DOI: https://doi.org/10.54393/fbt.v6i1.232

FUTURISTIC BIOTECHNOLOGY
https://fbtjournal.com/index.php/fbt
ISSN (E): 2959-0981, (P): 2959-0973
Vol 06 Issue 01, (Jan-Mar, 2026)

Original Article

OPEN ACCESS

Multi-Epitope-Based Vaccine Design Against Newcastle Disease Virus: Targeting Nucleoprotein Using Immunoinformatics

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ARTICLE INFO

Keywords:
Avian Paramyxovirus-1, Multi-epitope Vaccine, Epitope Prediction, Immunoinformatics, Newcastle Disease Virus, Nucleoprotein

How to Cite:
Cheema, L. A., Iftikhar, A., & Lodhi, U. F. (2026). Multi-Epitope-Based Vaccine Design Against Newcastle Disease Virus: Targeting Nucleoprotein Using Immunoinformatics: Multi-Epitope-Based Vaccine and Newcastle Disease Virus: Targeting Nucleoprotein . Futuristic Biotechnology, 6(1), 43-50. https://doi.org/10.54393/fbt.v6i1.232

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Received Date: 5th February, 2026
Revised Date: 21st March, 2026
Acceptance Date: 25th March, 2026
Published Date: 31st March, 2026

ABSTRACT

Avian paramyxovirus-1 (APMV-1) is the virus that causes Newcastle disease (ND), a highly infectious chicken illness that causes substantial financial losses globally. **Objectives:** To retrieve the amino acid sequence of the nucleoprotein of APMV-1, identify immunogenic B-cell and T-cell epitopes, design a multi-epitope vaccine using suitable linkers and an adjuvant, and evaluate its interaction with chicken immune receptors along with immune response simulation. **Methods:** The nucleoprotein sequence of NDV was retrieved from public databases. Immunoinformatics tools were used to predict B-cell and T-cell epitopes binding to MHC-I and MHC-II molecules. Selected epitopes were evaluated for antigenicity and allergenicity. The construct of the vaccine was designed by using the most antigenic 5 MHC-1, 4MHC-II, and all predicted B-cell epitopes of the NDV Nucleoprotein, along with suitable linkers, and by incorporating the B-subunit of the heat-labile enterotoxin (LTB) as an adjuvant. Interaction analysis with chicken immune receptors showed highly negative scores, which suggests strong and favorable binding between the vaccine construct and the TLR 4 receptor. Immune simulation was performed to assess the immunogenic potential of the construct. **Results:** Several B-cell and T-cell epitopes with high antigenicity and favorable immunological properties were identified. These epitopes were assembled into a multi-epitope vaccine construct with suitable linkers and an adjuvant. Interaction analysis indicated stable binding with chicken immune receptors, and immune simulation predicted a strong immune response. **Conclusions:** The designed multi-epitope vaccine shows potential as a candidate against Newcastle Disease, although experimental validation is required.

INTRODUCTION

A danger to the global chicken industry, Newcastle disease (ND) is a highly contagious viral illness that impacts both domestic and wild bird species. The disease was first reported in 1926 in Java, Indonesia, and Newcastle, England, and has since reached the entire world and become one of the leading causes of economic loss, especially in developing nations where poultry farming is one of the main sources of livelihood [1]. Newcastle disease virus (NDV) is the causative agent of Newcastle disease in birds, and it is a virus of the genus Avulavirus and family Paramyxoviridae [2]. The disease severity will vary with the strain, host species, environmental conditions, and the age of birds. Velogenic strains have the capacity to induce 100 percent death in a span of a couple of days in highly susceptible birds like chickens [3]. The virus is transmitted through direct and indirect contact, and it can survive across a wide range of environmental conditions, which also contributes to its transmission [4]. NDV has a one-stranded negative-sense RNA genome with six structural proteins, which are hemagglutinin-neuraminidase, fusion protein, phosphoprotein, nucleoprotein, matrix protein, and RNA polymerase [5]. The nucleoprotein (NP) is one of them, and it plays a major role in viral replication and genome encapsidation. It is a ribonucleoprotein complex that is necessary in the process of transcription and replication and has a highly conserved

