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RESEARCH ARTICLE

Single-Domain Antibody Fused with Chicken IgG Fc-Fragment Protects Goslings against Novel Goose Astrovirus (nGAstV)

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Previously, nanobodies (Nbs) targeting ORF2 protein of novel goose astrovirus (nGAstV) were identified and tested for their specificity and binding affinity to nGAstV. However, their ability to neutralize and prevent the viral infection in goslings remained unexplored, especially those fused with Fc domain of chicken IgG (cFc). In the present study, using nGAstV ORF2 as target antigen, Nb-positive clones from already developed Nb phage library reacting with nGAstV were obtained through three rounds of panning and enrichment. Four Nbs based on sequence variation and solubility were selected, fused with cFc, and genetically engineered to produce chimeric nanobodies. The specific reactivity of these Nbs-Fc with nGAstV was assessed by IFA and WB analysis, the affinity and IC_{50} of Nbs-Fc were determined by bio-layer Interferometry tests and iELISA, respectively. And the neutralizing activity of one nanobody with good biological activity (Nb9-2-cFc) was assessed by cell infection and *in vivo* antiviral assays in goslings. All the four Nbs-Fc were successfully expressed and confirmed through SDS-PAGE. These Nbs exhibited strong binding affinity to nGAstV, as demonstrated by IFA and WB analysis. Among these, Nb9-2-cFc showed the highest affinity (K_d=59.6 p*M*) and neutralization activity (IC_{50}=15.75ng/mL) against nGAstV. Further, *In vitro* assays revealed that this Nb could reduce virus proliferation by blocking the binding of ORF2 to its receptor. *In vivo* evaluation of prophylactic activity in goslings revealed that Nb9-2-cFc provided significant protection against nGAstV infection by reducing viral load and histopathological damage in target organs. These findings highlight Nb9-2-cFc as a promising candidate for emergency prevention and treatment against nGAstV infection in goslings.

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INTRODUCTION

Astrovirus disease, caused by astroviruses, affects wide range of animals, leading to gastroenteritis, encephalitis in farmed animals, fatal hepatitis in ducks, gout in geese, runting-stunting syndrome (developmental retardation), and cystic enteritis in avian species which ultimately may result in death (De Benedictis *et al.,* 2011; Raji *et al.*, 2022; Bi *et al.*, 2023). These viruses pose serious threats to human health and agricultural development (Fu *et al.*, 2023). Recently, outbreaks of gout, primarily caused by novel goose astrovirus (nGAstV), have been reported in coastal and inland provinces of China (Chen *et al.*, 2020; He *et al.*, 2022). This disease, associated with a mortality rate exceeding 50%, has resulted in significant economic losses in China's goose industry (Yang *et al.,* 2018). Currently, there are no commercial vaccines or antiviral drugs available against nGAstV. Additionally, the lack of commercially available serological test kit, and ineffective epidemiological investigations often result in delayed clinical treatment, further exacerbating economic losses in this industry.

Novel astrovirus strain was first isolated from deceased geese exhibiting visceral uric acid deposits (Zhang *et al.*, 2018). The nGAstV genome spans 7.0kb and includes a 5'-untranslated region (UTR), a 3'-UTR, a poly-A tail, and three open reading frames (ORF1a, ORF1b, and ORF2) (Cui *et al.,* 2022; Xu *et al.*, 2023). ORF1a encodes a serine protease and a viral genome-

linked protein (VPg), while ORF1b encodes an RNA-dependent RNA polymerase (RdRp), essential for genomic replication (Li *et al.,* 2022). ORF2 encodes capsid precursor proteins crucial for virion assembly and eliciting host immune responses (Jiang *et al.,* 1993; Arias and DuBois, 2017; Ren *et al.,* 2020; Zhu and Sun, 2022).

Antibody-based therapies targeting nGAstV represent a crucial focus in combatting this virus (Revets *et al.,* 2005; Salvador *et al.,* 2019). Nanobody (Nb) is a single-domain antibody derived from variable domain of heavy-chain-only antibodies (VHH) found in camelids. Nbs have been reported to be promising candidates as virus-neutralizing agents (Hamers-Casterman *et al.,* 1993; Huo *et al.,* 2020; Liu *et al.,* 2020; Tang *et al.,* 2023). They are valued for their small molecular size (approximately 2.5 nm in diameter and 4 nm in height) and low molecular weight (~15kDa), which contribute to high solubility, stability, cost-effectiveness, and low toxicity compared to conventional antibodies (Zou *et al.,* 2015; Wang *et al.,* 2019; Ackaert *et al.,* 2021; Ykema and Tao, 2021).

Additionally, genetically linking the Fc-domain of an immunoglobulin to nanobodies (chimeric nanobodies) have several extra advantages, including the reconstitution of antibody Fc functions, enhanced effector activity, enhanced specificity, half-life, stability and solubility (Bannas *et al.,* 2017; Hong *et al.,* 2019). Although, various research has explored the diagnostic and therapeutic applications of human Fc-fused Nbs in medicine (Bannas *et al.,* 2017; Tang *et al.,* 2023). However, very limited work has been done on the use of chicken Fc fused Nb as diagnostic and therapeutic marker (Vanmarsenille *et al.,* 2019).

Previously, Nbs against the nGAstV ORF2 protein were produced in alpacas, and the binding affinity of some selected Nbs with nGAstV was evaluated through ELISA and Western blotting (Wang *et al.,* 2023). In the present study, chicken Fc-fused Nbs (Nb-cFc) were tested in goslings to determine their therapeutic and prophylactic efficacy against nGAstV infection. Nbs showing high binding affinity with nGAstV were identified through panning, fused with cFc, and genetically engineered. The binding affinity of selected Nb-cFc to nGAstV and their virus neutralization potential were assessed through several assays. The present study may provide insights into the potential of Nb-cFc as diagnostic markers and emergency prevention for nGAstV infections in goose.

MATERIALS AND METHODS

Plasmids, cells and virus: Nanobody phage library (produced against nGAstV ORF2 protein in two-year-old alpacas; Wang *et al.,* 2023), CHIGG-Fc eukaryotic expression vector, novel Goose Astrovirus (nGAstV), Chicken Liver Cancer Cells (LMH), and human embryonic kidney cell 293F (HEK-293F) were kindly provided by the Animal Virus Molecular Ecology Innovation team, Chinese Agricultural Sciences at Lanzhou Veterinary Research Institute. *E. coli* DH5α receptor cells were obtained from Dalian Bao Biological Engineering Co. LTD, while *E. coli* ss320 electrocompetent cells and M13 helper phage were purchased from NEB.

Enrichment and panning of nGAstV-specific recombinant phages: To identify nanobodies showing high binding affinity to nGAstV, the enrichment and panning of recombinant phages were performed as described by Wang *et al.* (2023). Briefly, the purified ORF2 protein (1μg/well) was coated onto an ELISA plate, with 1% polyvinyl alcohol (PVA) used as an antigen-free control. The primary Nb phage library (100μL) was added to each well and incubated at room temperature for 2h. After incubation, the solution was discarded and elution was performed using 0.1 *M*/L HCl for 5min, followed by neutralization with an equal volume of Tris-HCl. The eluent was transferred to logarithmic phase NEB5αF bacteria and cultured at 37°C with shaking at 200rpm for 1h. Afterwards, M13-assisted phage rescue was conducted for an additional hour. A suitable amount of bacterial solution was then tenfold diluted and plated for titration. The remaining bacterial solution was cultured overnight and concentrated to obtain the first round of the phage Nb display library. This process was repeated for three rounds of phage panning. After the third round of phage panning, the bacteriophage Nb library was diluted and cultured overnight at 37°C. From this, 96 clones were randomly selected, transferred to Amp-Kan medium, and cultured overnight at 37°C with shaking at 200rpm. Then, the culture solution was centrifuged at 4000×g for 10min, and the supernatant was collected for Nb detection using iELISA. The purified ORF2 protein (100ng/well) was used as an antigen, while HRP-labeled rat anti-M13 antibody (diluted 1:6000) served as secondary antibody. To identify positive clones, P/N ratio was calculated as the ratio of OD_{450nm} values of sample to negative control.

