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ORIGINAL ARTICLE

The heterologous expression of novel recombinant protein composed of HN and F moieties of Newcastle disease virus and immunogenicity evaluation in mouse model

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ABSTRACT

Background and Objectives: The rapid spread of Newcastle disease (ND), driven by extensive commercial exchange in the poultry industry, necessitates urgent preventive measures. Although effective vaccines against the Newcastle disease virus (NDV) have been used since 1940, recent outbreaks and the limitations of current vaccines highlight the need for improved solutions. Advances in synthetic biology, reverse vaccinology, molecular biology, and recombinant DNA technology over the past 20 years have led to the development of recombinant vaccines, which offer enhanced protection and broader immunogenic coverage against NDV. This study aimed to express the immunogenic domains of Hemagglutinin Neuraminidase (HN) and Fusion (F) glycoproteins, linked to the heat-labile enterotoxin B subunit (LTB) bio-adjuvant, to develop an effective and reliable recombinant vaccine for NDV.

Materials and Methods: In this study, the L(HN)2F protein, composed of the LTB bio-adjuvant and the immunogenic regions of the doubled Hemagglutinin Neuraminidase (HN-HN) and Fusion (F) epitope, was expressed in *Escherichia coli*. Subcutaneous injection was used to evaluate the humoral immune response in mice and the result was compared with B1 vaccine.

Results: The induction of strong humoral immune responses proved the strong immunoreactivity of the recombinant protein.

Conclusion: The IgG elicited by the recombinant proteins was comparable to that of the commercial B1 vaccine against NDV, indicating its potential as a viable candidate for further development and evaluation as a recombinant vaccine against NDV.

Keywords: Newcastle disease; Hemagglutinin neuraminidase; Fusion (F) glycoprotein; Immune response; LTB bio-adjuvant

INTRODUCTION

Newcastle Disease (ND) is a highly contagious ailment that affects both wild and domestic birds posing a significant threat to the global poultry industry. Since its first detection in Newcastle, England, it has threatened the poultry industry. Over the last two decades, several outbreaks have been caused by genotype VII of the Newcastle Disease Virus (NDV) (1, 2). From 2013 to 2018, sixty countries reported an

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outbreak of the disease in domestic birds (3).

NDV encodes six structural proteins in order 3'-NP-P-M-F-HN-L-5', in Which NP encapsulates viral RNA and the L protein acts as an RNA-dependent RNA polymerase. The L segment connects to the P protein and establishing a structure to recognize the RNP for initiating transcription and replication (4). Hemagglutinin Neuraminidase (HN) and Fusion (F) are two surface glycoproteins of NDV that are important factors in ND virulence and infectivity. HN mediates the attachment of the virus to its sialicacid-containing receptors and the stalk segment of HN can improve fusion by interacting with the F glycoprotein (5).

Prevention and early detection play a major role in successfully controlling ND disease in poultry. The most widely used ND vaccines are the LaSota, B1 and VG/GA (6). These vaccine viruses have tropisms different from those of the poultry host cell, with the highest rate belonging to the LaSota strain. VG/GA is typically used as an enterotropic vaccine, while the B1 strain is the most attenuated vaccine for low-risk situations (6). Live vaccines do not provide humoral immunity, can be applied through mass vaccination strategies, and may lose storage activity at temperatures above 4°C (6, 7). NDV genotypes may change due to mutations from live attenuated vaccines (3). Inactivated vaccines are an alternative but are mainly incapable of producing long-lasting immunity and have high production costs (4, 6).

Different platforms, including bacteria, plants, and yeast, are used to express recombinant protein-based vaccine candidates against NDV (8). There is a critical need to develop a safe, effective, and dynamic vaccine due to natural changes in the viral genome. In the last decade, using recombinant technology to develop vaccines has increased. Proteins produced with this technology are generally inexpensive, widely available and safer than vaccines produced based on live viruses (9).

Several attempts have been made to develop a recombinant NDV vaccine, principally based on expressed F and HN genes. Immunization results showed that these recombinant proteins could protect chickens against NDV challenges. There are several recombinant and subunit vaccines, including the expression of full-length or partial F protein (3, 10, 11), HN protein (12) and simultaneous expression of both HN and F glycoprotein (13, 14). Subunit vaccines based on recombinant antigenic proteins are effective but have fewer immunogenic properties compared to live or attenuated vaccines containing several essential and non-essential immunostimulatory elements (15).

To overcome this and improve the efficiency and immunity of subunit vaccines, adjuvants can be added as immune system modulators (16). Heat-labile enterotoxin (LT) of toxigenic *Escherichia coli* consists of an A subunit with toxic and enzymatic activity and a pentamer of non-toxic heat-labile enterotoxin B subunit (LTB) with a pentameric structure, proven to have adjuvant properties. In animal and human studies, LTB has been used as an adjuvant mixed or linked with specific antigens and successfully improved both humoral and cellular immune responses. (17, 18). LTB can bind to GM1 ganglioside receptors on the eukaryotic cells' surface, thereby increasing antigen uptake (7, 19).

