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The heat-labile enterotoxin B subunit bio-adjuvant linked to Newcastle disease virus recombinant hemagglutinin neuraminidase elicited a humoral immune response in the animal model

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ABSTRACT

Newcastle disease is a highly contagious viral infection primarily affecting poultry, leading to significant economic losses worldwide due to its high morbidity and mortality rates. Given the severity of the disease and its impact on the poultry industry, there is an urgent need for a preventative approach to tackle this issue. Developing an efficient and effective vaccine is a valuable step toward reducing the burden of this virus. Consequently, investing in preventive measures, such as vaccination programs, is a top priority to mitigate the economic losses associated with Newcastle disease and protect the livelihoods of those relying on the poultry industry. Despite many vaccines against this viral disease, it still infects many wild and domestic birds worldwide. In this work, chimeric proteins, composed of the recombinant B subunit of *Enterotoxigenic E. coli* with one or two HN (Hemagglutinin-neuraminidase) subunits of NDV (LHN and LHN2, respectively), expressed using *E. coli* host. *In-silico*, *in-vitro*, and *In-vivo* procedures were performed to evaluate the immunogenicity of these proteins. The sera from immunized mice were analyzed using Western Blotting and ELISA. The LHN2 protein with an extra HN subunit elicited a higher antibody titer than the LHN protein ($P<0.05$). Both products could effectively elicit an immune response against NDV and can be considered a component of Newcastle disease vaccine candidates.

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Introduction

Newcastle disease virus (NDV) is an important riddle for the poultry industry, which can afflict many domestic and wild birds. Birds' meat supplies 30% of animal protein in the human diet (1), and Newcastle disease harms the economy and food safety (2). Considering its high morbidity and mortality rate and economic impacts, any positive cases of this disease should be directly notified to the OIE organization (3). Positive cases of ND can be observed with various clinical presentations, including respiratory, neurological, gastrointestinal, and reproductive symptoms; in some cases, the virus could induce about 90 percent mortality among infected birds. The severity of clinical signs varies depending on the host's immunity, environmental factors, and the strain of NDV. Based on viral pathogenicity, this virus is classified into three main strains from low to high: lentogenic, mesogenic, and velogenic (4). The viral genome contains a negative-sense RNA that encodes six main structural proteins. Among NDV proteins, hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins involve host-virus interaction. HN protein identifies and binds the virus to the sialic acid receptor on the cell membrane, and the F protein plays a critical role in fusion and entering the viral genome into the host cell (5). This virus can be detected with molecular methods, including PCR assay (6). ND prevention is usually through early diagnosis and commercial vaccines. Live attenuated and inactivated viral vaccines are commercially available and have been used widely since the 1950s. Live attenuated vaccines against Newcastle disease, including B1, LaSota, and VG/GA, are not feasible in some developing countries due to the high cost, lack of cold chain transportation, unavailable facilities, and insufficient protection. On the other hand, inactivated forms of vaccines cannot elicit a long-term immune response which is very important in laying hen and parents' stock. Furthermore, poultry injected with these vaccines cannot be consumed instantly after injection, and efficient immunity with this type of vaccine usually depends on an extra dosage with live attenuated vaccines (2). Hence, corrective measures should be considered to tackle these issues, and a practical alternative is to replace the former commercial vaccines with new ones. Generally, several strategies enhance a vaccine's immunogenicity and specificity, including using a proper adjuvant and designing a recombinant protein vaccine containing a conserved moiety of immunogens instead of their entire length. In particular, recombinant protein-based vaccines offer several advantages, including effectiveness, safety, and inexpensiveness, and those vaccines can induce an adequate immune response against various viral diseases. Different heterologous systems including plant-based recombinant protein vaccine candidates (7, 8), *Lactobacilli* (9), *E. coli* (10-13), and *Pichia pastoris* (14) based on either HN or F structural proteins were used for expressing recombinant

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NDV vaccine candidates. Generally, subunit and recombinant vaccines are effective but less immunogenic than live attenuated vaccines containing the entire viral particle. Therefore, adjuvants have to be used to boost their immunogenicity. In general, various chemical, synthetic or biological adjuvants can be used to enhance the immunogenicity of vaccines. Bio-adjuvants, such as binding moiety of some bacterial entero-toxins, generally perform through improving antigen presentation. An example of a vaccine bio-adjuvant is the B subunit of Labile toxin from enterotoxigenic *E. coli* (ETEC) or LTB protein which has been proven and used in recombinant vaccines (15). In this study, we have investigated and compared the efficiency of LHN and LHN2 chimeric vaccine candidates to elicit immune responses against NDV in BALB/c mice. Furthermore, In-silico steps were used to predict the immunogenicity of two novel recombinant chimeric vaccine candidates composed of one or two rHN components and rLTB as the adjuvant part. LHN2 construct was designed to prove the claim that doubling the HN component can improve the antigenicity and, subsequently, the immunogenicity of the candidate vaccine in comparison with the LHN protein. The results of this study provide the basis for the production of an innovative chimeric recombinant vaccine candidate against NDV.

