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Original Article

Development of an Effective Multiepitope Vaccine Against Infectious Laryngotracheitis Virus Based on Envelope Glycoproteins by Immunoinformatics Approaches

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ABSTRACT

Introduction: Infectious laryngotracheitis (ILT) is a significant respiratory disease that impacts poultry populations worldwide, known as ILT virus (ILTV). This viral disease presents considerable challenges not only to poultry health but also to the broader food industry, resulting in substantial economic losses and posing a risk to food security. Effective control and prevention of ILT are paramount, and vaccination strategies have emerged as critical measures in mitigating the impact of this disease. The envelope glycoproteins of ILTV are essential for the virus's ability to enter host cells, making them potential targets for vaccine development. However, to date, there has been a lack of comprehensive research focused on the evaluating these glycoproteins for their immunogenic potential in the context of ILTV vaccination.

Materials & Methods: This study employs advanced bioinformatics tools to systematically analyze the antigenicity, sensitization, conservation, and intracellular localization of linear B-cell epitopes derived from the envelope glycoproteins of ILTV.

Results: Through this rigorous analysis, we identified four highly antigenic epitopes. To enhance their immunogenicity, we engineered multiple configurations of these linear B-cell epitopes using epitope -folding techniques. Subsequently, we developed four multi-epitope vaccine candidates, each designed to incorporate two distinct adjuvants to bolster the immune response. Among these candidates, construct 1 exhibited optimal interactions with Toll-like receptors TLR2, TLR3, TLR4, and TLR5, resulting in significant levels of IFN-γ and IL-2 production. Following this, we performed codon optimization on this construct, which was then reverse -transcribed and successfully cloned into the pET-28a vector.

Conclusion: This critical step lays the groundwork for future in vitro and in vivo investigations aimed at evaluating the efficacy of the developed vaccine candidates in preventing ILT in poultry, ultimately contributing to enhanced biosecurity and economic stability in the poultry industry.

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1. Introduction

The Infectious laryngotracheitis virus (ILTV) is classified within the Herpesviridae family and the Alphaherpesvirinae subfamily, specifically referred to as Gallid herpesvirus-1 (GaHV-1). Its genome consists of a linear double-stranded DNA measuring around 150 kilobases, with a guanine-cytosine (GC) content of 48.16%. The genome encodes for unique long (UL) and unique short (US) regions, as well as two inverted repeat (IR) sequences. Various glycoproteins, such as gC, gD, gE, gG, gH, and gI, are present on the envelope of ILTV, and these proteins are essential for binding to and subsequent penetration of the host cell membrane [1].

The trigeminal nerve is recognized as the key site for latency concerning the wild-type strain of the ILTV and its vaccines. The prevalence of ILT has been reported to reach up to 100%. However, mortality rates exhibit considerable variability, ranging from 1% to 70%, influenced by multiple factors such as the virulence of the viral strain and the herd's immunity status [2].

ILTV is responsible for considerable respiratory illness in avian species, as it replicates within the trachea and the epithelial cells of the upper respiratory tract, leading to a latent infection in both the trigeminal nerve and the trachea. The disease presents with various clinical signs, including conjunctivitis, enlargement of the infraorbital sinuses, nasal discharge, bloody mucus, coughing, shortness of breath, weight loss, and diminished laying productivity [3]. The prevention and management of ILT is a significant global concern, particularly in regions where ILTV is endemic. A variety of vaccines have been formulated to address ILTV, such as live attenuated vaccines derived from continuous passage in embryonated eggs or tissue cultures, recombinant viral vectored vaccines, and vaccines based on recombinant deletion mutations. Nonetheless, these vaccines have not always been successful in eliciting robust immunity [4]. Additionally, a multi-epitopic vaccine centered on glycoprotein B has been established, yet other envelope glycoproteins were not taken into account. In this study, our initial goal was to create a multi-epitopic vaccine that considers all envelope glycoproteins as critical virulence factors for the entry of pathogens into host cells, utilizing immunoinformatic tools. It is expected that the vaccine formulated from this investigation will soon undergo efficacy testing in both in vitro and in vivo settings.