Vaccines have historically played a crucial role in combatting, eliminating, and eradicating numerous infectious agents such as Newcastle disease. Despite the existence of live attenuated and inactivated viral vaccines against Newcastle, the virus continues to pose a threat to the poultry industry. To combat NDV variety strains, prevent hazards of virus cultivation for vaccine production, and develop an effective vaccine against NDV, a recombinant vaccine design is essential (20).

In this study, a recombinant *l(hn)2f* construct has already been designed by in silico assembling conserved epitopic regions of the HN and F proteins of the NDV added to LTB as bio-adjuvant (21). After the heterologous expression of recombinant proteins in *E. coli* hosts, the immunogenic response of all proteins versus the vaccine strain of the NDV was determined in an animal model by the ELISA test.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. *E. coli* BL21 (DE3) (Pasteur Institute, Iran) and pET-28a (+) (Novagen, USA) were used in this study. Luria-Bertani (LB) media with the appropriate antibiotic (kanamycin 50 µg/ml) was used for recombinant bacterial culture and selection (22).

Design and construction of recombinant plasmids. The synthetic recombinant peptide amino acids (rL(HN)2F) were retrieved from Gene Bank (accession no. MH023426) and used in constructing all re-

combinant plasmids. The rL(HN)2F peptide is a chimeric protein comprising the heat-labile enterotoxin B subunit (LTB) as a bio-adjuvant, which enhances the immunogenicity of the vaccine. It includes two copies of neutralizing epitopes from the Hemagglutinin Neuraminidase (HN) protein and neutralizing epitopes from the Fusion (F) protein of Newcastle disease virus (NDV). These components are linked by a short peptide linker (EAAAK) to maintain structural integrity and facilitate protein assembly. Additionally, a 6× histidine tag was incorporated at the 5' end of the construct to aid in the purification process. This design was aimed at maximizing the immunogenic potential of the recombinant protein and simplifying its purification and characterization. Bioinformatic analysis were performed to select suitable epitopic regions and linkers (23). To express each fragment separately (*ltb*, *hn*, *hn-hn* and *f* genes), the segments were amplified using specific primers (Table 1) with the *Bam*HI (GGATCC) and *Hin*dIII (AAGCTT) restriction endonuclease recognition sites at the 5' and 3' ends of the genes. Each amplified fragment and the chimeric synthesized *l(hn)2f* were cloned into pET-28a (+) expression vector under the control of T7 promoter between *Bam*HI and *Hin*dIII restriction sites separately. The recombinant pET-28 plasmids were transformed into an *E. coli* BL21 (DE3) competent expression host cells. Authentic recombinant colonies, confirmed by PCR, digestion and sequencing, were stored at -70°C. The construct and attachment site of the primers used in this work are schematically shown in Fig. 1A.

Expression and purification of the recombinant proteins. *E. coli* BL21 (DE3) strains carrying recombinant pET28a-*ltB, hn, hn-hn, f,* and *l(hn)2f* plasmids were cultured in LB broth supplemented with 50 µg/ml kanamycin at 37°C. When the optical density at 600 nm (OD600) reached 0.7, protein expression was induced by adding 1 mM IPTG (Sigma) and incubating for an additional 4 hours at 37°C. After induction, cells were harvested by centrifugation at 3500 × g for 10 minutes at 4°C. The cell pellets were re-suspended in 1 ml lysis buffer (50 mM NaH2PO4, pH 8.0; 300 mM NaCl; 0.2 mg/ml lysozyme) and incubated on ice for 2 hours to facilitate cell lysis. Following incubation, the lysate was clarified by centrifugation at 15,000 × g for 30 minutes at 4°C. The supernatant and pellet were analyzed separately using 12% SDS-PAGE to confirm the presence of recombinant proteins. Purification of recombinant proteins was carried out using nickel–nitrilotriacetic acid (Ni–NTA, Qiagen) resin under denaturing conditions at room temperature. The supernatant was applied to the Ni–NTA column pre-equilibrated with binding buffer (50 mM NaH2PO4, pH 8.0; 300 mM NaCl; 8 M urea). Proteins were eluted with an increasing gradient of imidazole (20-500 mM) in the elution buffer (50 mM NaH2PO4, pH 8.0; 300 mM NaCl; 8 M urea; 500 mM imidazole). Eluted proteins were analyzed by 12% SDS-PAGE to verify their purity. Protein concentrations were determined using Bradford's assay (24). For further purification and to remove urea, proteins were dialyzed against PBS (pH 7.5) overnight at 4°C. (25).

Immunization protocol. Forty-five female BALB/c mice (5-6 weeks old, Razi Vaccine and Serum Research Institute, Iran) were randomly assigned to seven groups (A to G) for immunization. The study involved four immunization rounds at one-week intervals. Groups A-E: Each group was immunized subcutaneously in the back of the neck with 15 µg of purified recombinant proteins: rLTB, rHN, rHN-HN, rF, or rL(HN)2F, respectively, mixed with complete Freund's adjuvant (CFA, Sigma, USA). Following the initial immunization, each group received two additional injections of 10 µg of the same protein

Table 1. The list of primer sequences used in this study.