Materials and Methods

In-silico Analyses

SnapGene software Version 6.2 was used to simulate the PCR and cloning steps and, subsequently, the immunogenicity for the *lhn2f* fragment (gene bank accession number MH023426) (16) as the source of *lhn* and *lhn2* fragments. The Expacy database translated the DNA sequences to the amino acids. The ProtParam was triggered to calculate the physicochemical parameters of both LHN and LHN2 proteins, including isoelectric point, amino acid arrangement, grand average of hydropathicity (GRAVY), half-life, and molecular weight (17, 18). Three online tools, including I-TASSER, Robetta, and SCRATCH, were used to predict the best 3D structure of proteins (19-21). The most suitable 3D models were selected based on Z-score, Ramachandran plot, and overall quality factor (22, 23). The allergenicity of the chimeric recombinant proteins was anticipated using AllerTOP v. 2.0 and AlgPred servers (24, 25). Finally, the Antigenicity of the designed proteins was predicted by VaxiJen and ANTIGENpro online servers (26, 27).

Bacterial strains and growth condition

In the present study, BL21 (DE3) and DH5a strains of *E. coli* bacteria have been used for expression and cloning procedures, respectively, and cultured using Luria-Bertani (28) medium in 37°C. The construct was firstly cloned into pTG19-T and then was sub-cloned into the pET28a vector (Novagene, USA). Ampicillin (50 µg/ml) or Kanamycin (50 µg/ml) was added to the culture medium depending on the plasmid antibiotic resistance gene.

Constructs preparation

The *lhn2f* synthetic gene (gene bank accession number MH023426) (16) and specific primers (Table 1) were used to amplify *lhn* and *lhn2* constructs which contain a recombinant gene with this order: mature part of the B subunit of LT toxin from enterotoxigenic *E. coil* attached to recombinant HN proteins of NDV with one or two repeats by four and five repeats of EAAAK linker, respectively (Fig 1). The PCR products were cloned to the pTG19-T/A vector. The authentic recombinant plasmids were digested using *Bam*HI/*Hin*dIII enzymes, and the genes were sub-cloned into the pET28a expression vector digested with the same enzymes. The recombinant pET28a plasmids were transformed into competent *E. coli* BL21 (DE3) expression hosts by the heat-shock protocol. All the cloning procedures were verified through enzymatic digestion, colony PCR, and sequencing. Finally, 32 amino acids were added to the beginning of the expression cassette by the pET28a vector, including six His-tags which is used to purify proteins using Ni-NTA affinity chromatography.

Recombinant protein Expression and purification

The expression host harboring recombinant pET28a vector was grown in Luria-Bertani broth (50µg/ml kanamycin, OD_{600} = 0.3) and induced by 1mM isopropyl-β-D-galactopyranoside (IPTG). Consequently, protein content was analyzed on Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE) 15% at different time intervals post-induction (0, 4, 6, 10, 12, and 16 hours) to find the best time for recombinant protein expression. The proteins were purified in denaturing conditions (8 molar Urea) and a Ni-NTA affinity chromatography system (Qiagen, USA) followed by dialysis in phosphate-buffered saline (PBS, pH7). Bradford assay was conducted to evaluate protein concentration. The final concentration for LHN and LHN2 purified proteins were calculated at 844

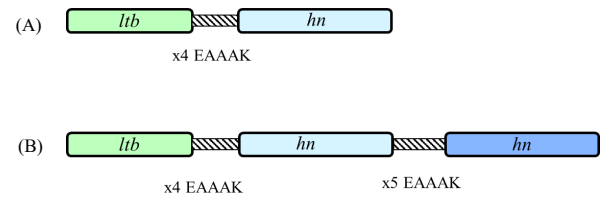

Figure 1. Schematic view of constructs. A): lhn, B): lhn2.

Table 1. Primers used for amplification of *lhn* and *lhn2* fragments. The Restriction enzyme sites are underlined.