Sequence analysis and expression of recombinant Nbs: Seven positive clones with high P/N values, as shown in Fig. 1, were selected, and PCR amplification of Nb sequences was performed using primers listed in Table 1. The PCR products were purified and ligated into the self-built CHIGG-cFc eukaryotic expression vector (consisting of sequence of Fc domain of chicken IgG). The constructed vector was transformed into DH5α cells, which were then cloned on ampicillin-containing agar plates. From each plate, 3 clones were randomly chosen, resulting in a total of 21 plasmids. DNA from the clones was extracted and sequenced for sequencing and phylogenetic analysis. Plasmids showing significant sequence differences were transfected into HEK-293F cells for expression. The expressed Nbs were purified using Protein A and their expression was analyzed through SDS-PAGE electrophoresis.

Indirect immunofluorescence assay (IFA): The binding affinity of selected chicken IgG Fc-fused Nbs to nGAstV was evaluated using IFA by following the protocol described by Zhang *et al.* (2021). Briefly, LMH cells were cultured in T75 cell culture flasks for 48h, digested with trypsin (0.25%), and evenly plated in 12-well plates at a 1:3 ratio. Once the cells reached confluence, they were infected with diluted nGAstV at a multiplicity of infection (MOI) of 0.01 and incubated at 37°C for 72h. After incubation, cells were washed three times with PBS and fixed with 1mL of 4% paraformaldehyde per well for 10-20min at room temperature. The paraformaldehyde was

Table 1: List of primers used for different PCR amplifications

Primer	Primer sequence (5′→3′)
Primers for amplification of Nbs	
Nb-F	TCCAGTGTGGTGGAATTCGCCACCAAAAAGAATATCGCATTTCTTCTTGCATCT
Nb-R	TCTAGACTCGAGTCAGTGATGGTGGTGGTGGTGGTGGTGTGAGGAGACGGTGACCTGGG
Primers for identification of nGAstV	
nGAstV-F	AAGAATGTYGATAGTACCCCT
nGAstV-R	TACCCCAGAGTGATTCTAAGTTGG
Primers for amplification of nGAstV ORF2 mRNA	
nGAstV-qF	ACAGCAGGATTTATCAGA
nGAstV-qR	CCTTGTCCAGTTGTATTC

then discarded, and cells were rinsed three times with 1mL of PBS. Subsequently, 1mL of 0.5% Triton-X100 was added to each well, incubated for 10min at room temperature, and then discarded. Cells were again washed twice with 1mL of PBS for 5min. Following washing, 1mL of 3% BSA as blocking solution was added to each well and incubated for 1h at room temperature. The blocking solution was discarded, and chicken IgG Fc-fused Nbs (diluted 1:100) were added as the primary antibody. FITC polyclonal goat anti-chicken IgY (IgG) (Shandong Landu Biotechnology Co., LTD.; Cat. No. LD-EK-164; dilution 1:5000) served as the secondary antibody. After staining with DAPI, the samples were examined and photographed using a confocal laser microscope.

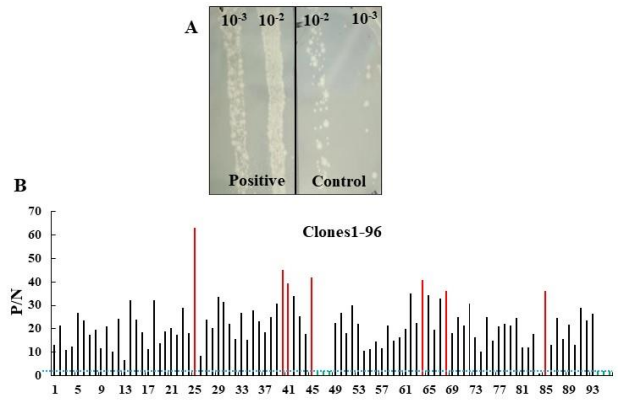

Fig. 1: Panning and enrichment of nGAstV-specific nanobody phage display library. (A) Enrichment display after three rounds of panning; (B) Affinity detection of Nanobodies with nGAstV using ORF2 Protein, evaluated based on P/N ratio.

Western blot analysis: Western blot (WB) analysis was also performed to evaluate the binding affinity of Nb to nGAstV. Briefly, nGAstV, nGAstV ORF2 and nGAstV P2 proteins were used as test antigens in SDS-PAGE, while inactivated Newcastle disease virus (NDV) was used as negative control. After the membrane transfer step, followed by blocking with 5% skim milk for 1h, the membrane was incubated with purified Nbs (1 mg/mL stock; 1:100 dilution) in 5% skim milk at room temperature for 1 h. After washing membrane three times in TBST for 5-10min each, the membrane was incubated with HRP labeled rabbit anti-chicken IgY (IgG) (Wuhan Amerjet Technology Co., LTD; Cat. No. 303-035-008; dilution 1:5000) for 1h. ECL method was used to visualize signals.

Virus titration: LMH cells were cultured in DMEM containing 10% fetal bovine serum. When the cells reached 80% confluence, nGAstV was inoculated. After 3-5 days of culture, the virus was collected by centrifugation at 8000rpm for 10min. The supernatant containing virus was filtered through a sterile 0.22μm filter to remove debris and any other bacterial contaminant. The filtrate was then mixed with PEG8000 solution (2:1), and incubated on ice for 1h. The mixture was centrifuged at 2000×g for 2h, and the pellet was resuspended in an appropriate amount of PBS. The virus was further purified through sucrose density gradient centrifugation, and virus was confirmed through PCR using primers listed in Table 1. LMH cells were seeded in 96-well plates, and when the cells reached 80% confluence, nGAstV was serially diluted from 10^{-1}-to 10^{-8} times and then added to LMH cells. Five replicates were made for each dilution, with 100μL per well. Cytopathic effects were observed after 72h of infection through IFA using Nb9-2-cFc as primary antibody and FITC polyclonal goat anti-chicken IgY (IgG) (dilution 1:5000) as secondary antibody. To assess the infectivity of nGAstV, $TCID_{50}$ was calculated by the Reed and Muench method (Lei *et al.*, 2021)

Biolayer Interferometry (BLI): The binding affinity between nGAstV ORF2 and Nbs (Nb4-3-cFc, Nb4-2-cFc, Nb9-2-cFc, and Nb5-1-cFc) was assessed using a Gator instrument (Probe Life). Briefly, recombinant Nbs at a concentration of 125 nmol/L were immobilized on the probes. The probes were exposed to PBST buffer (10m*M* PBS, pH 7.4, with 0.02% Tween) containing ORF2 at various concentrations (ranging from 100n*M*/L to 3.125n*M*/L) for association, followed by immersion in buffer (10 m*M* PBS, pH 7.4, with 0.02% Tween) for dissociation. The wave shifts obtained were analyzed using Gator software and the equilibrium dissociation constant (Kd) was determined by global fitting of the association and dissociation phases using a 1:1 Langmuir binding model, providing the binding kinetics and affinity between ORF2 and each Nb.