Primers	Sequence* 5' → 3'	Restriction site
LHN2F Forward	TTGCTTGGATCCATGGCGCCGCAAAG	*Bam*HI
LHN2F Reverse	GTCGACAAGCTTTTAGTGATGATGATGATGATGC	*Hin*dIII
LTB Reverse	GTCGACAAGCTTTTAGTTTTCCATGCTAATC	*Hin*dIII
HN-HN Forward	ATAATAGGATCCATGGTGGCGAACTACC	*Bam*HI
HN Reverse	TCGACAAGCTTTTAACCGCCGAAACGAC	*Hin*dIII
HN-HN Reverse	GTCGACAAGCTTTTAGCCACCAAAACGAC	*Hin*dIII
F Forward	GAGCTCGGATCCATGATCGTGGTTACCGGCGACAAG'	*Bam*HI

in incomplete Freund's adjuvant (IFA, Sigma, USA). The final booster consisted of 2 µg of the recombinant protein administered intraperitoneally without adjuvant. A detailed immunization schedule is provided in Table 2. Group F (Negative Control): Mice were injected with PBS under the same schedule as groups A-E. Group G (Positive Control): Mice were immunized with 100 µg, 80 µg, 50 µg, and 30 µg of a commercial NDV vaccine (B1 strain) at the same time intervals as the experimental groups. Antisera were collected from each mouse group after the second, third, and final immunizations (1st, 2nd, and 3rd boosters). Blood samples were obtained by cardiac puncture, allowed to clot at room temperature, and then centrifuged at 3000 × g for 10 minutes. The sera were pooled from each group, aliquoted, and stored at -70°C until further analysis.

Table 2. Immunization schedule for (A to E) groups

Group	A	B	C	D	E
Protein	rLTB	rHN	rHN-HN	rF	rL(HN)2F
1st injection	15 µg	15 µg	15 µg	15 µg	15 µg
1st booster	10 µg	10 µg	10 µg	10 µg	10 µg
2nd booster	10 µg	10 µg	10 µg	10 µg	10 µg
3rd booster	2 µg	2 µg	2 µg	2 µg	2 µg

Western blotting analysis. Purified protein samples (10 µg) were separated by 12% SDS–PAGE and were transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, Germany). Each recombinant protein was detected with related antiserum from the immunized mice (anti-LTB, anti-HN, anti-HN-HN and anti-F with 1:5000 and anti-L(HN)2F 1:2000 dilution and1:5000 anti B1) as primary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:2000; Sigma, Germany) antibodies were used as secondary antibodies. The chromogenic reaction visualized specific protein-antibody complexes using 3, 3′-Diaminobenzidine (DAB Reagents; Sigma). This procedure was repeated with one-step anti-6xHis-tag antibodies (according to the manufacturer's guidelines, Sigma) for all recombinant proteins.

Evaluation of humoral immune responses to recombinant proteins by ELISA. ELISA was performed to determine the specificity and titration of the antibodies as previously described. The purified rLTB, rHN, rHN-HN, rF and rL(HN)2F (200 ng each) were used as antigens. The specific antibodies against rLTB, rHN, rHN-HN, rF and rL(HN)2F recombinant proteins and antibody against B1 vaccine strain were serially diluted from 1:50 to:400000. The diluted sera containing antibodies against each recombinant protein from the immunized mice (1:6400) and antibodies to mouse IgG labeled with horseradish peroxidase were added to corresponding wells as a primary and secondary antibodies. The colorimetric reaction was visualized using O-phenylenediamine (OPD) and the responses were stopped by Sulfuric acid (2 M) (26). Finally, the cross-reactivity between the rL(HN)2F recombinant protein (200 ng) with antibodies against the B1 vaccine, as well as with antibodies against the each recombinant proteins rLTB, rHN, rHN-HN, rF, and rL(HN)2 were analyzed.

Statistical analysis. All statistical data was analyzed using the SPSS program (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 9.0 software (Graph Pad Software, Inc., San Diego, CA). The means of all data were compared using the one-way ANOVA test to determine differences. The differences were considered significant if the p-value was less than 0.05 ($p < 0.05$).

RESULTS

Construction of recombinant expression vectors. To amplify the recombinant individual fragments (*ltb*, *hn*, *hn-hn* and *f*), the chimeric *l(hn)2f* structure (23) was used as a template. The PCR products (*ltb*, *hn*, *hn-hn* and *f*) and the synthetic gene *l(hn)2f* were purified and ligated into the pET28a (+) expression vector separately. The presence of desired fragments in expression vectors, were analyzed by PCR technique and using specific primers (Fig. 1 B and C).