2. Materials and Methods

2.1. Retrieval of the protein sequences

Gallidalphaherpesvirus-1 (Genebank: GCF_000847005.1) was used as a reference strain to predict the effective vaccine candidates against ILTV. The workflow of this study and the individual steps are shown in Figure 1.

2.2. Identification of target proteins

Final target proteins were selected based on assessment of virulence, subcellular localization, and transmembrane topology. Virulence and envelope proteins were selected based on the literature [1]. The subcellular localization of all viral proteins in host cells and virus-infected cells (with emphasis on secretory and envelope glycoproteins) was verified using the Virus -mPLoc server [5]. In addition, the topology of the proteins was determined using the TMHMM server, which mainly determines the position of the transmembrane helices, and the N- and C-terminals [6, 7].

2.3. Library of conserved high score B-cell epitopes

2.3.1 Identification of linear B-cell epitopes

The sequence of each protein was entered into the BepiPred database [8] with a threshold of ≥0.6. This database predicts continuous B-cell epitopes from the amino acid sequence using a hidden Markov model [9].

2.3.2. Evaluation of suitable linear B cell epitopes

TMHMM server and 3D structural modelling were used to determine surface-exposed linear B-cell epitopes of envelope proteins. Surface-exposed epitopes were identified using Jmol software, version 14.6.4, which is used to determine chemical structures in 3 dimensions (Molecular Modelling) [10].

Antigenicity was determined with a cut-off ≥0.5 using the VaxiJen webtool [11]. Allergenicity of antigenic epitopes was determined with a cut-off ≥0.3 using Algpred 2.0 [12]. Epitope conservation was assessed using the IEBD epitope conservancy analysis web tool [13].

2.4. Epitope shuffling and construction of the multi-epitope vaccines

In this study, we developed a multiepitope vaccine against ILTV. Four promising epitopes with an antigenicity value > of 0.99 were selected for further analy-