Microneutralization assay: Serial dilutions of Nbs were mixed with equal volumes of 100 $TCID_{50}$ of virus and incubated for 1h at 37°C (Gao *et al.* 2016). The mixture was subsequently added to cultured LMH cells. Viral infection was quantified by indirect ELISA using Nbs targeting ORF2 protein of nGAstV. The final concentrations of Nbs that inhibited infection by 50% (IC_{50}) were determined using GraphPad Prism 9.0 software.

Cell viability analysis: To assess the cytotoxic effect of Nbs, cell viability was measured using a cell counting kit-8 (CCK-8) assay (Beyotime, China) with slight modifications (Xiao *et al.*, 2014). Briefly, LMH cells were

seeded (1×10^5 cells per well) in 96-well plates and incubated at 37°C in a 5% CO_2 humidified incubator. After the cells formed confluent monolayers, they were washed with PBS and treated with different concentrations of Nb9-2-cFc in 3% FBS medium, followed by incubation for 24h. Then, CCK-8 reagent (10µL) was added to each well, and the plates were incubated at 37°C for 1h. To assess the cell viability, the absorbance was measured at OD_{450nm} using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA). The results were expressed as the percentage of the optical density of treated cells relative to the untreated control cells, which was considered as 100% viability.

Reduction of nGAstV by Nb9-2-cFc: To evaluate the neutralizing effect of Nb9-2-cFc on reducing nGAstV infection, LMH cells were cultured in 12-well plates at a density of 5×10^5 cells/mL, with 1mL per well, and incubated for 48h at 37°C with 5% CO_2. LMH cells were inoculated with a mixture of nGAstV at a MOI of 0.01 and Nb9-2-cFc at concentrations of 5µ*M* and 10µ*M* (1:1) diluted in Opti-MEM for 1h. The inoculum was then removed, and the cells were washed three times with sterile 0.01*M* PBS preheated to 37°C. Then, 1mL of Nbs diluted in Opti-MEM to concentrations of 5µ*M* or 10µ*M* was added to the cell culture plates. The plates were further incubated at 37°C with 5% CO_2. Cells were harvested at 48h and 72h post-treatment to analyze viral ORF2 protein content using WB analysis, and viral infection was assessed using IFA. For this analysis, Nb9-2-cFc was used as primary antibody. For WB analysis, HRP labeled rabbit anti-chicken IgY (IgG) served as secondary antibody, while FITC polyclonal goat anti-chicken IgY (IgG) was used as secondary antibody for IFA. Concurrently, cell supernatants were also collected to measure progeny virus titers and $TCID_{50}$ was calculated by the Reed and Muench method (Lei *et al.*, 2021).

Flow cytometry assay (FCM): To investigate the neutralization mechanism of Nb, flow cytometry was performed. Briefly, 25µL of Nb9-2-cFc (at concentrations of 2.5, 5, and 10µ*M*) was added to a 96-well cell culture plate, followed by an equal volume of nGAstV ORF2 protein (0.15µ*M*). The plate was shaken for 30min at room temperature and then incubated statically for 10min at 37°C. LMH cells were digested with trypsin, and the digestion was halted with complete DMEM medium. The cells were washed twice with PBS and adjusted to a concentration of 5×10^6 cells/mL in PBS. Subsequently, 50µL of this cell suspension was added to each well of the 96-well plate. After shaking for 10min at room temperature, the plate was incubated for 30min at 37°C. The contents of each well were transferred to flow cytometry tubes, followed by the addition of 1mL of PBS. The tubes were centrifuged (500g, 4°C, 6min) to remove the supernatant, and the cells were washed three times with PBS. The primary antibody against His-tag, (dilution 1:1000) was added to the flow tubes. The tubes were wrapped in aluminum foil and shaken for 10min at room temperature, followed by a 30min incubation at 37°C. After centrifugation (500g, 4°C, 6min), the supernatant was removed by centrifugation, and FITC-labeled anti-mouse secondary antibody (dilution 1:1000) was added to each flow tube (200µL/tube). The tubes were wrapped in aluminum foil and placed in a shaking incubator for 10min at room temperature, followed by a 30min incubation at 37°C. The cells were washed three times with PBS, resuspended in 200µL of PBS, transferred to a 96-well cell culture plate, and analyzed by flow cytometry.

Emergency prevention efficacy in goslings: Eighty (80) one-day-old healthy goslings were obtained from a commercial hatchery in Guangdong Province. The goslings were raised in specific pathogen-free (SPF) environment without any immunization and fed ad libitum. To confirm the goslings free from nGAstV prior to inoculation, the cloacal swabs were collected, and the virus was detected through PCR using primers given in Table 1. To assess prophylactic efficacy, the goslings were randomly divided into four groups, each consisting of 20 goslings. The experimental design for evaluating the prophylactic efficacy of Nb9-2-cFc has been presented as Fig. 7A. The Nb1 and Nb2 groups were intraperitoneally injected with 12.5 and 25µ*M* of purified Nb9-2-cFc, respectively. After 24h of administration, the Nb1, Nb2, and nGAstV groups were challenged with 0.5mL of nGAstV ($10^{5.1}EID_{50}$/0.2 mL) through subcutaneous injection in the enterocoelia. The PBS group served as negative control and was inoculated with equal volume of PBS at the same injection site. The nGAstV group, which only received nGAstV challenge, served as untreated control. Cloacal swabs were collected from goslings on days 3, 5, 7, and 9 post-challenge and viral load was assessed using qPCR. Following this, the goslings were sacrificed, and lungs and kidney samples were collected and preserved in 10% neutral buffered formalin for histopathological examination. The tissue sections were prepared using the method described previously and stained with hematoxylin and eosin (Wei *et al.*, 2020).

Quantitative real-time PCR: Cloacal swabs from goslings were washed three times with PBS, and the total RNA was extracted using TRIzol RNA reagent (TaKaRa, China). Reverse transcription was carried out using a PrimeScript RT master mix (TaKaRa, China) by following the manufacturer's instructions. A series of ORF2 plasmids with known concentrations were used as the standard, and qPCR amplification was performed for each standard using the primers given in Table 1.

Statistical analysis: The quantitative data have been presented as mean ± standard deviation (SD). Statistical significance was assessed using Student's two-tailed t-test or analysis of variance (ANOVA) using GraphPad Prism software (version 9.0, USA). Differences between groups were considered statistically significant at the following levels: *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$.

Ethics statement: The study protocol was approved by the Ethics Committee of Gansu Agricultural University (approval no. GSAU-Eth-VMC-2024-040). All of the procedures conducted in this research were in accordance with the Declaration of Helsinki and the relevant regulations in China.

RESULTS

Enrichment and panning of specific recombinant phages: After three rounds of enrichment and panning, the result showed significant enrichment of the specific recombinant phages, with a concentration of 10^{-3} compared to the antigen-free control. After culturing, 93 clones were randomly selected for Nbs detection through ELISA, alongside three antigen-free controls. The cutoff P/N ratio based on the ratio of OD values of sample to negative control was calculated as ≥2.1. A total of 89 positive clones with a PN ratio of ≥2.1 were identified (Fig. 1).