Primer	Sequence (5′-3′)	TM	Restriction site	PCR product length (bp)
L Forward	TTGCTTGGATCCATGGCGCCGCAAAG	67.95	*Bam*HI	
HN Reverse	TCGACAAGCTTTTAACCGCCGAAACGAC	66.59	*Hin*dIII	LHN 611 bp
HN2Reverse	GTCGACAAGCTTTTAGCCACCAAAACGAC	66.68	*Hin*dIII	LHN2 900 bp

µg/ml and 464 µg/ml, respectively.

Western Blotting

Western blotting was performed to verify protein expression. The transferred protein on the polyvinylidene difluoride (PVDF) membrane was separately incubated with an anti-His-tag antibody conjugated to Horse Radish Peroxidase (HRP) with the dilution of 1: 2000 recommended by the manufacturer (Sigma Aldrich) or immune serum derived from BALB/c mice immunized with commercial B1 live attenuated virus vaccine strain ((Razi Vaccine & Serum Research Institute, Iran) with the dilution of 1: 5000. followed by another step which was coated with an anti-mouse commercial antibody conjugated to Horse Radish Peroxidase (HRP) with the dilution of 1: 5000. The last sera were used as the specific antibody conducted from a previous study.

Immunization protocol

Female BALB/c mice (six-week-old weighing 25-30 grams) were prepared from the Pasteur Institute of Iran. Six animals per group were categorized as LHN, LHN2, negative and positive controls. For groups 1 and 2, immunization was performed on days 0, 14, 21, and 28 with 20, 10, 5, and 2 µg of LHN and LHN2 purified proteins. Animals from the negative control (group 3) received PBS, while the positive control (group 4) received B1 commercial Newcastle vaccine strain. Blood samples were collected within a week after booster doses, and sera were used for immunological analyses.

ELISA Assay

IgG antibody titer in mice sera was measured against LHN and LHN2 proteins by indirect ELISA. For indirect ELISA, the proteins were coated on each well and then the specific antibody (mice sera) and anti-mouse commercial antibody conjugated to Horse Radish Proxidase (HRP) with the dilution of 1: 5000 were used.

Statistical analysis

GraphPad Prism version 9.0 was used to draw charts and graphs. All statistical analyses were carried out by SPSS.16.0 software. Antibody titers of various groups were compared using a one-way ANOVA assay provided by SPSS software, and the P values less than 0.05 were considered statistically significant.

Results

Bioinformatics analysis

The final construct consists of 106 amino acids from LTB and 71 amino acids from HN proteins fused by EAAAK linker repeats (16). The physio-chemical characterization of both proteins is presented in Table 2.

The 3D models predicted by Robetta's server were selected for further bioinformatics analysis (Fig. 2). ERRAT server (Fig. 3) and Z-Score (Fig. 4) were used to determine the overall quality of models chosen for LHN and LHN2 proteins, and (Table 3.) Ramachandran plot for

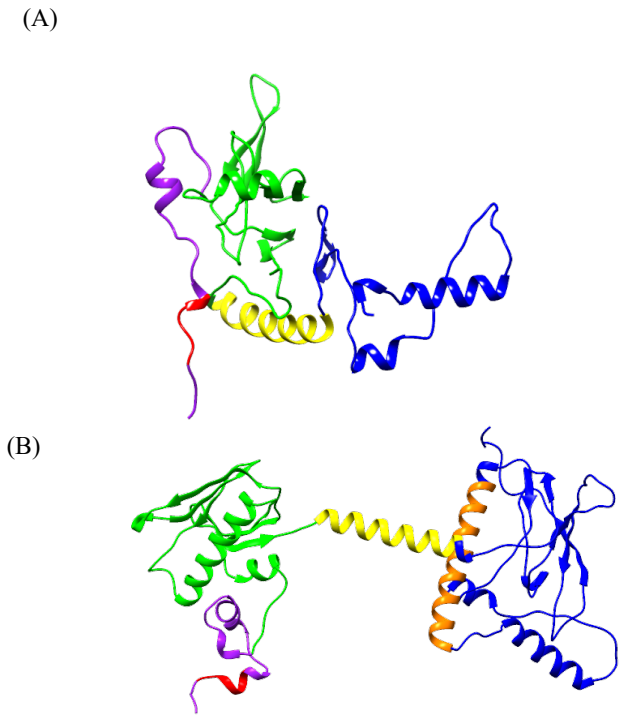

Figure 2. The 3D model prediction by the Robetta server. A): Ribbon view of 3D models for LHN, B): Ribbon view of 3D models for LHN2 (The red regions represent 6 His-tags, the purple represents the 32 amino acids that were added to the construct, the yellow and light brown represent the 4x EAAAK and 5x EAAAK linkers, the green region represents LTB, the blue represents the HN and HN2 moieties.