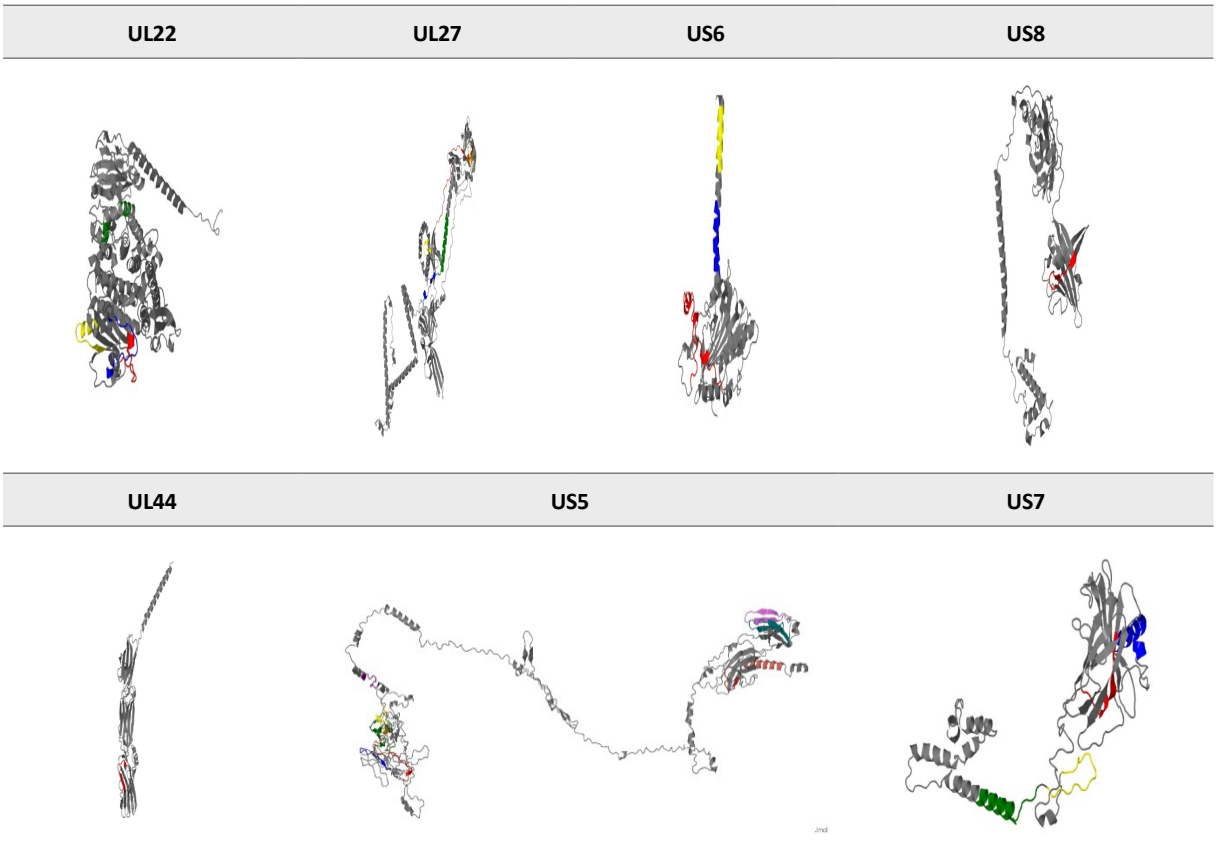

Figure 1. Determination of localization of linear B cell epitopes on the tertiary structures of envelope glycoproteins of ILTV using Jmol software

sis. Epitope shuffling was performed to investigate and compare the arrangement of epitopes in different modes. Naked constructs were designed by arranging an adjuvant (ISQAVHAAHAEINEAGR/ SIINFEKL) + linker (EAAAK) + first epitope + linker (GPGPG) + second epitope + linker (GPGPG) + third epitope + linker (GPGPG) + fourth epitope (epitope shuffling was performed).

Next, the production of antibody class-specific B-cell epitopes was studied using the IgPred server developed by Raghava's group [14]. After epitope shuffling, multiepitope vaccines were selected based on antigenicity, allergenicity, preservation, and induction of different antibody classes.

2.5. Prediction of tertiary structure of multi-epitope vaccines

The tertiary structure (3D) of the constructs was modeled using the Robetta tool [15] as a protein structure prediction server. The stability of the tertiary structures was validated using the ProSA web server [16]. A Ramachandran plot of the chimeric proteins was then generated using the Zlab Ramachandran Plot Server. Figure 2 shows the energetically allowed and disallowed dihedral angles based on the Van der Waal radius of the side chains [17].

2.6. Molecular docking of multi-epitope vaccines with toll-like receptors

Molecular docking was performed to assess the interaction between the chimeric proteins and Toll-like receptors 1, 2, 4, and 6 using pyDockWEB [18]. Out of four multi-epitope vaccines, all four constructs were individually modeled against the specified Toll-like receptors to predict and compare their binding affinities and potential for immune.

2.7. Physicochemical characterization of multi-epitope vaccines

The number of amino acids, estimated half-life, molecular weight, theoretical protein isoelectric (pI), aliphatic index, and instability index were analyzed using Expasy ProtParam Server [19]. The predicted functional class and functional score were examined using VICMpred server [20].