Sequence analysis and expression of Nbs: Seven positive clones with high P/N values, as shown in Fig. 1, were selected for PCR amplification from bacterial solutions. The PCR products were subsequently purified and cloned into the CHIGG-cFc eukaryotic expression vector. Sequence analysis using DNAMAN software revealed significant differences among the sequences of all seven Nb clones, as illustrated in Fig. 2A and 2B. Based on sequence variation and soluble nature of these Nbs, a total of four Nb clones (Nb4-3-cFc, Nb4-2-cFc, Nb5-1-cFc and Nb9-2-cFc) were selected and confirmed by SDS-PAGE analysis. Each Nb exhibited the expected band size of 40kDa, as shown in Fig. 2C.

Binding affinity of selected chicken IgG Fc-fused Nbs to nGAstV: IFA results demonstrated that all four Nb clones specifically reacted with nGAstV (Fig. 3A) but did not exhibit reactivity with NDV and LMH cells (controls). WB analysis indicated that Nb4-2, Nb4-3, and Nb9-2-cFc specifically recognized both the ORF2 protein and nGAstV, without reacting with NDV. Surprisingly, Nb-5-1-cFc did not show any reactivity with either the ORF2 protein or nGAstV. Since the ORF2 protein primarily consists of P1 and P2 proteins, Nb9-2-cFc was able to recognize P2 protein, whereas Nb4-2 and Nb4-3 did not show reactivity (Fig. 3B).

nGAstV proliferation and titration: PCR amplification of ORF2 gene from purified viral culture had shown a band of 1000bp, which was consistent with the size of the intended target fragment (Fig. 4A), indicating that nGAstV was successfully purified. Following the inoculation of varying concentrations of nGAstV to LMH cells, significant lesions were observed in the LMH cells after 72h of infection by IFA method (Fig. 4B), indicating that nGAstV proliferation was successful. The $TCID_{50}$ value was determined to be 4.27 $Log_{10}TCID_{50}$/mL, as calculated using the Reed-Muench method.

Affinity detection of recombinant Nbs: The binding kinetics of the four Nb clones, which showed good specificity towards nGAstV ORF2 protein, were evaluated using biolayer interferometry (BLI), and the equilibrium dissociation constant (K_d) was calculated. The results demonstrated that Nb9-2-cFc exhibited favorable binding activity with nGAstV ORF2 protein, with an equilibrium dissociation constant (K_d) of 59.6p*M*/L (Fig. 5A, Table 2).

Since the Nbs had shown the reactivity with the ORF2 protein, the neutralizing activity of the four Nb clones was further analyzed using nGAstV. As shown in Fig. 5B, the Nb9-2-cFc neutralized nGAstV in a dose-dependent manner, displaying high neutralizing activity with an IC_{50} value of 15.75ng/mL. However, the other three Nbs did not exhibit high neutralizing activity (Table 2). Additionally, the cytotoxicity of Nb9-2-cFc in LMH cells was also assessed using a cell counting kit-8 (CCK-8) assay. As shown in Fig. 5C, the viability of LMH cells was comparable to that of the untreated control cells up to a concentration of 50μ*M* of Nb9-2-cFc, suggesting that Nb9-2-cFc is non-toxic to LMH at concentrations below 50μ*M*.

nGAstV reduction by Nb9-2-cFc: The WB analysis revealed that the levels of nGAstV progeny virus ORF2 protein at 48h post-treatment of LMH cells with 5μM and 10μM Nb9-2-cFc did not differ significantly from the control group treated with vehicle only. However, by 72h post-infection, the expression levels of nGAstV ORF2 protein in LMH cells were significantly reduced following treatment with 5μM and 10μM Nb9-2-cFc as compared to the viral treatment group. The WB strips and the quantitative assessment of bands intensity, measured through grey scale analysis (Arbitrary Units; AU), in different groups have been presented in Fig. 6A and B, respectively.

IFA results revealed that a significant reduction of nGAstV infection was observed following Nb9-2-cFc treatment, with highest virus reduction observed in the cells treated with 10μ*M* of Nb at 72h post-treatment. In contrast, a high proliferation of nGAstV was detected in untreated LMH cells (Fig. 6C). The quantitative assessment of fluorescence intensity measured through grey scale analysis of LMH cells (AU) in different groups has been presented in Fig. 6D. Similarly, significantly lower progeny virus titers were observed in the supernatants of LMH cell cultures treated with Nbs at both 48 and 72h of post-treatment, with lowest titer observed in cultures treated with 10μ*M* of Nb at 72h post-treatment (Fig. 6E) as compared to non-treated Controls.

FCM analysis further indicated that the fluorescence intensity detected from the cells received 5 and 10μ*M* of Nb9-2-cFc was similar to the background level, suggesting that ORF2 binding to the cell surface receptor was minimal at these concentrations. However, at a lower concentration of 2.5μ*M* Nb9-2-cFc, the relative fluorescence intensity increased in infected cells, indicating enhanced ORF2 binding to cells. These findings suggest that Nb9-2-cFc neutralizes nGAstV by blocking the binding of ORF2 to its receptor (Fig. 6F).

Emergency prevention efficacy of Nb9-2-cFc in goslings: *In vivo* prophylactic efficacy of Nb9-2-cFc against nGAstV was tested in goslings (Fig. 7A). Cloacal swabs from goslings were collected after every 48h starting from day 3 after the nGAstV challenge and qPCR was performed to determine viral load through expression analysis of ORF2 mRNA (Fig. 7B). In the nGAstV group, viral shedding first appeared on day 3 after challenge, peaked on day 5 and then decreased by day 9. While no viral shedding was observed in the control group. In the Nb1 group (12.5μ*M* Nb9-2-cFc), viral shedding first appeared on day 4, with significantly lower ORF2 mRNA