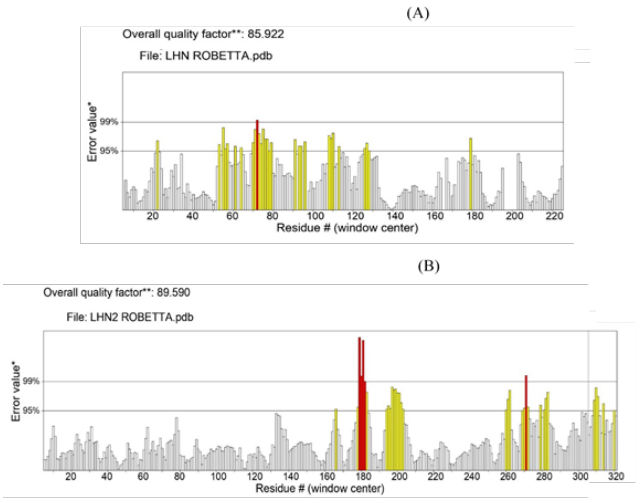

Figure 3. The overall quality factor of proteins predicted by ERRAT server. A): LHN, B): LHN2. (White bars represent the low error rate, yellow represents the error rate between 95 and 99 percent, and red bars represent misfolded areas).

Table 2. The physico–chemical characterization of the proteins.

Protein	Length	Amino acids*	Theoretical Pi	Instability index	Half-life of protein	GRAVY index	The aliphatic index
LHN	611 bp	229 aa	8.98	36.96	30 hours	-0.593	60.57
LHN2	900 bp	325 aa	8.98	34.75	30 hours	-0.612	58.06

The length of amino acids are calculated considering the added sections to the beginning of the construct by the pET28a vector

Table 3. The quality score of the predicted 3D model of proteins.

Recombinant proteins	Z-Score (overall model quality)	ERRAT2 (Overall model quality factor)
LHN	-5.07	85.922
LHN2	-8.83	89.590

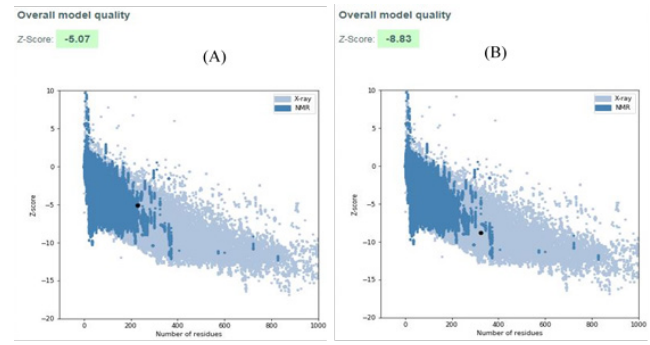

Figure 4. Overall model quality of the proteins 3D models by Prosa-web (Z-Score). A): LHN, B): LHN2.

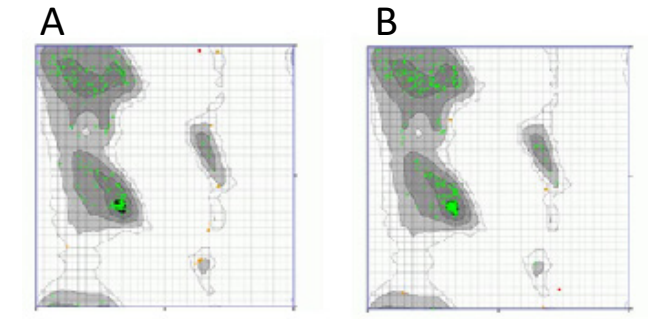

Figure 5. Ramachandran plot of the proteins 3D models predicted by PROCHECK and Zlab Servers. (A): LHN, **(B):** LHN2.

LHN was predicted by the Zlab server (Fig 5). Using VaxiJen and ANTIGENpro servers, both recombinant proteins were predicted to have antigenic properties. AlgPred and AllerTOP v 2.0 servers determined both proteins as non-allergens.

Cloning, expression, and purification of chimeric proteins

The expressed LHN and LHN2 proteins (10 hours after induction) were purified using Ni-NTA affinity chromatography systems. The purified proteins were dialyzed and screened on 15% SDS-PAGE (Fig. 6. A-B). The final concentration of LHN and LHN2 proteins calculated by Bradford's assay were 844 and 464 mg per 100 ml culture media, respectively. The expression of both proteins was confirmed through the western blotting method by anti-His tags (data not shown) and the antisera derived from the B1 strain of Newcastle disease virus (Fig. 6. C).