Expression and purification of recombinant proteins in *E. coli*. The recombinant proteins, rL(HN)2F (52 KDa), rLTB (13 KDa), rHN (10 KDa), rHN-HN (22 KDa) and rF (17 KDa), harboring C-terminal 6×His-tag were expressed in *E. coli* Bl21 (DE3) and the expected bands were visualized by 12% SDS-PAGE. Fig. 2A displays the purified and dialyzed recombinant proteins on an SDS-PAGE. As indicated, the proteins are positioned at their corresponding molecular weights, which demonstrates correct expression and proper folding without any degradation. The

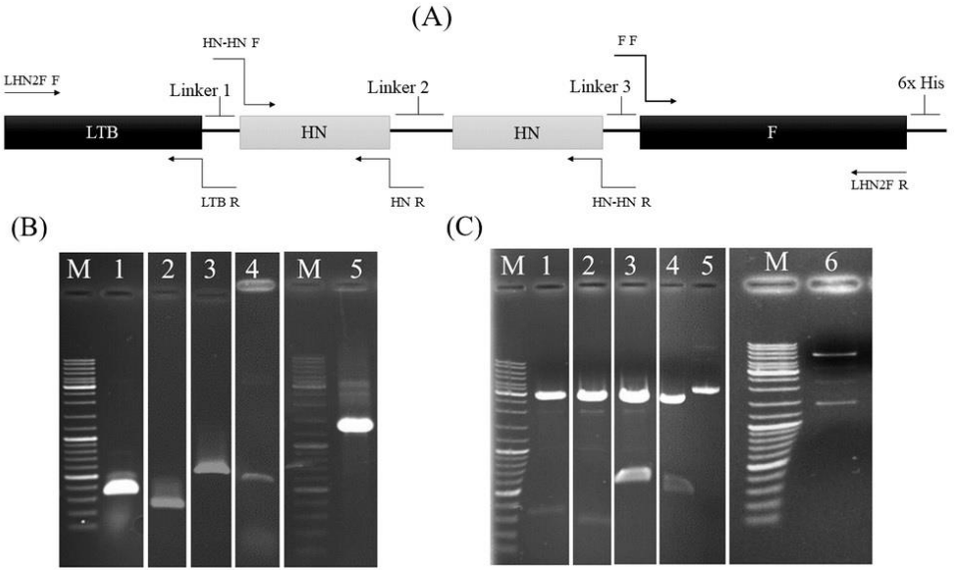

Fig. 1. Schematic view of chimeric L(HN)2F protein and gel electrophoresis of the cloning steps.
(A) Diagram of chimeric L(HN)2F; the LTB -HN, HN-HN and HN-F connections were created by four, five and four repeats of EAAAK linkers, respectively. The presence of the different fragments in pET28a (+) was confirmed by (B) Verification of Constructs by PCR, lane 1. *ltb* (L(HN)2F F and LTB R ~ 350 bp), lane 2. *hn* (HN-HN F and HN R ~ 250 bp), lane 3. *hn-hn* (HN-HN F and HN-HN R ~ 550 bp), lane 4. *f* (F F and L(HN)2F R ~ 450 bp) and lane 5. *L(hn)2f* (L(HN)2F F and L(HN)2F R ~ 1400 bp) and (C) Verification of Constructs by Restriction Enzyme Digestion (*Bam*HI/ *Hin*dIII). Lane 1. *ltb*, lane 2. *hn*, lane 3. *hn-hn*, lane 4. *f*, lane 5 negative control (undigested plasmid) and lane 6. *L(hn)2f*. M: DNA 100 bp Ladder Mix (Thermo).

concentration of the purified proteins was estimated by standard Bradford protein assay (AB595) and the average yield for recombinant rL(HN)2F, rLTB, rHN, rHN-HN and rF was estimated as 1232.65, 302, 392, 744. and 297 µg/ml, respectively.

***In vivo* immunogenicity of proteins.** After immunizing mice with the purified proteins, sera were analyzed using Western blot to confirm the presence and accuracy of the recombinant proteins produced in bacterial systems. Additionally, the cross-reactivity of the humoral immune response to all recombinant fragments was confirmed with anti-LTB, anti-HN, anti-HN-HN, anti-F, anti-L(HN)2F, and commercial anti-6x His tag antibodies. The results are presented in Figs. 2B-D. Bands corresponding to the molecular weights of the proteins are visible on the PVDF membrane, indicating their complete, integrity and accurate expression. The distinct bands corresponding to the proteins of interest suggest that the recombinant proteins maintain their structure and have not undergone significant degra- dation.

Additionally, the cross-reactivity observed with specific antibodies indicates that the immune response generated in mice is robust and recognizes the recombinant proteins effectively. This suggests that the proteins are likely properly folded and can present their epitopes to the immune system.