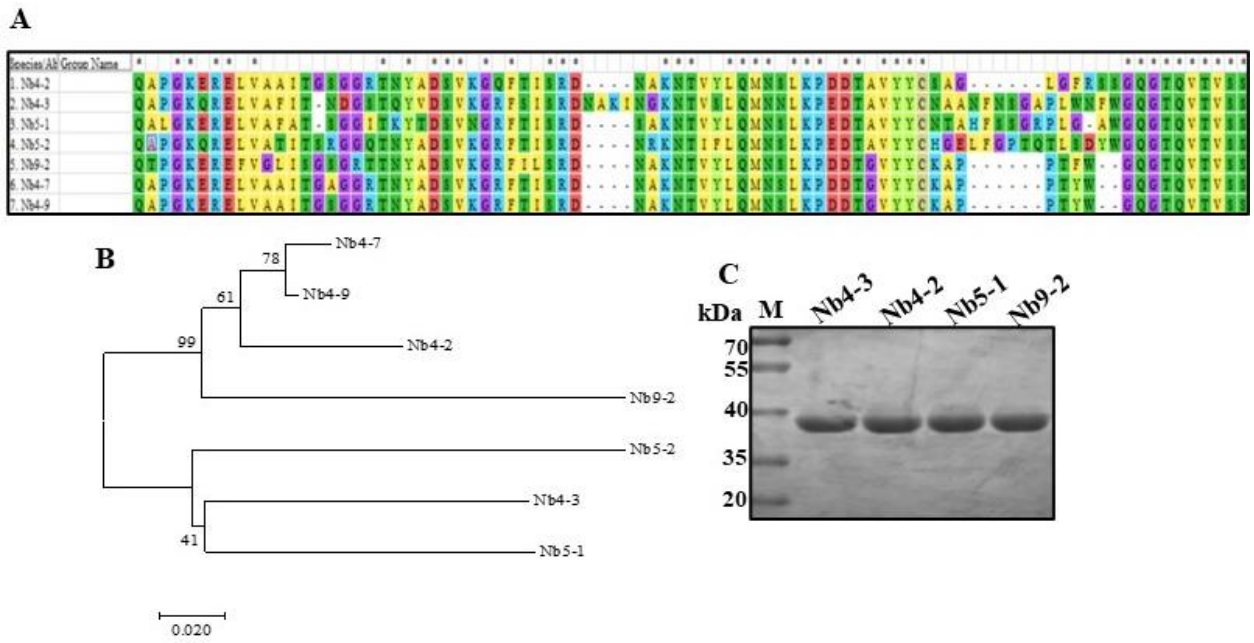

Fig. 2: Sequence analysis and expression of Nbs. (A) Sequence analysis of selected Nbs; (B) Phylogenetic tree showing the evolutionary relationships among the selected Nbs; (C) SDS-PAGE identification of purified Nbs-cFc. The predicted molecular weight of cFc-fused nanobodies was approximately 40kDa. (M: Protein marker; lanes 2-5: four screened Nbs).

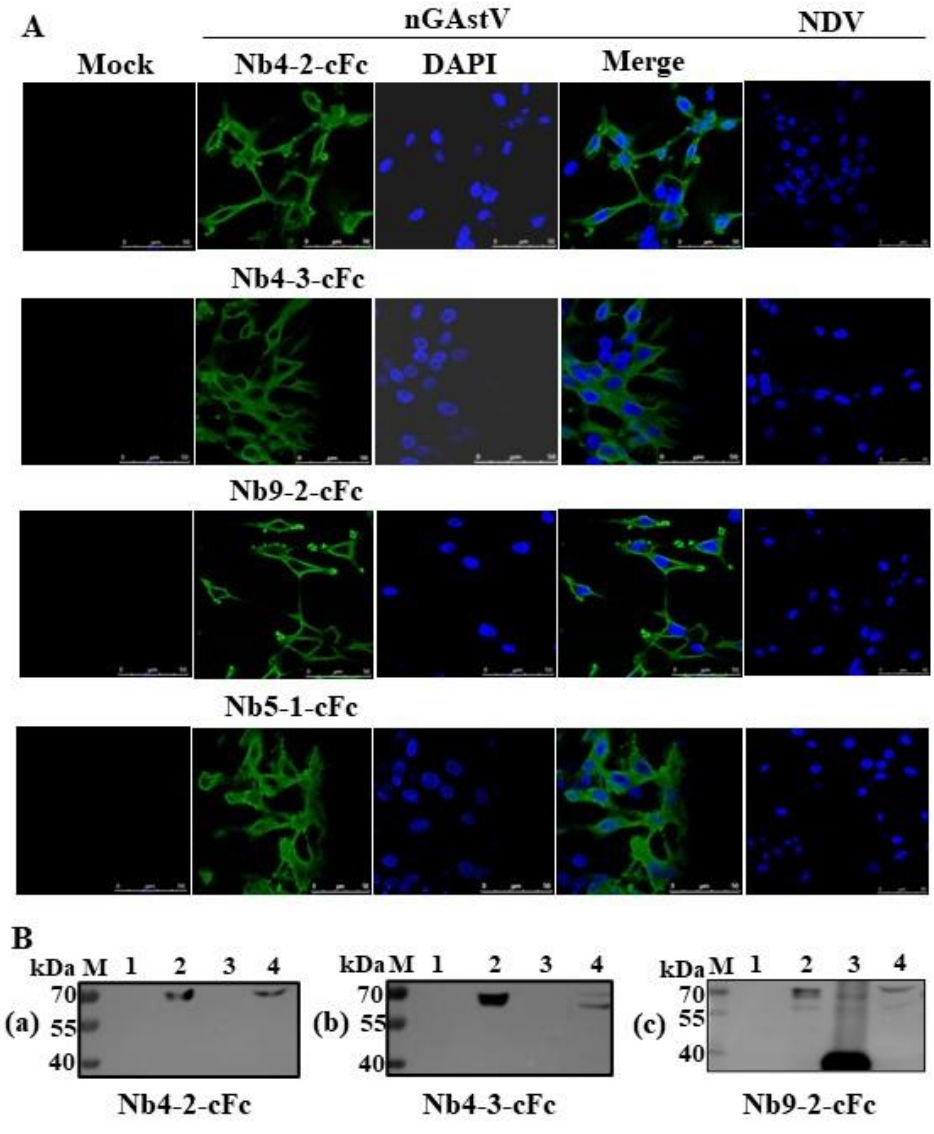

Fig. 3: Specificity detection of Nbs. (A) IFA analysis; (B) Western blot analysis. (a) WB analysis using Nb4-2-cFc as primary antibody. (b) WB analysis using Nb4-3-cFc as primary antibody (c) WB analysis using Nb9-2-cFc as primary antibody (1: NDV; 2: nGAstV ORF2 protein (70kDa); 3: nGAstV P2 protein (40kDa); 4: nGAstV (70kDa; M: Marker).

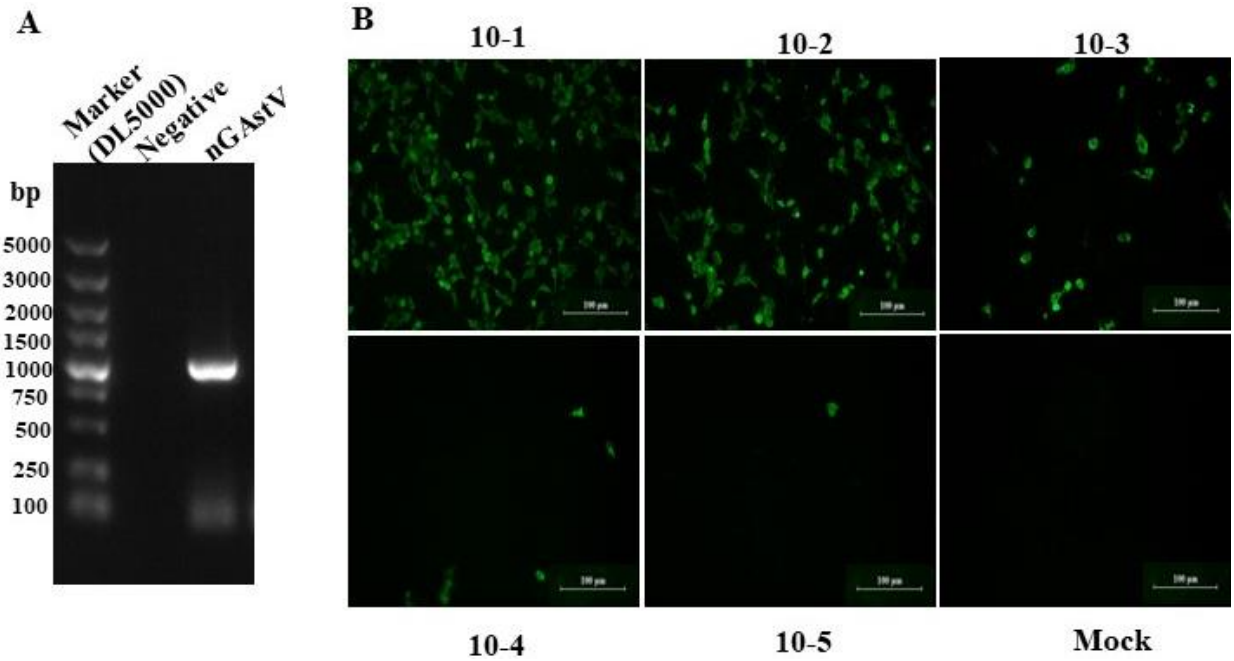

Fig. 4: nGAstV proliferation analysis. (A) PCR identification of nGAstV; (B) Cytopathic effects observed in LMH cells infected by nGAstV. In IFA, Nb9-2 was used as primary antibody, while FITC Polyclonal Goat Anti-Chicken IgY (IgG) (1:5000) with green fluorescence was used as secondary antibody.