Immunological analysis

The indirect ELISA test has been used to detect IgG titers against recombinant chimeric proteins. In both LHN and LHN2 groups, the antibody titers were shown a slight increase after each booster dose. The IgG titers against LHN and LHN2 after the second, and third boosters were compared, and the ELISA results showed that the boosters of LHN2 could elicit higher antibody titers compared with the same boosters in the LHN group ($p<0.05$). The IgG titer against LHN was doubled after the third booster (Fig. 7. A). Recombinant proteins were cross-reacted with antibodies against LTB, HN, HN2, LHN2F and B1 vaccine strains (Fig. 7. B).

Discussion

The Newcastle disease poses a significant hazard that intimidates the poultry industry. Given the high costs and logistical complexities associated with existing vaccination methods, there is a critical need to develop a more efficient and cost-effective approach for protecting against ND (1). Recombinant vaccines have garnered increasing attention in recent years due to their superior safety profile and demonstrated effectiveness in preventing infectious

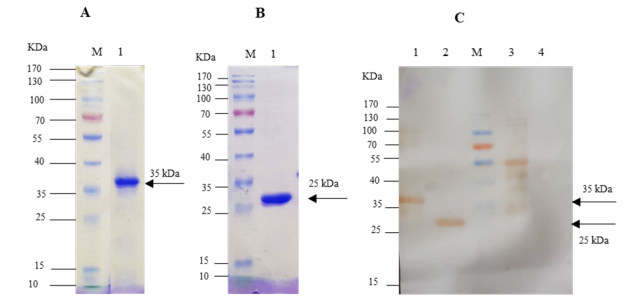

Figure 6. SDS-PAGE and Western-Blotting analysis of LHN and LHN2 constructs. A): LHN2 (~ 35 kDa), B): LHN (~ 25 kDa) C): Western blot analysis of LHN2 (line 1), LHN (line 2), and LHN2F (line 3, positive control) proteins confirmed by the reaction with serum from immunized mice with B1 vaccine strain: lane 4: negative control (Bovine serum albumin protein) In all cases the line M is Page Ruler protein ladder (Thermo Scientific).

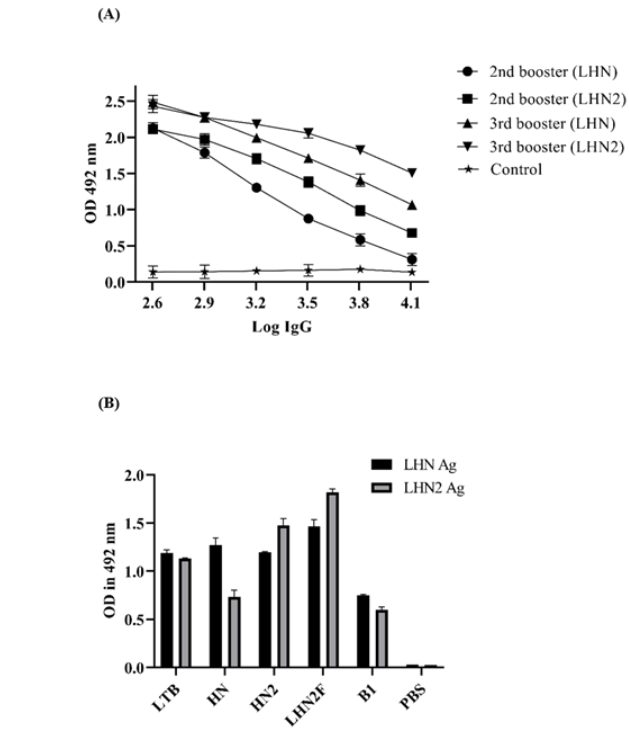

Figure 7. Total IgG antibody responses against LHN and LHN2 chimeric antigens. A): Antibody response against LHN and LHN2 after 2nd and 3rd injection, (B): cross-reactivity for LHN and LHN2 antigens with different antibodies.