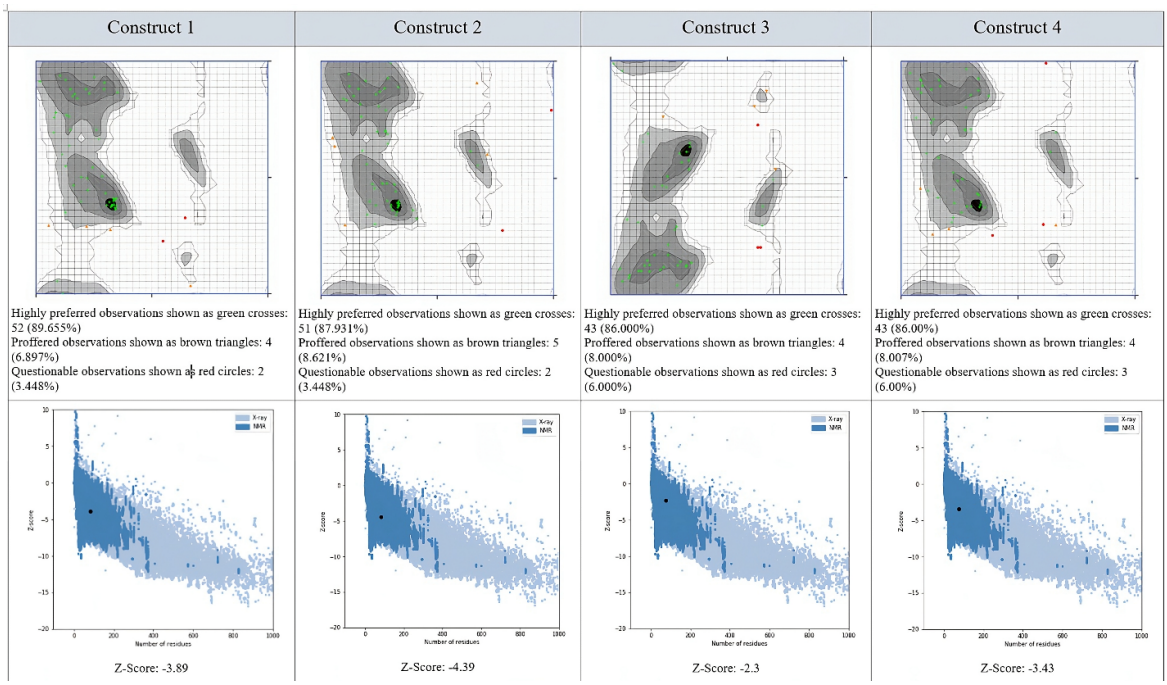

Figure 2. The Ramachandran plots of four multiepitope constructs

Note: The plots show that more than 96% of all amino acids of the chimeric proteins are located in the highly preferential zone (shown as green crosses). The ProSA web plots show that the Z-scores of the four multi-epitope constructs are in the range of the native conformations revealed by NMR spectroscopy (dark blue) and x-ray crystallography (light blue).

2.8 Immune simulation analysis of multi-epitope vaccines

The immunosimulation potential of the best multi-epitope construct based on molecular docking was determined using the C-ImmSim web server [21]. This database was used to predict the immunosimulation potential for induction of IFN-γ, IL -2, Th1, IgM, IgG1, and IgG2 (Figure 3).

2.9. Codon optimization and in silico cloning

Some codon adaptation tools were used to adapt the codon usage. We chose *Escherichia coli* strain BL21 as the host for cloning the selected construct. The cleavage sites of the restriction enzymes SalI and BglII were determined for the vector and the selected construct using the CLC Main Workbench ver. 6 tool. The construct was then cloned into the pET-28a (+) vector between SalI and BglII. In addition, His tag was attached to the C-terminal site of the pET-28a (+) vector for protein purification (Figure 4).