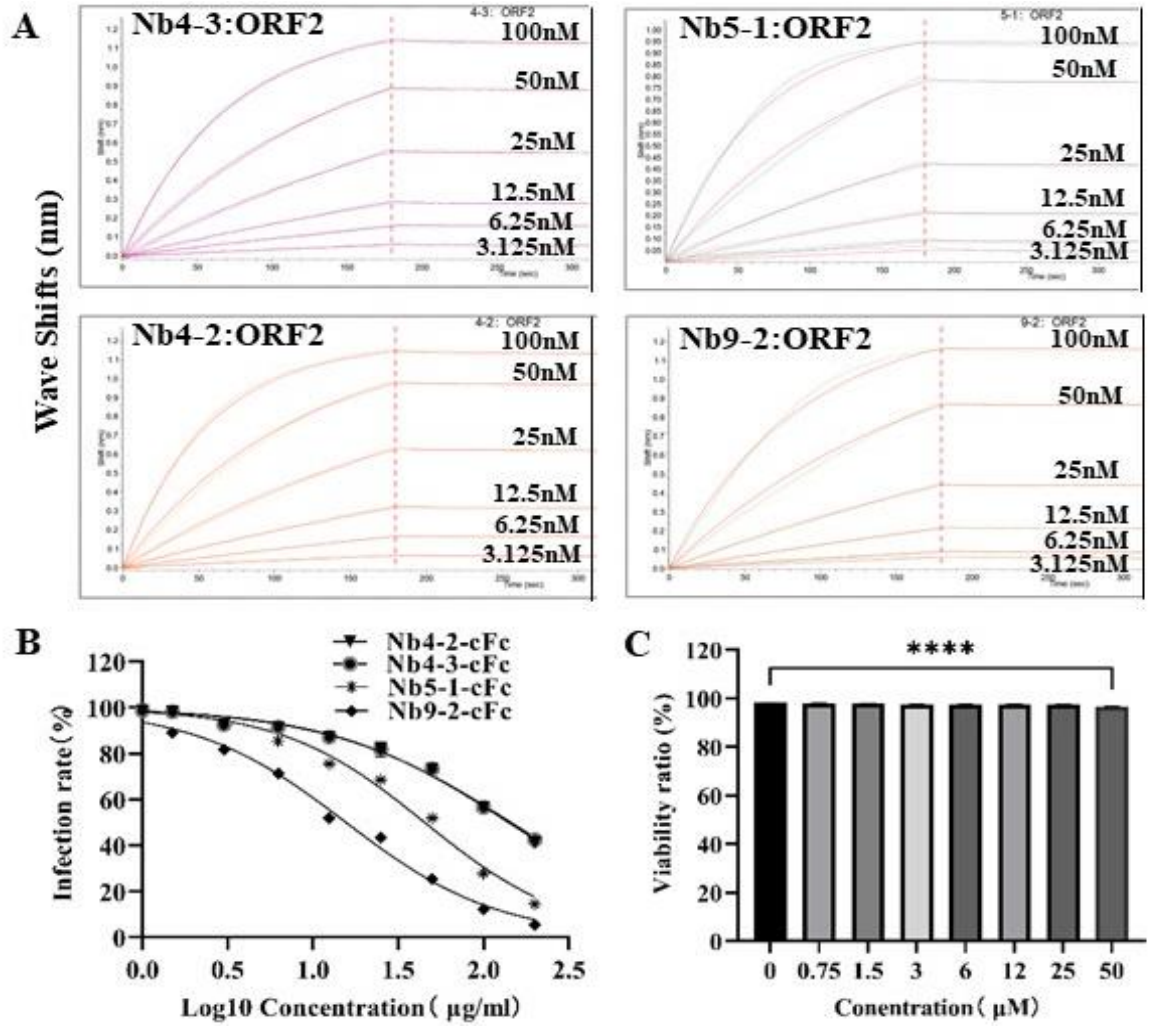

Fig. 5: Identification of recombinant Nb affinity. (A) Biolayer interferometry (BLI) sensorgrams showing the binding kinetics of Nb to nGAstV ORF2 protein. Curves before red dashed lines represent the association phase, while those after represent the dissociation phase; (B) IC_{50} determination, showing that the Nb9-2-cFc neutralized nGAstV in a dose-dependent manner; (C) Cytotoxicity assessment of Nb9-2-cFc, demonstrating that the viability of LMH cells inoculated with Nb9-2-cFc was comparable to that of the untreated control cells up to a concentration of 50μM.

