pandas. Their high neutralizing potency validates the H protein as a critical target and underscores the importance of using a eukaryotic expression system for immunogen design to elicit antibodies against native conformational epitopes. The immediate application of these antibodies lies in their potential as a passive immunotherapy to control active CDV outbreaks in captive populations. For long-term impact, future work will focus on precisely mapping the neutralizing epitopes, elucidating the detailed mechanism of action, and engineering these murine antibodies (e.g., through chimerization) to reduce immunogenicity risks for clinical use in pandas. This work ultimately contributes to a broader strategy of developing targeted biologics for the conservation of endangered wildlife.

Declarations

List of abbreviations

CD: Canine distemper; CDV: Canine distemper virus; mAbs: Monoclonal antibodies; N: Nucleocapsid; P: Phosphoprotein; M: Membrane protein; F: Fusion protein; H: Hemagglutinin; L: Large protein; ELISA: Enzyme-linked immunosorbent assay; TMB: Tetramethylbenzidine; OD: Optical density; CPE: Cytopathic effect; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; WB: Western blot; IFA: Immunofluorescence assay.

Ethics Approval and Consent to Participate

All animal experiments were approved by Institutional Animal Care and Use Committee of the Chengdu Research Base of Giant Panda Breeding protocol #2022005.

Consent for Publication

Not applicable.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article and its supplementary files.

Competing Interests

The authors declare that they have no conflict of interests.

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Author contributions

Yunli Li: performed experiment design, data analysis and drafted the manuscript. Jianzhao Wu, Jiaqi Li and Jianhong Zeng: performed experiments and data curation. Lin Li, Xiaoyan Su and Jianzhong Wang: contributed in methodology, investigation and conceptualization. Xianzhu Xia and Rong Hou: contributed in resources and supervision. Na Feng and Songrui Liu: supervised the study and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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