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structure among the genotypes of NDV [6]. The NP is highly conserved and, therefore, a good candidate for vaccine development because mutations in essential residues may cause debilitating effects on viral replication. Besides, an earlier study revealed that immunodominant nucleoprotein epitopes can be manipulated without affecting structural integrity, indicating that they can be used as an advanced vaccine approach [7]. The vaccines against ND that are currently available are mostly live attenuated vaccines and inactivated vaccines. Even though live vaccines are highly immunogenic, they are more susceptible to mishandling and may regain their virulence, whereas inactivated vaccines are relatively safer but less immunogenic and necessitate booster doses [8]. Furthermore, the introduction of genetically diverse strains of NDV has increased the threat to the long-term efficacy of the traditional vaccines, provoking the issues of partial protection and continuous outbreaks [9]. Thus, the necessity of better vaccination measures has become more than obvious. Recent developments in reverse vaccinology and immunoinformatics have given new possibilities in vaccine design. In contrast to the traditional techniques, immunoinformatics allows the detection of highly immunogenic B-cell and T-cell epitopes through computational means, thus saving time, cost, and excessive use of large-scale laboratory work [10]. The use of multi-epitope vaccines, which are formed by joining the chosen cytotoxic T-lymphocyte (MHC-I), helper T-lymphocyte (MHC-II), and B-cell epitopes, has proven to be effective in the induction of cellular and humoral immunity [11].

These vaccines are more specific, safer, and have wider coverage of immunity. Although NDV vaccine development has been done in a number of ways, the research has not concentrated on the development of a multi-epitope vaccine against conserved nucleoprotein using a whole immunoinformatics strategy. The current research seeks to fill this gap by developing a multi-epitope vaccine against NDV that is developed using the nucleoprotein sequence. The proposed research will encompass the retrieval and physicochemical analysis of the nucleoprotein sequence, prediction and screening of immunogenic B-cell and T-cell epitopes, construction of a multi-epitope vaccine based on appropriate linkers and adjuvants, and in silico assessment of its structural stability, interaction with immune receptors, and simulation of the immune response. This practice can help lead to the formation of a more efficient and widely protective vaccine candidate against Newcastle disease.

METHODS

This study was an in silico immunoinformatics-based experimental research conducted to design a multiepitope vaccine targeting the nucleoprotein of Newcastle disease virus (NDV). The study was conducted in the Department of Biotechnology, Kinnaird College for Women. The research was completed over a period of approximately four months, from March 2025 to June 2025. This was the entire amino acid sequence of the Nucleoprotein (Accession No. AVN98140) of Newcastle disease virus (NDV) that was obtained in FASTA format from the NCBI database. The selected sequence was submitted from Pakistan and chosen due to the critical role of nucleoprotein in viral replication and transcription. The physicochemical characteristics of the recovered sequence, such as molecular weight, amino acid composition, instability index, aliphatic index, theoretical isoelectric point (pI), estimated half-life, and grand average of hydropathicity (GRAVY), were assessed using the ExPASy ProtParam server. The MHC-I binding prediction tool available at Immune Epitope Database (IEDB) was used to predict cytotoxic T-lymphocyte (CTL) epitopes. The ANN 4.0 method was selected for epitope prediction, and peptide length was set to 9–10 mers. The study chose to use Human HLA alleles instead of the chicken-specific MHC-1 alleles due to their unavailability on the IEDB dataset. According to some studies, human class I homologous alleles can bring about an immune response similar to chicken BF alleles [12, 13]. Epitopes with IC50 values lower than 100 nM and percentile rank less than 1.0 were considered strong binders [14]. For helper T-lymphocyte (HTL) epitope prediction, the MHC-II binding tool of IEDB was used with the NN-align 2.3 prediction method. The peptide length was set to 15 mers. Human HLA-DR reference alleles were selected based on their similarity to chicken alleles [15]. Epitopes exhibiting IC50 values below 100 nM and percentile rank below 1.0 were shortlisted for further analysis. Linear B-cell epitopes were predicted using the BepiPred Linear Epitope Prediction 2.0 tool available at IEDB. The default threshold value of 0.5 was applied, and residues scoring above this threshold were selected as potential B-cell epitopes. All predicted B-cell epitopes were included in the final vaccine construct. Screening of predicted T-cell epitopes was performed based on antigenicity and allergenicity. The Antigenicity was evaluated by VaxiJen v2.0 server with a threshold of 0.5, and the Allergenicity was evaluated by AllerTOP v2.0. The epitopes whose antigenicity scores exceeded 0.5 and were non-allergenic were considered reliable for eliciting an immune response. High antigenicity and binding affinity were used to finalize the top five MHC-I and four MHC-II epitopes. The IEDB population coverage tool was used to estimate global and regional immune responsiveness through population coverage analysis of the chosen MHC-I and MHC-II epitopes [16]. The complete MHC-I, MHC-II, and B-cell epitopes were combined to form a multi-epitope