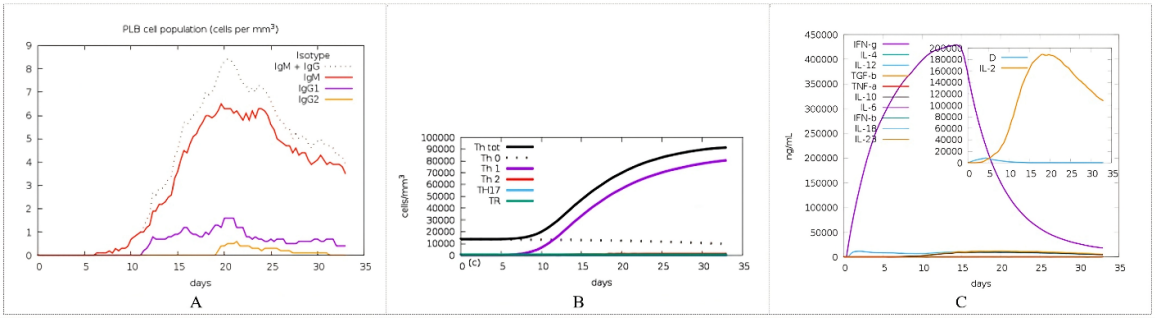

Figure 3. The analysis of the immunosimulation using the C-ImmSim server

A) The number of B lymphocytes in plasma, divided by isotypes, (IgM, IgG1 and IgG2) shows increased levels of IgM and IgG1; B) Population of T helper cells shows increased Th1 cell population; C) Concentration of cytokines and interleukins, shows increased levels of IFN-γ and IL -2

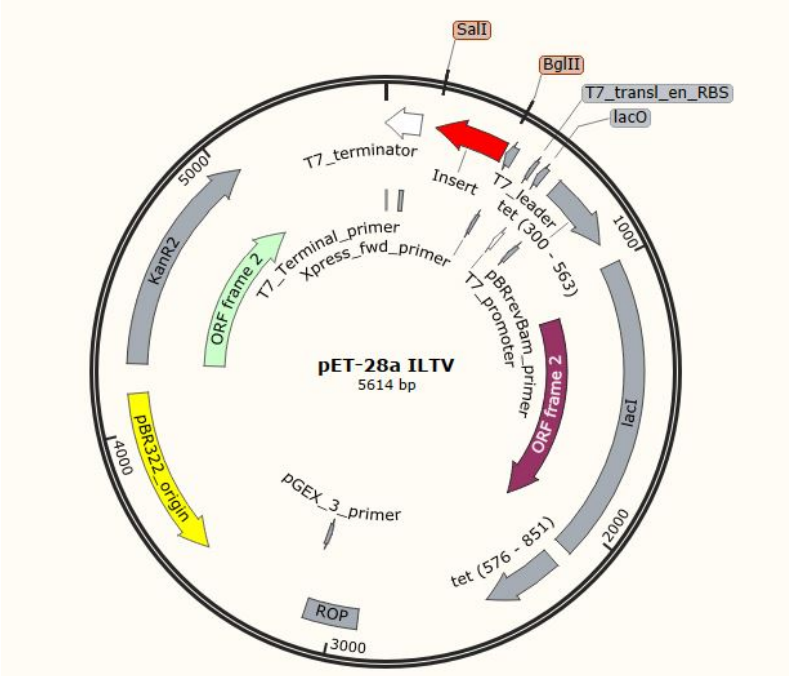

Figure 4. In silico cloning of construct 1 of the multiepitope vaccine into the pET-28a expression vector using the restriction enzymes SalI and BglII. The red arrow shows the construct and the black circle shows the pET-28a vector

3. Results

3.1. Identification of the virulent envelope glycoproteins

Of the 79 proteins belonging to ILTV, nine were virulent envelope glycoproteins according to a literature review [1]. The subcellular localization of nine proteins was confirmed by Virus-mPLoc and TMHMM server.

3.2. High scoring library of B cell epitopes

Glycoprotein L has no linear B- cell epitopes. Among the eight remaining envelope glycoproteins, 55 linear B- cell epitopes were identified. Two epitopes were excluded because of their low antigenicity, and six epitopes were not surface- exposed. A total of 47 linear epitopes were selected and their antigenicity, allergenicity, and conservation were evaluated. In addition, Jmol software showed that all 47 selected epitopes were surface-exposed (Figure 1). Four epitopes were selected for epitope shuffling because of their high antigenicity value. These four epitopes belonged to the J, C, H, and B envelope glycoproteins, respectively.