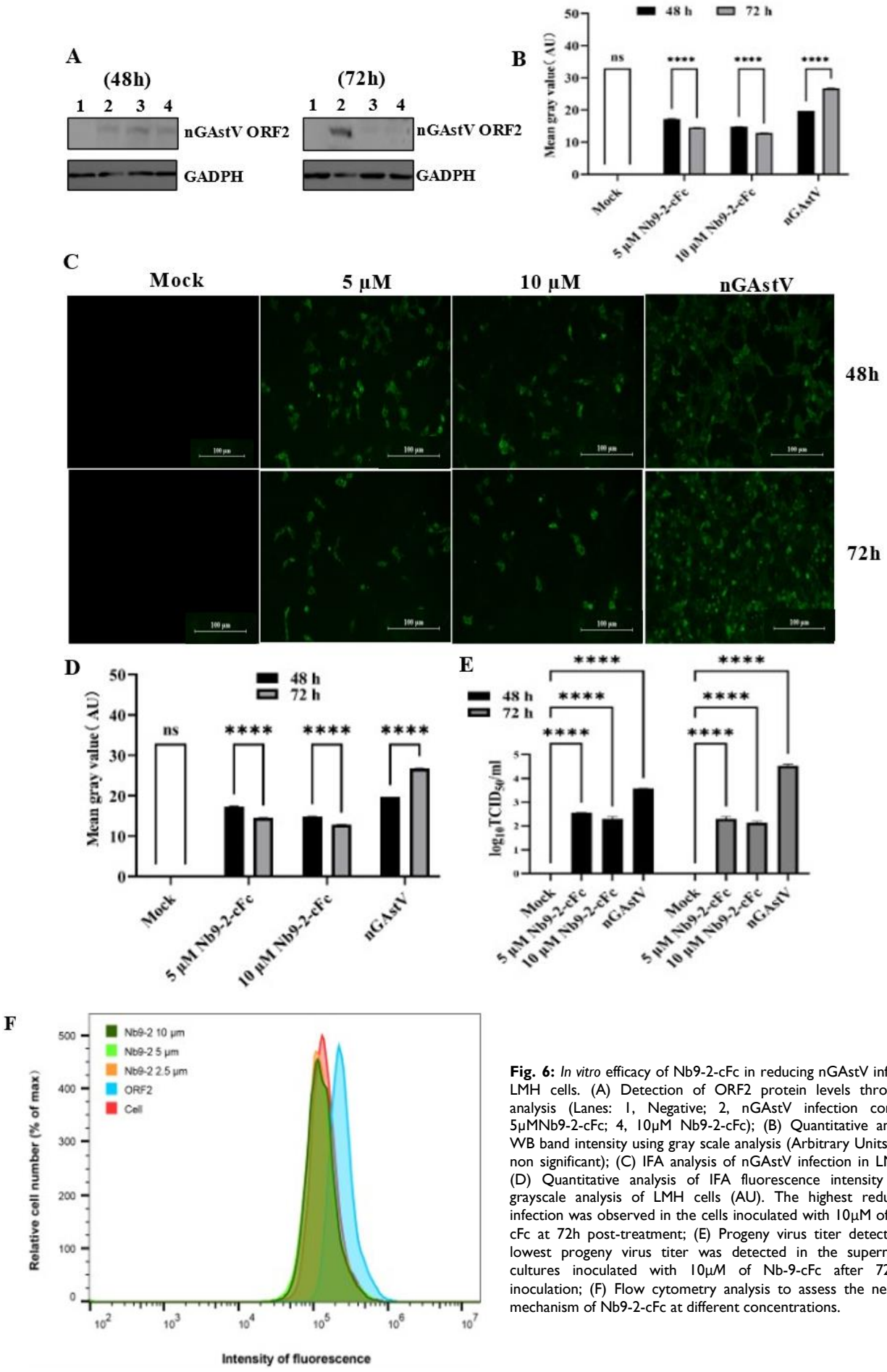

Fig. 6: *In vitro* efficacy of Nb9-2-cFc in reducing nGAstV infection in LMH cells. (A) Detection of ORF2 protein levels through WB analysis (Lanes: 1, Negative; 2, nGAstV infection control; 3, 5μMNb9-2-cFc; 4, 10μM Nb9-2-cFc); (B) Quantitative analysis of WB band intensity using gray scale analysis (Arbitrary Units, AU; ns, non significant); (C) IFA analysis of nGAstV infection in LMH cells; (D) Quantitative analysis of IFA fluorescence intensity through grayscale analysis of LMH cells (AU). The highest reduction in infection was observed in the cells inoculated with 10μM of Nb-9-2-cFc at 72h post-treatment; (E) Progeny virus titer detection. The lowest progeny virus titer was detected in the supernatant of cultures inoculated with 10μM of Nb-9-cFc after 72h post-inoculation; (F) Flow cytometry analysis to assess the neutralizing mechanism of Nb9-2-cFc at different concentrations.

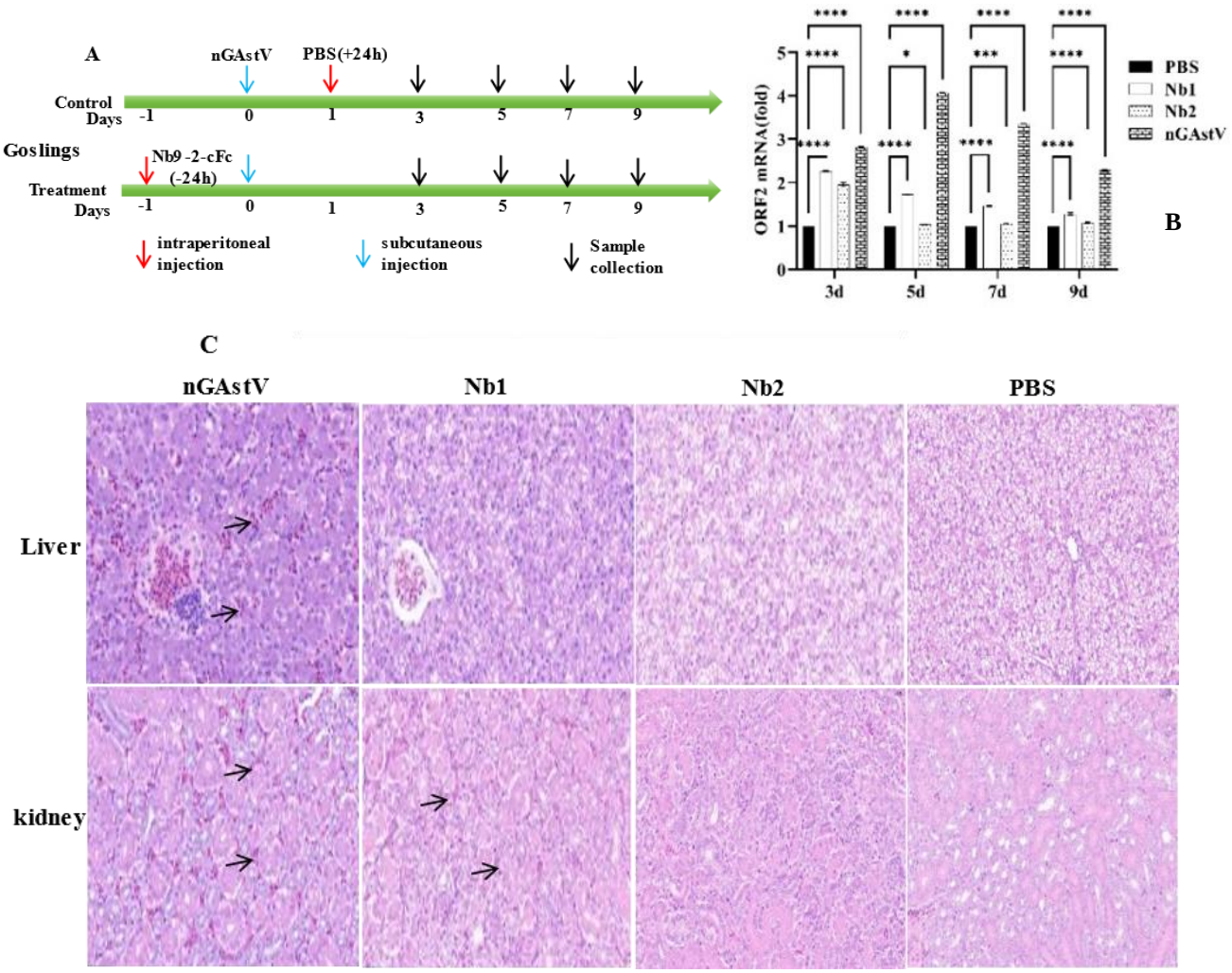

Fig. 7: Emergency prevention efficacy of Nb9-2-cFc in goslings challenged with nGAstV. (A) Experimental design for evaluating the prophylactic efficacy of Nb9-2-cFc. Goslings were treated with Nb9-2-cFc 24h before viral challenge with $10^{5.1}$ EID_{50} (lethal dose) of nGAstV; (B) ORF2 mRNA expression levels of nGAstV in different groups measured through qPCR; (C) Histopathological Examination of liver and kidney tissues from goslings in different groups on day 3 post-inoculation. Black arrows indicate edema, hemorrhage or inflammatory infiltration. (Nb1: 12.5μ*M* of purified Nb9-2-cFc; Nb2: 25μ*M* of purified Nb9-2-cFc; nGAstV: Virus infection only; PBS: no treatment, no infection).