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vaccine. Appropriate linkers were also used to ensure structural integrity and appropriate presentation of the epitopes. The adjuvant was conjugated to the B-cell epitopes with the EAAAK linker, B-cell and MHC-I epitopes were conjugated with the GPGPG linker, and MHC-I and MHC-II epitopes were linked with the AAY linker [17]. As an adjuvant to increase immunogenicity, the heat-labile Escherichia coli enterotoxin B-subunit (LTB) was included at the N-terminal. Moreover, a 6x histidine tag was added to the C-terminus of the vaccine, which helps in its purification.

The physicochemical properties, solubility, toxicity, antigenicity, and allergenicity of the final construct were evaluated using ProtParam, SOLUPROT, ToxinPred, VaxiJen v2.0, and AllerTOP v2.0, respectively. Secondary structure prediction was performed using PSIPRED and GOR IV methods. SWISS-MODEL was used to model the tertiary structure, and Ramachandran plot analysis was used to validate it. Molecular docking was performed using ClusPro against the chicken TLR4 receptor (PDB ID: 3MU3), and interaction analysis was conducted using PDBsum. TLR4 was chosen as it plays a crucial role in the innate antiviral immunity initiation and promotion of the Th1-biased response by activating MyD88- and TRIF-dependent pathways, resulting in the production of cytokines and IFN-7. The C-IMMSIM server was used to perform immune simulation [18]. Lastly, codon optimization was carried out by using the GenSmart Optimization tool, and in-silico cloning was done into the pET28a (+) plasmid through SnapGene software.

RESULTS

The entire amino acid sequence of the nucleoprotein (Accession No. AVN98140) of the Newcastle disease virus was obtained from the NCBI database and analyzed physicochemically through the ExPASy ProtParam tool. The nucleoprotein had 489 residues of amino acids and weighed 53,426.18 Da. The calculated instability index was 37.87, which showed that the protein was stable because a value of less than 40 suggested structural stability. The theoretical isoelectric point (pI) was determined to be 5.47, which implies that the protein is slightly acidic. The aliphatic index (75.87) was moderate in terms of thermostability, and the GRAVY score of -0.359 indicated a hydrophilic nature and excellent interaction with aqueous conditions. These characteristics confirm that the nucleoprotein is a stable and suitable candidate for vaccine development (Table 1).

Table 1: Physicochemical Properties of the Nucleoprotein of Avian Paramyxovirus-1

Parameters	Results
Molecular Weight	53426 .18
Number of Amino Acids	489
Instability Index	37.87
Aliphatic Index	75.87
Estimated Half-Life (*E. coli*)	>10 Hours
Grand Average of Hydropathicity (GRAVY)	-0.359

A large number of potential MHC-I epitopes were predicted using the IEDB MHC-I binding tool. After screening based on IC50 values (<100 nM), antigenicity (>0.5), and non-allergenicity, 63 epitopes met the criteria. Among them, the five epitopes with the highest antigenicity scores were selected for vaccine construction. All selected epitopes demonstrated strong binding affinity and were predicted to be non-allergenic. Similarly, MHC-II epitope prediction yielded multiple candidates. After applying the same screening parameters, 21 epitopes were identified as strong binders. From these, four epitopes exhibiting high antigenicity and low IC50 values were selected for incorporation into the final vaccine construct. These epitopes are expected to stimulate helper T-cell responses effectively (Table 2).