3.3. Epitope shuffling and construction of multi-epitope vaccines

Epitope shuffling was performedand different epitope arrangements were compared based on antigenicity, al-

Table 1. Four multi-epitope vaccines were developed using two best arrangements of epitopes through epitope shuffling (2 and 6) and two different adjuvants

Construct Number	Sequence
Construct 1	ISQAVHAAHAEINEAGREAAAKVEVKFSNDGEVGPGPGDLGYIGEDGIGPGPGYQVRDLETGQIRPGPGPGEATIQPRKF-SNDP
Construct 2	ISQAVHAAHAEINEAGREAAAKVEVKFSNDGEVGPGPGYQVRDLETGQIRPGPGPGDLGYIGEDGIGPGPGEATIQPRKF-SNDP
Construct 3	SIINFEKLEAAAKVEVKFSNDGEVGPGPGDLGYIGEDGIGPGPGYQVRDLETGQIRPGPGPGEATIQPRKFSNDP
Construct 4	SIINFEKLEAAAKVEVKFSNDGEVGPGPGYQVRDLETGQIRPGPGPGDLGYIGEDGIGPGPGEATIQPRKFSNDP

Formula: Adjuvant (ISQAVHAAHAEINEAGR/ SIINFEKL) + Linker (EAAAK) + epitope 1+ Linker (GPGPG) + epitope 2 + Linker (GPGPG) + epitope 3 + Linker (GPGPG) + epitope 4.

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Table 2. Molecular dockings of four multi-epitope vaccines against ILTV with TLR1, TLR2, TLR4 and TLR6, using pyDoc WEB server

Constructs of Multi-epitope Vaccines	TLR1				TLR2				TLR4				TLR6			
	Electrostatics	Desolvation	VdW	Total	Electrostatics	Desolvation	VdW	Total	Electrostatics	Desolvation	VdW	Total	Electrostatics	Desolvation	VdW	Total
Construct 1	-27.798	-6.876	79.970	-26.677	-52.025	19.426	22.009	-30.398	-10.352	-19.185	85.864	-20.950	-28.493	0.516	-8.744	-28.851
Construct 2	-23.191	-0.359	37.183	-19.832	-24.389	11.144	-35.151	-16.761	-19.504	-17.285	28.465	-33.943	-26.201	5.302	-19.010	-22.799
Construct 3	-28.998	1.092	20.556	-25.850	-34.648	13.296	-14.570	-22.809	-4.374	-30.994	97.087	-25.659	-23.144	-2.633	33.262	-22.451
Construct 4	-12.267	-12.089	19.201	-22.436	-38.257	13.412	-7.262	-25.570	-15.526	-18.638	92.818	-24.882	-24.632	-6.851	6.976	-30.785

VdW: Van der Waals force.

Note: Bold cases indicate better interactions with TLRs.

lergenicity, and induction of Ab classes. Finally, the two best epitope arrangements (2 and 6) were selected. Using these two models and two adjuvants (ISQAVHAAHAEINEAGR/ SIINFEKL) four constructs were designed (Table 1).

3.4 Prediction of the tertiary structure of multiepitope vaccines

The tertiary structures of the multiepitope constructs were predicted using the Robetta web server [15]. The resulting 3D structures were validated using ProSA web analysis and Ramachandran plots (Figure 2). All four multi-epitope vaccines models showed that more than 86% of the amino acids were located in the preferred zone. The ProSA-Web plot showed that the Z-score of the multiepitope vaccines falls within the range of native conformations, as determined by NMR spectroscopy (dark blue) and x-ray crystallography (light blue).

3.5. Molecular docking of multiepitope vaccines with Tlrs

Molecular docking was performed to predict the interactions of the chimeric proteins with TLR 1, 2, 4 and 5. For each type of multiepitope vaccine, docking results from four models were compared, and construct 1 was selected for further analysis (Table 2).

3.6. Physicochemical characterization of multiepitope vaccines

The number of amino acids, estimated half-life, molecular weight, theoretical pI, aliphatic index, instability index, predicted functional class, and functional score for all four constructs were estimated and considered for further analysis (Table 3).