Table 2: Affinity kinetics of Nbs to nGAstV ORF2

Units	Nb4-2-cFc	Nb4-3-cFc	Nb9-2-cFc	Nb5-1-cFc
Equilibrium dissociation constant determined through biolayer interferometry (BLI)				
K_d (pmol/L)	427	365	59.6	127
IC50 for Nbs determined through Microneutralization assay				
IC_{50} (ng·μL-1)	140.1	144.9	15.68	45.15

expression level as compared to the nGAstV group. In the Nb2 group (25μ*M* Nb9-2-cFc), a comparatively low viral load was observed on day 3, while no viral shedding was detected in cloacal swabs collected from the Nb2 group on day 5 onward. The findings suggest that Nb9-2-cFc at a 25μ*M* concentration may reduce nGAstV infection in goslings.

Histopathological analysis of liver and kidney showed obvious histopathological changes at day 7 post-infection (Fig. 7C). In the control group, the hepatocytes were radially arranged around central vein, and the cells were evenly distributed, with no inflammatory infiltration. In the nGAstV group, severe regional lymphocytic phlebitis, lymphocytic inflammatory cell infiltration, and periphlebitis were observed in the liver and kidney of the goslings. The Nb1 group had less inflammatory cell infiltration in the liver and kidney of the goslings, while Nb2 group showed no significant abnormalities in the liver. These results suggest that Nb9-2-cFc exhibits promising prophylactic effects against nGAstV infection.

DISCUSSION

The viral capsid protein encoded by the ORF2 reading frame, predominantly composed of P1 and P2 proteins, plays crucial roles in viral adsorption, replication, and immune stimulation (Zhang *et al.* 2021). Therefore, ORF2 protein serves as an ideal diagnostic target. In a previous study, Nbs were produced in alpacas and specific Nbs showing binding with nGAstV ORF2 protein were identified. Then, Nbs were genetically engineered and their binding affinity to nGAstV ORF2 protein was assessed through ELISA and Western blotting (Wang *et al.*, 2023). However, neutralizing activity and the ability to prevent viral infections were not addressed. In the present study, the same Nbs phage library was screened, and specific Nbs were identified and enriched through three rounds of panning by using nGAstV ORF2 protein as antigen.

Due to the short half-life of the Nbs, which limits its clinical application, several researchers have conjugated Fc domain of an immunoglobulin with the VHH domain

and found that the resultant chimeric nanobodies reinstate antibody Fc functions with enhanced effector activity along with enhanced specificity, half-life, stability and solubility (Bannas *et al.*, 2017; Hong *et al.*, 2019). In human medicine, numerous studies have explored the application of Fc conjugated Nbs in cancer diagnosis, therapeutics and immunotherapies (Bannas *et al.*, 2017; Tang *et al.*, 2023; Li *et al.*, 2024). In this study, chicken Fc-fused Nbs were tested for the first time in goslings to evaluate their therapeutic and prophylactic efficacy against nGAstV infection.

For production of nanobodies, four selected sequences with variations were ligated in a self-built CHIGG-cFc eukaryotic expression vector. CHIGG-cFc vector with multiple cloning sites (MCS) at the C-terminus allows the expression of recombinant protein of interest fused to the IgG-Fc region. Since the N-terminus of MCS is inserted into the secretion signal sequence, Fc fusion chimeric proteins can be secreted into the culture supernatant. While retaining the original protein activity, Fc fusion chimeric proteins have the advantages of long half-life, high expression in mammalian cells, and easy purification with Protein A mediator. The expressed Nb-cFc were having molecular weight of approximately 40kDa, as confirmed through SDS-PAGE. These findings are consistent with previous studies, in which chimeric Nbs were having sizes around 40kDa (Qasemi *et al.*, 2016; Jia *et al.*, 2023).

Further analysis of Nb binding affinity to nGAstV through IFA showed that all four selected Nbs exhibited strong reactivity with nGAstV, with no cross-reactivity to Newcastle disease virus (NDV) or LMH cells used as controls. WB analysis results revealed that Nb4-2-cFc and Nb4-3-cFc specifically recognized the ORF2 protein, while Nb9-2-cFc recognized both the ORF2 and P2 protein. However, Nb5-1-cFc did not recognize any protein, suggesting that it may be conformationally bound to ORF2 protein (Harmsen and De Haard, 2007; Muyldermans *et al.*, 2013). These findings align with previous research where specific nanobodies demonstrated high-affinity recognition of viral proteins (Qasemi *et al.*, 2016).

The affinity of four nanobodies to nGAstV ORF2 protein was further evaluated using biolayer interferometry assays (BLI). Similar to the results of IFA and WB, the BLI results indicated that Nb9-2-cFc exhibited high binding activity to nGAstV ORF2 protein (K_d=59.6 p*M*/L). Nb9-2-cFc also displayed high neutralizing activity, as evidenced by its low IC50 value (15.75ng/mL). In contrast, the other three Nbs did not show significant neutralization, indicating that these clones have distinct functional capabilities. This result supports findings from previous studies emphasizing the variability in neutralizing efficiency among different Nbs (Harmsen and De Haard, 2007).

To assess *In vitro* efficacy of Nb9-2-cFc in reducing nGAstV infection in LMH cells, a virus neutralization assay was performed, and infection of nGAstV in LMH cells was assessed though IFA and fluorescence intensity measurements. The results demonstrated a dose-dependent reduction in nGAstV infection, with lowest titer at a concentration of 10μ*M*. Additionally, flow cytometry (FCM) analysis demonstrated that Nb9-2-cFc neutralizes the virus by blocking the binding of ORF2 to its receptor, which aligns with neutralization mechanisms in antibody-based therapeutics (Xue *et al.*, 2019; Bhattacharya *et al.*, 2023). Similarly, several studies have demonstrated the virus neutralizing activity of Nbs which exhibit binding affinity to viral proteins (Chen *et al.*, 2022; Zhu *et al.*, 2022; Bhattacharya *et al.*, 2023).

In vivo prophylactic efficacy of Nb9-2-cFc against nGAstV was also assessed in goslings. After 24h of administration of Nbs, goslings were challenged with lethal dose of nGAstV and viral load was determined using qPCR by amplifying ORF2 protein. A significantly reduced virus shedding was observed in goslings received Nb9-2-cFc, with highest virus clearance in those inoculated with 25μ*M* concentration. A decline in the expression of ORF2 protein in Nb inoculated goslings further demonstrated that the protective efficacy of Nb may be due to inhibition of viral proliferation because of blocking the binding of ORF2 to its receptor. Histopathological analysis also revealed that the goslings inoculated with Nb9-2-cFc (25μ*M*) exhibited reduced inflammatory cell infiltration in the liver and kidney. These results are consistent with previous studies demonstrating the prophylactic potential of Nbs in viral infections (Liu *et al.*, 2019; Huang *et al.*, 2022; Hoang *et al.*, 2024).

Conclusions: This is the first report on the development of chicken Fc fused chimeric nanobody against nGAstV in goslings. Overall, a specific nanobody Nb9-2-cFc was identified as a promising antiviral agent against nGAstV. This chimeric Nb specifically binds to ORF2 protein, preventing nGAstV to bind with its receptors. Its strong binding affinity, high specificity, and effective neutralization suggests that this candidate may be used as both a diagnostic marker and emergency prevention therapy. However, future research should be carried out to determine the potential synergistic effects with other antiviral agents to enhance its clinical applicability.

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Authors contributions YG executed the research and prepared original draft; ZC conducted software analyses; JG handled data curation; QZ reviewed and edited the manuscript; JY provided supervision, and FA managed project administration; and XL assisted in funding acquisition. All authors have read and approved the final version of the manuscript

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