Table 2: The Best MHC-I Epitopes for Incorporation into the Vaccine Construct and Selected MHC-II Epitopes of the Nucleoprotein Predicted by IEDB

Start	End	Epitopes	Length	IC50	Antigenicity	Allergenicity
Best MHC-I Epitopes						
93	101	KQNEATLAV	9	58.27	0.6563	Non-allergen
264	273	LTAFFLTLKY	10	19.34	0.9616	Non-allergen
263	272	GLTAFFLTLK	10	25.57	0.9659	Non-allergen
265	273	TAFFLTLKY	9	62.62	1.1361	Non-allergen
66	74	KPLRQGALI	9	75.54	1.1535	Non-allergen
MHC-II Epitopes						
5	19	FDEYEQLLAAQTRPN	15	3.1	0.5736	Non-allergen
216	230	AIQLTIRHSLAVRIF	15	1.4	0.6596	Non-allergen
331	345	YSFAMGMASVLDKGT	15	9.7	0.7133	Non-allergen
217	231	IQLTIRHSLAVRIFL	15	2.8	0.5928	Non-allergen

The BepiPred 2.0 tool was used to predict linear B-cell epitopes with a threshold of 0.5. Residues with a result of higher than

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this value were regarded as possible B-cell epitopes. Several areas within the nucleoprotein were seen to be immunogenic. The most important predicted B-cell epitopes are summarized to make them clear and concise. These epitopes are likely to cause intense humoral immunity by enhancing the production of antibodies (Table 3).

Table 3: Selected B-Cell Epitopes of the Nucleoprotein Predicted by IEDB

Sr. No.	Epitopes	Start	End	Length
1	FDEYEQLLAAQTRPNGTHGGGEKGSTL	5	31	27
2	DPED	44	47	4
3	ANK	64	66	3
4	KQNEA	93	97	5
5	FTNNVPQFNNRSGVSEERAQR	107	127	21
6	RACSN	137	141	5
7	TAGVEDDAPED	147	157	11
8	ETADESETRRINKYMQQGRIQKKYIL	184	209	26
9	RNTAGGSST	239	247	9
10	QK	292	293	2
11	KQ	295	296	2
12	L	300	300	1
13	RMKGE	302	306	5
14	DQMSFA	318	323	6
15	GTGKYQFARDF	344	354	11
16	AQGSSINED	369	377	9
17	A	379	379	1
18	LTPA	384	387	4
19	RR	389	390	2
20	SEEISGMDIPTQQVGVLTGLSDEGPRASQGGPS-KTQGQPDAGDGETQFLDLMRAVANSMREAPNP-TQGTPHLEPPPTPGPSQENDID	400	486	87

The selected B-cell, MHC-I, and MHC-II epitopes were incorporated into the final vaccine construct along with the heat-labile enterotoxin B subunit (LTB) as an adjuvant using appropriate linkers. The resulting construct contained 528 amino acid residues (Figure 1).

Figure 1: Simplified Depiction of the Vaccine Construct

The physicochemical analysis of the vaccine construct showed a molecular weight of 59,004.48 Da and a theoretical pI of 9.79. Structural stability was verified by the instability index (38.56). The aliphatic index (65.51) showed moderate thermostability, and the predicted solubility score of 0.874 by SOLUPROT indicated good expression in E. coli. The vaccine was anticipated to be non-allergenic and non-toxic, and the antigenicity score (0.4935) showed that an immunological response may be triggered by the vaccine. The GRAVY (Grand Average of Hydropathicity) value of 0.791 indicates that the vaccine is hydrophobic (Table 4).