3.7. Immunostimulant analysis of multi-epitope vaccines

Immunostimulant analysis showed that construct 1 could successfully simulate an immune response by in-

Table 3. Physicochemical characteristics of multi-epitope vaccines against ILTV

Protein Characteristics	Construct 1	Construct 2	Construct 3	Construct 4
No. of amino acids	84	84	75	75
Predicted functional class	Cellular process	Cellular process	Cellular process	Cellular process
Function score	1.175	1.175	1.122	1.122
Molecular weight (kDa)	8619.40	8619.40	7808.61	7808.61
Theoretical protein isoelectric (pI)	4.66	4.66	4.45	4.45
Asp + Glu	13	13	12	12
Arg + Lys	7	7	7	7
The estimated half-time	>10 hours (*E. coli*, in vivo)	>10 hours (*E. coli*, in vivo)	>10 hours (*E. coli*, in vivo)	>10 hours (*E. coli*, in vivo)
Aliphatic index	65.12	65.12	67.60	67.60
Instability index	9.44	9.44	2.23	2.23
Number of disulfides bounds	0	0	0	0
Ag overall prediction	0.9090 (probable antigen)	0.9090 (probable antigen)	1.0745 (probable antigen)	1.0745 (probable antigen)
Allergenicity	0.0348	0.0348	0.0342	0.0432
Alpha helix	17%	17%	4%	4%
Beta strand	25%	25%	32%	29%
Disordered	60%	60%	48%%	48%

ducing acceptable levels of Th1 cell populations, IgM, IgG1, IgG2, IFN-γ, and IL -2 (Figure 3 and Table 4).

3.8. Codon optimization and in silico cloning

Construct 1, which exhibitedthe best interactions with human TLRs and immune simulations, was selected for in silico cloning. While the construct contained 84 amino acids, the number of nucleotides of the possible DNA sequence of construct 1 after back translation was 252 nucleotides. The prepared DNA sequences were inserted into pET-28a vector between restriction sites SalI and BglII using CLC Main Workbench 6 software. The total length of the vector plus insert is 5614 bp (Figure 4).

4. Discussion

Food hygiene constitutes a major challenge affecting the global community. Consequently, the immunization of animals, especially in the poultry sector, against bacterial and viral infections is a pressing issue that warrants worldwide attention [22]. Given the high costs and lengthy processes associated with drug discovery, there has been a notable surge in vaccine-oriented prevention programs in recent years [23]. Recent progress in bioinformatics techniques has enabled the design of multi-epitope vaccines that leverage immunodominant protective epitopes against a range of viral serotypes, thereby minimizing both time and financial resources required for development [24, 25]. The ILTV represents a significant respiratory disease in the poultry industry,

Table 4. Immune simulation analysis of construct 1 using C-ImmSim server

Protein	IFN-γ (ng/mL)	IL-2 (ng/mL)	T-cell Population (cell/mm³)	B- cell Population (cell/mm³)		
			Th1	IgM	IgG1	IgG2
Construct 1	440000	190000	82000	6.5	1.8	0.8

contributing to notable economic losses through reduced egg production, weight loss in poultry, and elevated mortality rates [26]. The development of a potent vaccine appears to be the most efficient strategy for managing and preventing viral infections. However, vaccines investigated to date have shown inadequacies in terms of safety and their ability to provide effective immunization against ILTV [27, 28]. Live attenuated vaccines are limited by residual virulence, the risk of transmission to unexposed individuals, the potential for latent infections that may reactivate, the phenomenon of viral shedding, and the possibility of reversion to virulence after in vivo passage [29]. Moreover, recombinant vaccines that employ viral vectors are characterized by a subdued immune response Without appropriate adjuvants, these vaccines fail to achieve complete prevention of viral replication when exposed to viral challenges [30].

Envelope glycoproteins, located on the surface of the virus, act as ligands that bind to the host cell membrane. Additionally, findings from various studies indicate that these glycoproteins are capable of inducing significant immune responses [31, 32]. Consistent with the characteristics of other alphaherpesviruses, the entry of the virus into host cells requires five specific viral envelope glycoproteins: gC, gB, gD, and the gH/gL heterodimers, in conjunction with various cell surface receptors. Notably, the glycoproteins gB, gH, and gL are conserved across all herpesviruses and are indispensable for the processes of viral entry and cell fusion. Devlin et al. indicated that gG serves as a virulence factor in ILTV. Their findings suggest that the removal of gG from the ILTV genome results in diminished clinical manifestations and mortality rates, as well as an increased weight in specific pathogen-free (SPF) chickens infected with the virus. Furthermore, gG has been identified as a chemokine-binding protein (vCKBP) in various alpha-herpesviruses in vitro [33].