ELISA analysis. The ELISA analysis of serial dilution of anti-rL(HN)2F for different boosters was performed. The results in Fig. 3A indicate the sensitivity and specificity of the antibodies produced, showcasing their ability to recognize the rL(HN)2F antigen across various concentrations. Also, the cross-reactivity of anti-L(HN)2F IgG with other antigens, including rLTB, rHN, rHN-HN, rF and rL(HN)2F antigens and rL(HN)2F antigen with different antibodies, including anti-LTB, anti-HN, anti-HN-HN, anti-F and anti-L(HN)2F antibodies, were analyzed (Fig. 3 B and C). The cross-reactivity studies illustrated in Fig. 3B and C demonstrate the binding affinity of anti-L(HN)2F IgG to other recombinant antigens, such as rLTB, rHN, rHN-HN, and rF. This suggests that the antibodies generated against rL(HN)2F have the potential to recognize similar epitopes present in these other antigens, providing insights into the broader immunogenicity of the recombinant protein. Conversely, testing the reactivity of the rL(HN)2F

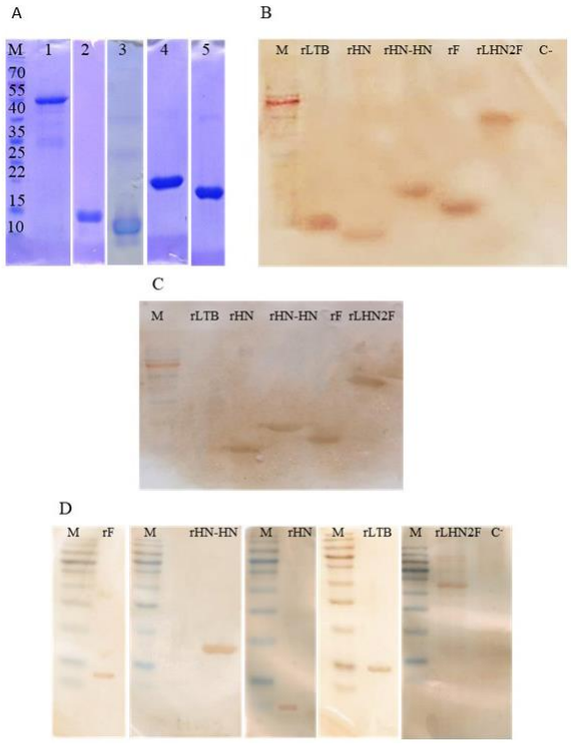

Fig. 2. SDS-PAGE and western blotting analysis of rL(HN)2F. (A) SDS-PAGE analysis of purified rL(HN)2F (lane 1), rLTB (lane 2), rHN (lane 3), rHN-HN (lane 4) and rF (lane 5). Western blotting analysis of purified recombinant proteins with (B) commercial anti-His-tag antibody, (C) anti-Newcastle B1 antibodies. (D) Western blotting analysis of purified recombinant rL(HN)2F, rLTB, rHN, rHN-HN and rF by their specific antibodies. M. protein molecular weight marker (KD). C-. Negative control (protein without His-tag).

antigen with various antibodies reinforces the specificity and effectiveness of the immune response, highlighting the relevance of these antibodies for potential diagnostic or therapeutic applications. Overall, these findings underscore the robust humoral immune response elicited by the recombinant proteins and their potential utility in immunological studies.

The antibody titer after the first, second, and third boosters for groups B and C was compared using indirect ELISA. As shown in Fig. 4, the ELISA results demonstrate the progressive increase in antibody titers in response to the rHN and rHN-HN recombinant proteins. The specific IgG antibodies against rLTB, rHN, rHN-HN, rF, rL(HN)2F proteins and B1 (as a positive control) were detected after immunization in the blood sera. The cross-reactivity analyses of sera from rL(HN)2F and B1 immunized mice were assayed against all purified recombinant proteins (Fig. 5A). The anti-L(HN)2F and anti-B1 IgG antibody responses from the mice groups were titrated by ELISA at 14-, 22- and 30 days post-first immunization (Fig. 5B).

DISCUSSION

The prevalence of Newcastle disease (ND) continues to challenge the efficacy of existing vaccines, raising questions about their ability to protect against clinical disease and prevent viral transmission among birds. Over the past six decades, existing vaccines have not fully prevented either infection or the shedding of the Newcastle disease virus (NDV) into the environment (27). Additionally, multiple outbreaks have been associated with mutations in vaccine strains that enhance virulence, underscoring the need for new vaccination strategies. Consequently, there is a clear necessity to develop vaccines based on alternative approaches that cover a broader spectrum of viral strains, exhibit enhanced safety profiles, and minimize side effects. Recombinant vaccines emerge as a promising solution, although they often exhibit inadequate immunogenicity and typically require natural or synthetic adjuvants to boost immune responses (28). Biological molecules like LTB (The *E. coli* heat labile toxin, B subunit) and cholera toxin B subunit (CTB) serve as effective bio-adjuvants due to their recognized ability to signal the immune system and promote general immune responses (7, 16). Given the significant global and economic impact of ND, with the critical roles of the viral glycoproteins F and HN in mediating pathogenicity, our research aimed to design and produce a novel recombinant vaccine candidate. This candidate incorporated specific epitopic regions derived from the F and HN genes alongside the bio-adjuvant LTB, resulting in the construct designated as L(HN)2F. The highly epitopic sequences of LTB (amino acids 1-104 without signal peptide), HN (296-366 aa), HN (296-366 aa), and F (42-182 aa) fragments were selected. To facilitate proper protein folding and maintain conformational integrity, EAAAK repeat linkers were used (23).