diseases (29). Different investigations have evaluated the feasibility of producing recombinant surface glycoproteins using the Newcastle disease virus's HN or F glycoprotein. In these studies, recombinant vaccine models were successfully developed via various platforms, including plants (7, 8), yeast (14), and bacteria (10). Despite considerable efforts, developing a safe and effective vaccine remains an ongoing challenge. Given the limitations of existing vaccines, there is a pressing need to develop more effective and safe alternatives (30). In this study, two novel vaccine models based on the use of HN and LTB proteins were presented. A computational strategy was employed to simulate the molecular procedures and protein expression steps in developing the vaccine model. Based on the calculated GRAVY index (-0.593 and -0.612), both HN and LTB proteins were classified as hydrophilic, suggesting a high likelihood of efficient interaction with immune cell surface receptors, an important feature for a suitable antigen. As determined by the Z-score, the quality of the 3D protein structures fell within the normal range for proteins of similar dimensions. Furthermore, several online tools predicted that both recombinant proteins were antigenic yet non-allergenic, indicating their safety for vaccine development. The final concentrations of the LHN and LHN2 proteins were determined to be 0.844 mg/mL and 0.464 mg/mL, respectively, which were higher than those reported in a similar study in the concentrations of rHN and rF were determined to be 0.37 mg/mL and 0.45 mg/mL, respectively (10). The production of the LHN and LHN2 recombinant chimeric proteins was confirmed by western blot analysis using antibodies derived from the B1 Newcastle disease virus strain. The immune response elicited by recombinant proteins was evaluated in BALB/c mice, given that mice are more cost-effective and less logistically challenging animal models than chickens in the early stages of experiments. Also, mice can mimic the immune response of chickens (31). The result of ELISA has shown high titers of IgG immune response after administering to BALB/c mice. Compared to similar studies, these results are consistent with previous findings that HN recombinant protein can induce a strong immune response against NDV in animal models. For example, a study by Wong et al. (2009) evaluated a recombinant HN protein in chickens and found that it induced a significant immune response (12). Another study by Motamedi et al. (2018) examined the immunogenicity of the recombinant HN and F proteins in mice. They induced a strong immune response in the BALB/c mice, which were not significantly different (10). However, this study is unique because it evaluated two different recombinant protein vaccine candidates (LHN and LHN2) that contain different numbers of HN parts. This approach allows for a comparison of the effectiveness of the two vaccine candidates and can provide insight into the optimal composition of NDV vaccines. Furthermore, the LTB was used as a bio-adjuvant in designing the vaccine candidate, which has several potential advantages compared to other vaccine adjuvants and has been shown to enhance antigen-specific immune responses, including both cellular and humoral responses. It is important to note that the in-line combination of an adjuvant with an antigen could eliminate the separate quality control, dose adjustment, and, more importantly, the effective presence of an adjuvant near the antigen molecule. In particular, LTB has been found to improve the immunogenicity of various vaccine antigens, such as the influenza virus. The cross-reactivity of the chimeric recombinant proteins with IgG antibodies against HN, HN2, LHN, LHN2, B1 vaccine strain, and LHN2F helps validate the specificity of recombinant LHN and LHN2 proteins. Understanding the cross-reactivity of these antibodies with the recombinant proteins is a crucial and indispensable part of formulating vaccine strategies (32). Expectedly, antibodies with higher similarity with the recombinant proteins exhibited better reactivity, demonstrated through a higher optical density in ELISA assay. Adding two HN subunits in the LHN2 protein significantly elicited a higher humoral immune response in the second, and third boosters compared with LHN protein with only one HN subunit ($p<0.05$). Accordingly, incorporating two HN subunits has significantly enhanced the vaccine's humoral immune response by augmenting the antigen dosage. The result of this study suggests that incorporating such enhancers may be a viable means of improving vaccine effectiveness. In conclusion, the results of this study provide promising evidence that LHN and LHN2 recombinant protein could produce a humoral immune response in mice and might be a viable approach for preventing NDV infection. Notably, LHN2 demonstrated a statistically significant increase in immune response compared to LHN, suggesting a potential advantage of LHN2 as a vaccine candidate for NDV. However, further research will be necessary to evaluate the immunoreactivity of these vaccine candidates in chicken models and field trials.

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Conflict of Interest
The authors state no conflict of interest.

Authors' contributions
Mehregan Rahmani has been involved in laboratory experiments, data analysis, writing, and article submission. Atena Mozafari has contributed to the gene design, Data analysis, and revision of the articles. Mahyat Jafari has contributed to the laboratory experiments. Ali Hatef Salmanian, the supervisor and designer of this project, has contributed to the article's design and writing revisions.

Ethical approval
All animal studies were carried out based on the protocols confirmed by the Animal Care Committee of the National Institute for Medical Research Development (NIMAD) on July 1st, 2018. The Ethics Committee approval ID is IR.NIMAD.REC.1397.225.