Studiesrevealed that the trachea of birds vaccinated with the gG deletion mutant of ILTV showed an increased presence of inflammatory cell infiltration compared to those vaccinated with wild-type ILTV. The gH glycoprotein serves as a principal envelope proteins in ILTV and is integral to the viral replication cycle. It is responsible for the binding and entry of the virus into host cells; thus, mutants lacking gH are unable to achieve infection in these cellular environments [34]. Given their surface localization, envelope glycoproteinsrepresent significant candidates for the creation of multiepitope vaccines targeting ILTV. Additionally, linear B-cell epitopes play a crucial role in humoral immunity, facilitating targeted immune response through the activation of antigen-specific antibodies. This research focuses on the development of multiepitope vaccines incorporating linear B-cell epitopes, taking into account factors such as antigenicity, allergenicity, stability, and surface accessibility on ILTV.

Cai Q et al., 2007, employed epitope shuffling to develop a chimeric polyepitope gene that encodes peptides capable of eliciting both B-cells and T-cells responses against *Plasmodium falciparum*. Their findings indicated that the administration of a polyepitope library serves as an effective strategy for screening and optimizing chimeric gene vaccines targeting a range of microorganisms. Given that one method to enhance immunogenicity involves assessing various peptide configurations, this study utilized this technique to create a highly effective multi-epitope vaccine [36]. We also used two adjuvants (ISQAVHAAHAEINEAGR/ SIINFEKL) to develop a promising vaccine against ILTV. SIINFEKL is a restricted peptide epitope of ovalbumin (OVA -I: OVA 257-264 aa) specifically recognized by T cells, while ISQAVHAAHAEINEAGR is a restricted peptide epitope of ovalbumin (OVA-II: OVA 323-339 aa). Adjuvants focus on immunodominant MHC epitopes, whereas EAAAK linkers have been added to maintain the function of specific epitopes so that they can function accurately following translocation into the host body [36]. Understanding the secondary and tertiary structure of any protein provides fundamental information about the function, dynamics, and interaction of one protein relative to others [37]. The Robetta tool's prediction of the three-dimensional structure of our protein construct provided essential information regarding the positioning of key amino acids.

To identify any potential discrepancies in the final vaccine model, we utilized several validation tools. Revisions were ultimately carried out to markedly enhance this structure. The analysis of the Ramachandran plot confirms the overall adequacy of the selected model's quality. This research employed computational and immunoinformatic techniques to create a multiepitope vaccine targeting ILTV, utilizing linear B-cell epitopes derived from envelope glycoproteins. The resulting chimeric vaccine demonstrated optimal interactions with TLRs and exhibited significant immunoreactivity, as evidenced by the induction of adequate Th1 cell populations, immunoglobulin levels, and cytokine production. Reverse transcription and in silico cloning of the construct into the pET-28a vector were successfully achieved. In summary, our results demonstrate that the multiepitope construct is a safe antigen, which could be a potential candidate for ILTV vaccine development, exhibiting stability and inducing adequate immune responses.

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Compliance with ethical guidelines

This article does not contain any studies involving humans or animals.

Data availability

All data generated or analyzed during this study are included in this published article.

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Authors' contributions

Conceptualization: Parisa Jamour, Maryam Meskini, and Asghar Abdoli; Experiments, data collection, analysis and interpretation: All authors; Writing the original draft: Behrouz Ebadi and Narjes Noori Goodarzi; Review and editing: Parisa Jamour, Behrouz Ebadi, and Maryam Meskini; Supervision: Asghar Abdoli.

Conflict of interest

The authors declared no conflict of interest.

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