After the expression of proteins, the initial purification of the target protein using Ni-NTA resin under denaturing conditions was performed. To address potential issues with protein folding, the dialysis was performed to increase the solubility and

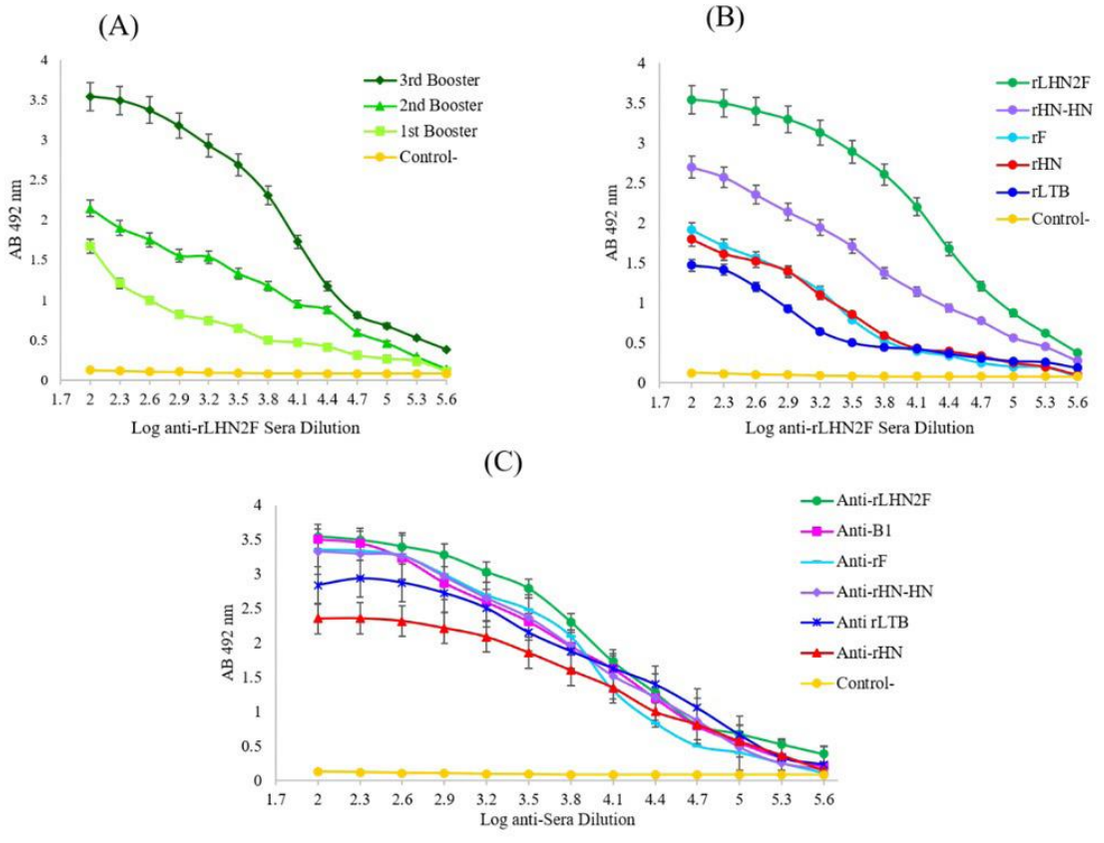

Fig. 3. ELISA assays of anti-sera serial dilutions of recombinant proteins. (A) The reaction of rL(HN)2F antigen with anti-L(HN)2F attained from different boosters and control mice. (B) The cross-reactivity of anti-L(HN)2F IgG with different antigens, including rLTB, rHN, rHN-HN, rF and rL(HN)2F antigens. (C) The cross-reactivity of rL(HN)2F antigen with different antibodies, including anti B1 vaccine strain, anti-LTB, anti-HN, anti-HN-HN, anti-F and anti-L(HN)2F antibodies. Sera from mice immunized with PBS+adjuvant was used as negative control ($p < 0.05$). The error bar is the standard deviation.

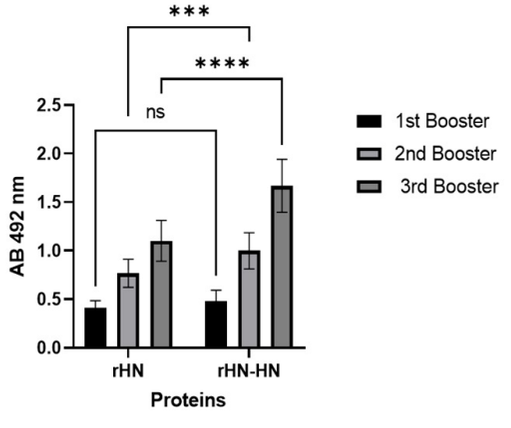

Fig. 4. Diagrams of IgG titration. Diagram of comparing humoral immune responses assessed by ELISA results of sera from mice immunized with rHN and rHN-HN recombinant proteins (B and C groups) after the first, second, and third boosters.

partially refold the protein. The subsequent analyses (ELISA and Western blotting) confirmed proper structure, folding and the preservation of conformational epitopes (29). Additionally, upon injection into an animal, the physiological conditions are likely to facilitate proper folding of conformational epitopes, enhancing epitope formation (30).