References

1. Ike AC, Ononugbo CM, Obi OJ, Onu CJ, Olovo CV, Muo SO, Chukwu OS, Reward EE, Omeke OP. Towards improved use of vaccination in the control of infectious bronchitis and Newcastle disease in poultry: understanding the immunological mechanisms. Vaccines 2021; 9(1): 20. DOI: 10.3390/vaccines9010020.
2. Dimitrov K M, Afonso C L, Yu Q, Miller P J, Newcastle disease

vaccines—A solved problem or a continuous challenge? Vet Microbiol 2017; 206: 126-136. DOI: 10.1016/j.vetmic.2016.12.019.
3. OIE, Chapter 2.3. 14. Newcastle disease. 2009, World Organisation for Animal Health Paris, France. 576-589.
4. Deist MS, Gallardo RA, Bunn DA, Kelly TR, Dekkers JC, Zhou H, Lamont SJ. Novel mechanisms revealed in the trachea transcriptome of resistant and susceptible chicken lines following infection with Newcastle disease virus. Clin Vaccine Immunol 2017; 24(5): e00027-17. DOI: 10.1128/CVI.00027-17.
5. Iorio RM, Mahon PJ. Paramyxoviruses: different receptors–different mechanisms of fusion. Trends Microbiol 2008; 16(4): 135-137. DOI: 10.1016/j.tim.2008.01.006.
6. Ammar A, Abd El-Aziz N, Abd El Wanis S, Bakry N, Molecular versus conventional culture for detection of respiratory bacterial pathogens in poultry. Cell Mol Biol 2016; 62(2): 52-56. DOI: 10.14715/cmb/2016.62.2.9.
7. Motamedi MJ, Ebrahimi MM, Shahsavandi S, Amani J, Kazemi R, Jafari M, Salmanian A-H, The immunogenicity of a novel chimeric hemagglutinin-neuraminidase-fusion antigen from Newcastle disease virus by oral delivery of transgenic canola seeds to chickens. Mol Biotechnol 2020; 62: 344-354. DOI: 10.1007/s12033-020-00254-y.
8. Boroujeni NA, Khatouni SB, Motamedi MJ, Afraz S, Jafari M, Salmanian A-H, Root-preferential expression of Newcastle virus glycoproteins driven by NtREL1 promoter in tobacco hairy roots and evaluation of oral delivery in mice. Transgenic Res 2022; 31(2): 201-213. DOI: 10.1007/s11248-021-00295-2.
9. Xiao Y, Wang L, Rui X, Li W, Chen X, Jiang M, Dong M. Enhancement of the antioxidant capacity of soy whey by fermentation with Lactobacillus plantarum B1–6. J. Funct. Foods 2015; 12: 33-44. DOI: 10.1016/j.jff.2014.10.033.
10. Motamedi MJ, Shahsavandi S, Amani J, Kazemi R, Takrim S, Jafari M, Salmanian A-H. Immunogenicity of the multi-epitopic recombinant glycoproteins of Newcastle disease virus: implications for the serodiagnosis applications. Iran J Biotechnol 2018; 16(4). DOI: 10.21859/ijb.1749.
11. Mayahi V, Esmaelizad M, Harzandi N. Designing a novel recombinant HN protein with multi neutralizing antigenic sites and auto tag removal ability based on NDV-VIIj for diagnosis and vaccination application. Indian J Microbiol 2018; 58: 326-331. DOI: 10.1007/s12088-018-0727-z.
12. Wong S, Tan W, Omar A, Tan C, Yusoff K. Immunogenic properties of recombinant ectodomain of Newcastle disease virus hemagglutinin-neuraminidase protein expressed in Escherichia coli. Acta Virol 2009; 53(1): 35. DOI: 10.4149/av_2009_01_35.
13. Sim J-S, Pak H-K, Kim D-S, Lee S-B, Kim Y-H, Hahn B-S, Expression and characterization of synthetic heat-labile enterotoxin B subunit and hemagglutinin–neuraminidase-neutralizing epitope fusion protein in Escherichia coli and tobacco chloroplasts. Plant Mol Biol Rep 2009; 27: 388-399. DOI: 10.1007/s11105-009-0114-3.
14. Kang X, Wang J, Jiao Y, Tang P, Song L, Xiong D, Yin Y, Pan Z, Jiao X. Expression of recombinant Newcastle disease virus F protein in Pichia pastoris and its immunogenicity using flagellin as the adjuvant. Protein Expr Purif 2016; 128: 73-80. DOI: 10.1016/j.pep.2016.08.009.