Table 4: Physicochemical Parameters of the Construct

Parameters	Results
No. of amino acids	528
Molecular weight	59004.48 Da
Theoretical pI	9.79
Estimated half-life (E. coli)	>10 hours
Index of Instability	38.56
Aliphatic index	65.51
Grand average of hydropathicity (GRAVY)	0.791

The secondary structure prediction showed that the vaccine construct was predominantly composed of alpha helices (51.52%), followed by random coils (38.83%), and extended strands (9.66%). Tertiary structure modeling using SWISS-MODEL indicated reliable structural quality, with 96.24 percent of residues located in the most favored regions of the Ramachandran plot. Molecular docking of the vaccine construct with the chicken TLR4 receptor using ClusPro resulted in the formation of 29 clusters with the lowest binding energy score of -596.6, indicating strong binding affinity (Figure 2).

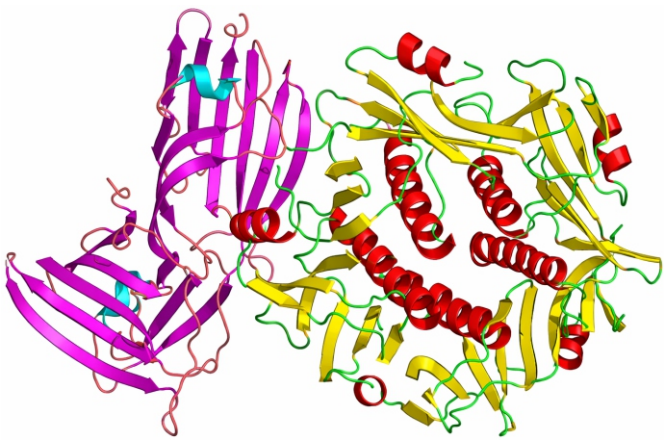

Figure 2: The Docked Complex of the Vaccine and the TLR 4 Receptor

Interaction analysis performed using PDBsum showed 8 hydrogen bonds, 2 salt bridges, and 73 non-bonded contacts between the vaccine and the receptor. The vaccine (chain A) had 10 interface residues and represents an interface area of 556 Å2, and it interacts with the receptor (chain B) that had 14 interface residues and an interface area of 475 Å2. The docked complex and interacting residues are shown (Figure 3).

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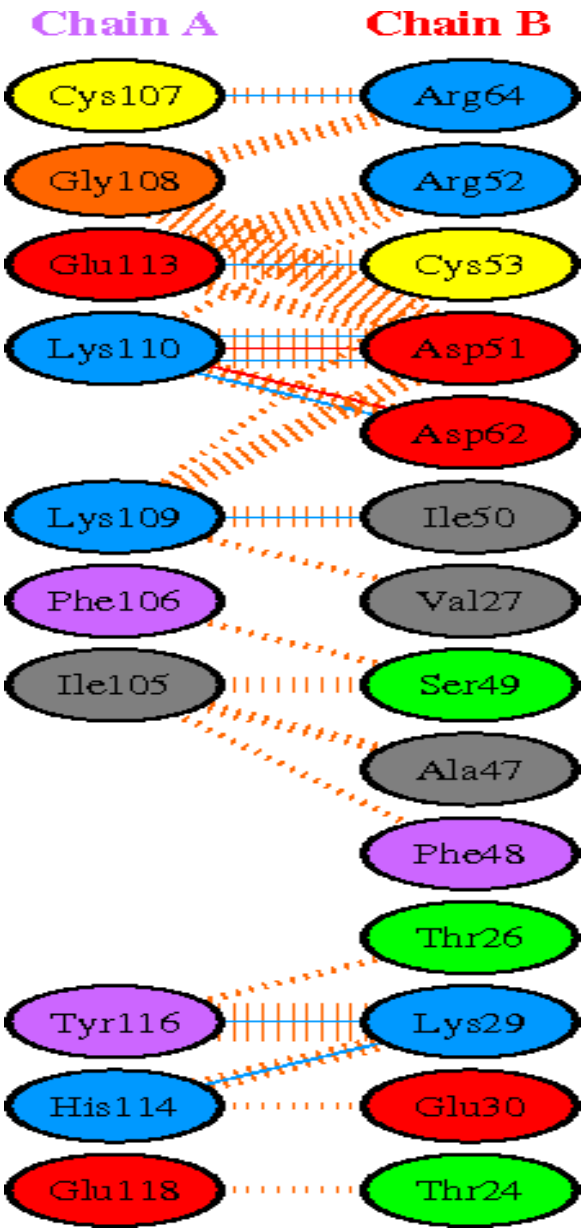

Figure 3: The Interaction among the Residues of Chain A and Chain B with 8 Hydrogen Bonds (Blue), 2 Salt Bridges (Red), and 73 Non-Bonded Contacts (Orange)

Immune simulation using the C-IMMSIM server demonstrated a significant rise in IgM and IgG antibody levels following antigen exposure. Increased populations of B cells and CD4+ T-helper cells were also observed, along with elevated cytokine production, indicating a favorable predicted immune response (Figure 4).