The high-level expression of each recombinant protein in *E. coli* led to the formation of inclusion bodies comprising different fragments (rLTB, rHN, rHN-HN, and rF) and the heteromultimeric protein L(HN)2F. This phenomenon may be attributed to codon optimization based on *E. coli* preference and the use of a strong T7 promoter that enhances transcription efficiency (27, 31). Mice immunized with these recombinant proteins demonstrated significant immune responses, resulting in a robust production of IgG antibodies across all groups. Notably, even with reduced antigen amounts in each booster, the anti-

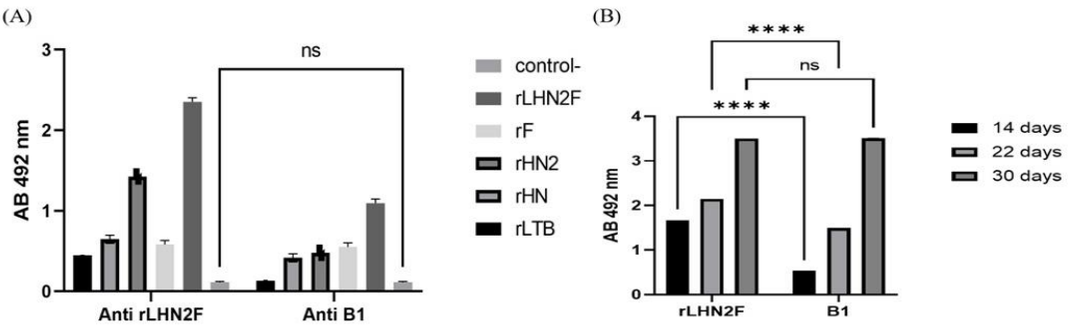

Fig. 5. Diagrams of IgG cross-reactivity. (A) Characterization and cross-reactivity analyses of sera from B1 vaccine and rL(HN)2F mice immunized versus all purified recombinant proteins by ELISA. Antibodies titration is calculated at AB492 nm and 1:6400 dilution. (B) Analysis of serum antibody response from the immunized mice with rL(HN)2F and B1 commercial vaccine by ELISA at various days post-first immunization. one-way ANOVA test was performed to analyze and compare treatment groups. Control mice were injected with PBS and adjuvant ($p < 0.05$). The error bar is the standard deviation.

body response improved, indicating successful stimulation of the humoral immune system. These results were consistent with several findings using pathogenic bacterial or viral chimeric proteins for animal immunological study (21, 27, 31, 32). Immunoblotting analyses using anti-6xHis tags and specific antibodies confirmed the presence of distinct bands at the expected molecular weights, signaling the expression of intact and full-length proteins. Despite structural complexities, these results suggest that the chimeric rL(HN)2F effectively presents the LTB, HN, HN-HN, and F domains for immune recognition. Additionally, cross-reactivity observed in Western blot and ELISA analyses established that antibodies generated against rL(HN)2F could successfully detect purified rLTB, rHN, rHN-HN, and rF proteins. Conversely, antibodies produced against these individual proteins also can recognize the rL(HN)2F construct, confirming the robust design of the chimeric gene and the successful performance of repeated peptide linkers.

Furthermore, the data indicated that the sera from mice immunized with the commercial B1 vaccine strain against NDV could detect rL(HN)2F, rHN, rHN-HN, and rF proteins, whereas rLTB proved undetectable. This behavior reinforces the accuracy and functionality of the rHN, rHN-HN, rF, and rL(HN)2F constructs, with the lack of detection of rLTB illustrating its external origin from the B1 NDV vaccine. The utilization of LTB was solely for its advantageous bio-adjuvant properties, aimed at stimulating a more effective immune response. As LTB is a protein produced by bacteria, it is not a B1 commercial NDV vaccine component. On the other hand, this protein has been used only for its intrinsic behavior as a bio-adjuvant and its ability to stimulate the immune system generally. This negative reaction could be considered an internal negative control and reaffirms the accuracy and efficiency of the rHN, rHN-HN, rF, and rLHN2F structures. According to the immunoblotting results, antibodies produced in the serum of mice immunized with the recombinant protein L(HN)2F demonstrated the capacity to detect various protein fragments, including rLTB, rHN, rHN-HN, and rF. Conversely, antibodies derived from these individual antigenic components were also able to recognize the L(HN)2F construct, reinforcing the premise of a robust immune response elicited by the chimeric protein (data not shown). The results from ELISA corroborated these findings, demonstrating that the L(HN)2F recombinant protein exhibited a strong immunogenic profile comparable to that of the commercial Newcastle vaccine (B1) strain. Immune sera from mice immunized with rL(HN)2F displayed the highest antibody titers against the various recombinant proteins. The ELISA assay quantified immune responses by analyzing immune serum from rL(HN)2F-immunized mice after the final injection across various dilutions against 200 ng of each pure recombinant protein. Results indicated that the highest antibody titers were produced in response to the rL(HN)2F protein, followed by rHN-HN, rF, rHN, and rLTB, respectively. Mice that received purified proteins demonstrated substantial production of specific IgG antibodies, particularly those immunized with rL(HN)2F, which exhibited