15. Kazemi R, Akhavian A, Amani J, Salimian J, Motamedi M-J, Mousavi A, Jafari M, Salmanian A-H. Immunogenic properties of trivalent recombinant protein composed of B-subunits of LT, STX-2, and CT toxins. Microbes Infect 2016; 18(6): 421-429. DOI: 10.1016/j.micinf.2016.03.001.
16. Mozafari A, Amani J, Shahsavandi S, Salmanian AH. A Novel Multi-Epitope Edible Vaccine Candidate for Newcastle Disease Virus: In Silico Approach. Iran J. Biotechnol 2022; 20(2): e3119. DOI: 10.30498/ijb.2022.298822.3119.
17. Gasteiger E, Hoogland C, Gattiker A, Duvaud SE, Wilkins MR, Appel RD, Bairoch A. Protein identification and analysis tools on the ExPASy server. Methods Mol Biol 2005; DOI: 10.1385/1-59259-584-7:531.
18. Laskowski R, MacArthur M, Thornton J. PROCHECK: validation of protein-structure coordinates. 2006; DOI: 10.1107/9780955360206000882.
19. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER Suite: protein structure and function prediction. Nat Methods 2015; 12(1): 7-8. DOI: 10.1038/nmeth.3213.
20. Kim D E, Chivian D, Baker D. Protein structure prediction and analysis using the Robetta server. Nucleic Acids Res, 2004. 32(suppl_2): W526-W531. DOI: 10.1093/nar/gkh468.
21. Cheng J, Randall A Z, Sweredoski M J, Baldi P. SCRATCH: a protein structure and structural feature prediction server. Nucleic Acids Res 2005; 33(suppl_2): W72-W76. DOI: 10.1093/nar/gki396.
22. Anderson R J, Weng Z, Campbell R K, Jiang X. Main-chain conformational tendencies of amino acids. Proteins: Struct Funct Bioinform 2005; 60(4): 679-689. DOI: 10.1002/prot.20530.
23. Wiederstein M, Sippl M J, ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res 2007; 35(suppl_2): W407-W410. DOI: 10.1093/nar/gkm290.
24. Sharma N, Patiyal S, Dhall A, Pande A, Arora C, Raghava G P, AlgPred 2.0: an improved method for predicting allergenic proteins and mapping of IgE epitopes. Brief Bioinformatics 2021; 22(4): bbaa294. DOI: 10.1093/bib/bbaa294.
25. Dimitrov I, Naneva L, Doytchinova I, Bangov I. AllergenFP: allergenicity prediction by descriptor fingerprints. J Bioinform 2014;30(6): 846-851. DOI: 10.1093/bioinformatics/btt619.
26. Zaharieva N, Dimitrov I, Flower D, Doytchinova I. Immunogenicity prediction by VaxiJen: a ten year overview. J Proteom Bioinform 2017; 10(11). DOI: 10.4172/jpb.1000454.
27. Magnan C N, Zeller M, Kayala M A, Vigil A, Randall A, Felgner P L, Baldi P, High-throughput prediction of protein antigenicity using protein microarray data. J. Bioinform., 2010. 26(23): 2936-2943. DOI: 10.1093/bioinformatics/btq551.
28. Nurzijah I, Elbohy O A, Kanyuka K, Daly J M, Dunham S, Development of plant-based vaccines for prevention of avian influenza and Newcastle disease in poultry. Vaccines, 2022. 10(3): 478. DOI: doi.org/10.3390/vaccines10030478.
29. Zakir F, Islam F, Jabeen A, Moni S S, Vaccine development: A historical perspective. Biomed Res 2019. 30(3): 452-455. DOI: 10.35841/biomedicalresearch.30-19-203.
30. Hu Z, He X, Deng J, Hu J, Liu X. Current situation and future direction of Newcastle disease vaccines. Vet Research, 2022; 53(1): 1-13. DOI: 10.1186/s13567-022-01118-w.
31. Lai K S, Yusoff K, Mahmood M. Functional ectodomain of the hemagglutinin-neuraminidase protein is expressed in transgenic tobacco cells as a candidate vaccine against Newcastle disease virus. Plant Cell, Tissue Organ Cult 2013; 112: 117-121. DOI: 10.1007/s11240-012-0214-x.
32. Izmirly A M, Alturki S O, Alturki S O, Connors J, Haddad E K. Challenges in dengue vaccines development: pre-existing infections and cross-reactivity. Front immunol 2020; 11: 1055. DOI: 10.3389/fimmu.2020.01055.