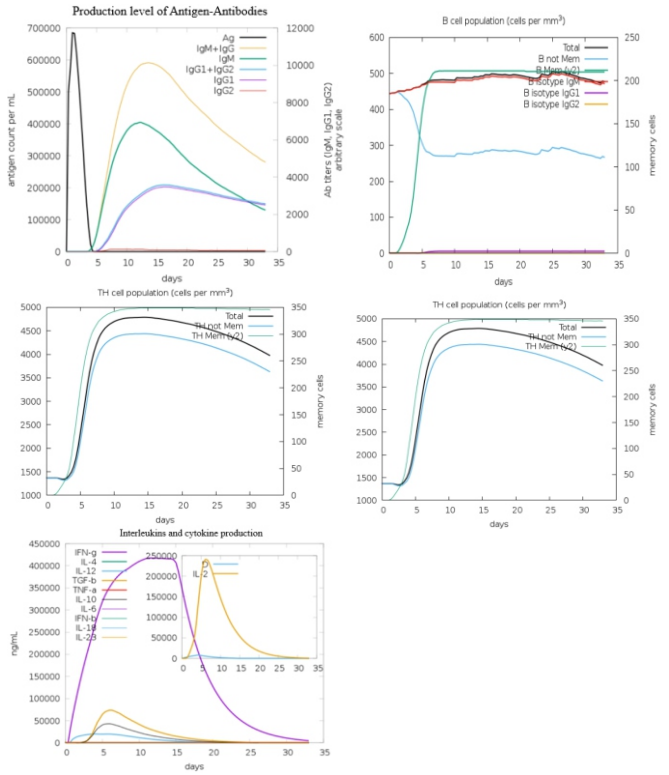

Figure 4: C-ImmSim Analysis Results Showing the Predicted Immune Response Following Exposure to Vaccine Construct

Codon optimization for *E. coli* expression resulted in an optimized GC content of 51.07% which falls within the recommended range (≈30–70%) for efficient gene expression in E. coli expression system. Furthermore, in-silico cloning into the pET28a (+) vector confirmed the theoretical feasibility of experimental expression (Figure 5).

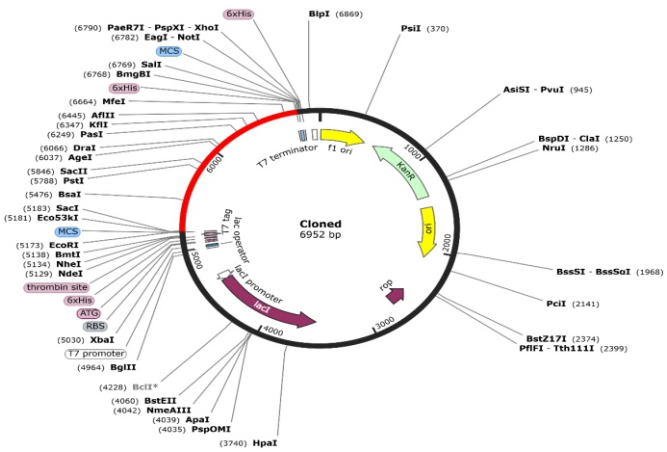

Figure 5: Optimized Vaccine Sequence Cloned into the pET28a (+) Vector, Resulting in a Construct of 6952 bp

DISCUSSION

Newcastle disease continues to pose a significant threat to the poultry industry worldwide, particularly in developing countries where poultry production contributes