measurable antibody levels even at dilutions as high as 1:400,000. Statistical analyses revealed significant differences in the antibody titers, particularly against the HN protein following the second and third booster doses in groups B and C. Also, the ELISA showed the appropriate stimulation of the immune system and the higher dilutions of the specific IgG antibody against rHN protein when rHN-HN pure protein was used and injected as an antigen. Statistical analysis showed significant differences in antibody titer in the second and third booster doses at the F and G groups. There was high significant reactivity in the anti LHN2F compared with anti-B1 at 14- and 22 days post-first immunization ($p < 0.05$). Also, the same titer of the anti-LHN2F and anti-B1 was monitored for 30 days. These observations imply that the rL(HN)2F-immunized group demonstrated a faster and more efficient antibody production compared to the group immunized with the commercial B1 vaccine. This accelerated humoral immune response could potentially provide an advantage in practical vaccination strategies, especially considering the need for timely immunological protection in poultry populations. As can be seen, the gradual production of antibodies in an immunized group with the rLHN2F protein is much better and faster than in the group immunized with the commercial vaccine (B1).

The research funding mentioned earlier highlights the challenges associated with heterologous expressing HN (hemagglutinin-neuraminidase) and the relatively low immunogenicity of HN in mouse models, which can limit its effectiveness as a vaccine candidate (13, 14). To address this limitation, we implemented duplication strategies for shorter HN epitopic sequences, effectively increasing the antigen dose through the inclusion of the HN-HN fragment. This approach resulted in nearly a twofold increase in recombinant protein yields (from 392 to 743.91 µg/ml), correlated with enhanced immunogenicity in the mouse model. ELISA results confirmed that rHN-HN elicited a superior antibody response, underscoring its potential as an effective vaccine candidate due to its ability to amplify antigen doses and focus on the most potent epitopic domains. As shown, when intraperitoneal injection of the immunized group with rHN protein (the last booster, which causes to activate clonal B cell effectively and robust response) is performed by the rHN-HN purified protein, the level of antibodies titer increases significantly. According to the ELISA results, when the rHN protein was used as the final injection (intraperitoneal) in a group of mice immunized with rHN-HN pure protein (G group), there was a decrease in the antibody titer. These outcomes reinforce the notion that a dual HN structure (HN-HN) can enhance the immunogenic profile of the HN component. Additionally, despite differences in antibody response to each component, the lack of statistically significant differences suggests that using flexible linkers successfully maintained the antigenic integrity of each component, which was also confirmed by the bioinformatic analyses. This study shows that the recombinant protein can trigger an immune response against all conserved and significant epitopes in any part of the rLHN2F protein (23).

The results of this study align with findings from prior research. Shahid et al. (2020) (27) expressed the HN protein of the Newcastle disease virus in *E. coli* BL21 (DE3) and assessed its immunogenicity in 10-day-old SPF chickens' post-purification. They reported that serum IgY levels peaked between 50 to 60 days, demonstrating a significant increase in antibody titers between days 10 and 60. Additionally, Motamedi et al. (2018) (33) expressed the rHNF epitope protein of the Newcastle disease virus in *E. coli* Rosetta-gami B (DE3). Their results indicated that very high dilutions of serum from animals vaccinated with the B1 vaccine could respond effectively to minimal amounts of purified recombinant protein (as low as 100 ng). Furthermore, analysis of serum cross-reactivity from mice immunized with the B1 vaccine revealed that antibodies against the B1 strain and the chimeric protein peaked 28 days post-immunization. Overall, it appears that recombinant proteins incorporating multiple immunogenic components (fusion) may play a crucial role in the development of new recombinant proteins for vaccine application. This suggests that LHN2F has the potential to be used as a recombinant vaccine candidate against various strains of the Newcastle disease virus.

CONCLUSION

In summary, this study successfully designed, constructed, and expressed a recombinant fused rL(HN)2F protein from Newcastle disease virus (NDV) containing LTB (L) as a bio-adjuvant in prokaryotic host cells. By adding two copies of HN

sequences, a bio-adjuvant, and highly conserved regions of two key pathogenic molecules and several novel epitopic parts of glycoproteins (HN and F) of NDV into the final protein structure L(HN)2F, the immune response against NDV was significantly enhanced in immunized mice compared to the B1 vaccine strain.

Ethical statement. The study was approved by an ethical committee of National Institute for Medical Research Development (NIMAD: IR.NIMAD.1397.225).

Conflict of interest. Atena Mozafari, Mehregan Rahmani, Yasaman Yasini Nasab, Shahla Shahsavandi, Mahyat Jafari and Ali Hatef Salmanian declare that they have no conflicts of interest.

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