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substantially to food security and economic stability [2]. Although conventional live attenuated and inactivated vaccines are widely used, their efficacy has been compromised due to the continuous emergence of genetically diverse NDV strains [7, 8]. Live vaccines such as B1 strains protect against classical genotypes but may offer limited immunity against highly virulent and evolving strains [19]. These limitations highlight the necessity for alternative vaccination strategies that are capable of inducing broader and long-lasting immunity. In the present study, a multi-epitope vaccine targeting the nucleoprotein of NDV was designed using immunoinformatics tools. The selection of nucleoprotein as a vaccine target is supported by its highly conserved nature and its essential role in viral genome encapsulation and replication. Mutations within critical residues of the nucleoprotein have been shown to impair RNA synthesis and viral replication, demonstrating its structural and functional importance [6]. Compared to surface glycoproteins that are prone to mutations, the conserved characteristics of nucleoprotein provide an advantage for developing broadly protective vaccines. Recent advancements in reverse vaccinology and immunoinformatics have revolutionized vaccine development by enabling the identification of highly immunogenic epitopes through computational approaches [9, 10]. Multi-epitope vaccines offer the advantage of stimulating both humoral and cellular immune responses by incorporating B-cell and T-cell epitopes within a single construct [11]. Similar immunoinformatics-based vaccine strategies have been successfully applied against other pathogens, demonstrating improved specificity, safety, and cost-effectiveness [20]. The use of suitable linkers such as EAAAK, GPGPG, and AAY in the present construct is consistent with previous studies emphasizing their role in maintaining structural flexibility, minimizing steric hindrance, and enhancing epitope presentation [21]. The incorporation of the heat-labile enterotoxin B-subunit (LTB) as an adjuvant further strengthens the immunogenic potential of the construct. Previous research has demonstrated that fusion of LTB with antigenic components can enhance antigen presentation and induce a rapid and robust immune response in poultry models [22]. While other adjuvants, such as avian β-defensin, have also been utilized in NDV vaccine constructs [12], the solubility and stability profile observed with LTB in the present study supports its selection. Physicochemical evaluation indicated that the designed vaccine construct is stable, non-allergenic, non-toxic, and suitable for expression in Escherichia coli. These findings are comparable with other multi-epitope vaccine studies, where favorable GRAVY scores, aliphatic index, and stability parameters were associated with effective vaccine candidates [23, 24]. Additionally, molecular docking studies demonstrated a strong and stable binding between the vaccine construct and the chicken TLR 4 receptor, indicating the vaccine's capacity to trigger natural immune reactions. Immune simulation analysis predicted significant antibody production, elevated B-cell and T-helper cell populations, and increased cytokine levels, indicating the potential to induce both cellular and humoral immunity.

However, certain limitations must be acknowledged. The present study is entirely based on computational predictions and lacks in-vitro and in vivo experimental validation. Moreover, the use of human HLA alleles due to the limited availability of chicken-specific alleles may introduce minor variability in epitope prediction. Future studies should focus on laboratory synthesis, expression, and purification of the vaccine construct, followed by experimental validation in animal models to evaluate its safety, immunogenicity, and protective efficacy under field conditions.

CONCLUSION

This study employed immunoinformatics tools to design a multi-epitope vaccine against Avian paramyxovirus-1, the cause of Newcastle disease in poultry. Predicted B- and T-cell epitopes from the viral nucleoprotein were combined with the *E. coli* heat-labile enterotoxin B subunit (LTB) as an adjuvant, producing a highly antigenic, non-toxic, and non-allergenic vaccine. Structural analyses showed high stability and strong TLR4 binding, while immunological simulations indicated robust cellular and humoral responses with immune memory formation. The vaccine construct was cloned into the pET28a (+) vector, highlighting its potential as an effective and economical alternative to conventional vaccines. Further experimental validation is needed before large-scale production and commercialization.

Authors' Contribution

Conceptualization: LAC
Methodology: LAC, AI
Formal analysis: LAC, AI, UFL
Writing and Drafting: LAC, AI
Review and Editing: LAC, AI, UFL

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

Source of Funding

The authors received no financial support for the research, authorship and/or publication of this article.

DOI: https://doi.org/10.54393/fbt.v6i1.232

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