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Research Article

Designing and Evaluation of a Multiepitope Vaccine against Avian Newcastle Disease Virus, Influenza Virus and Infectious Bronchitis Virus

Morteza Ghandadi[1,2]

[1]*Department of Pharmacognosy and Pharmaceutical Biotechnology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran.*
[2]*Pharmaceutical Sciences Research Center, Mazandaran University of Medical Sciences, Sari, Iran.*

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Abstract

Background: Poultry industries play an important role in the human food supply. Newcastle disease virus (NDV), Infectious bronchitis virus (IBV), and Avian influenza virus (AIV) can cause epizootic outbreaks in poultry industries and lead to extensive economic losses. Furthermore, these viruses can also infect wild birds, and avian influenza virus is a serious threat to humans. Here, a multiepitope vaccine has been designed to induce an immune response against IBV, NDV, and AIV using extensive bioinformatics tools.

Methods: To do so, the antigenic proteins, including the hemagglutinin of H5 and H7 subtypes of AIV, nucleocapsid and spike proteins of IBV, and fusion and hemagglutinin-neuraminidase proteins of NDV, have been studied to find immunodominant epitopes with MHC-I/MHC-II binding potential. Four antigenic and non-allergenic epitopes from each antigenic protein were connected to avian beta-defensin 1 as an adjuvant to construct the multiepitope vaccine. To investigate the potential of vaccine-induced activation of toll-like receptors (TLR-2, 5), the tertiary structure of the vaccine was modeled and docked to TLR-2/5 proteins.

Results: Evaluation of the physicochemical properties of the vaccine construct has demonstrated the stability and solubility of the vaccine upon overexpression. The vaccine construct demonstrated antigenicity and was specified as a non-allergenic protein. The vaccine can induce significant cellular and humoral immune responses, and TLR proteins can recognize the vaccine in its three-dimensional form.

Conclusion: Overall, the multiepitope vaccine designed in the present study against IBV, NDV, and AIV shows significant immunological potential that should be further investigated in wet laboratory experiments.

Introduction

Poultry industries are essential for the production of meat and eggs for human consumption. Various infectious diseases can be spread in bird farms, leading to epizootic outbreaks with socioeconomic implications. Infectious bronchitis virus (IBV) is a coronavirus that causes infectious respiratory disease in chickens (Gallus gallus). Although the virus can cause disease in birds of all ages, young chicks are more susceptible to severe illness. The main symptoms include tracheal rales, coughing, nasal discharge, and sneezing. Furthermore, IBV can affect the gut, kidneys, and reproductive system.[1] Infected birds excrete the virus through their feces and respiratory discharges. The virus is transmitted via aerosol, contaminated equipment and contaminated water and feed.[2] IBV infection causes significant economic losses in poultry industries due to its highly contagious nature and the severity of the disease. It has been reported that IBV infection leads to a reduction in egg production and quality. Additionally, mortality rates of up to 60% in chicks and poor weight gain in broilers have been observed because of the infection.[3] Various strategies, including inactivation and live attenuation have been employed to develop anti-IBV vaccines. Despite various vaccines against IBV serotypes, the disease has proven to be recurrent due to the high mutational rate as RNA virus. Furthermore, strong cellular immunity is not induced by inactivated vaccines and new antigenic variants can be developed by contribution of live-attenuated vaccines.[4] IBV has four structural proteins with antigenic potential including nucleocapsid (N), spike (S), membrane (M) and envelope (E). In the present study, the S and N proteins have been selected to design the multiepitope vaccine. Phosphorylated N has demonstrated highly immunogenic nature and conservancy among various strains (94-99%). Furthermore, the Spike protein is located on the surface of the virion and is responsible for inducing virus-neutralizing and HI antibodies.[5,6] Accordingly, the S and N proteins of IBV have been selected in the present study to

*Corresponding Author: Morteza Ghandadi, E-mail: ghandadi@yahoo.com

design the multiepitope vaccine.

Newcastle disease virus (NDV), also known as avian orthoavulavirus 1 causes Newcastle disease as another important avian disease affecting wild birds and domestic species all over the world.[7] Although NDV can infect humans, it rarely leads to mild fever and influenza like symptoms and most of the times is non-symptomatic. However, its impact can be significant in the poultry industry. Due to its significant socioeconomic importance and potential of development of an epizootic in the poultry industry, it has to be reported immediately to the World Organization for Animal Health (WOAH) upon detection.[8] The disease is mostly characterized by respiratory symptoms including gasping, coughing, sneezing, and rales. However, depression, nervous manifestations and diarrhea have also been reported. NDV is mainly transmitted through direct contact, and it has been detected in various parts of the infected birds' bodies.[8] The genome of NDV encodes six structural proteins: large polymerase protein, fusion (F) protein, hemagglutinin-neuraminidase (HN), phosphoprotein, matrix protein, and nucleocapsid protein. The HN and F proteins are exposed on the virus's envelope and can stimulate the immune system upon infection.[9] It has been reported that DNA vaccines based on the F or HN genes increase survival of birds with NDV infection through production of neutralizing antibodies, demonstrating the antigenic potential of these genes.[10] Various live-attenuated and inactivated vaccines have been employed in the poultry industry against NDV. Furthermore, state-of-the-art technologies, including antigenically matched vaccines have also been developed against NDV, however these vaccines cannot completely prevent the spread of phylogenetically divergent virulent NDV strains, and further improvements are still required.[8]

The next important virus affecting poultry industries, which can also be a threat to humans, is influenza virus A. Its infection leads to acute respiratory disease in several animals, including poultry, wild birds, pigs and humans.[11] The virus genotype is classified based on two surface glycoproteins neuraminidase (NA) and hemagglutinin (HA). These proteins are also involved in the immune response against influenza infection and are utilized in vaccine design.[12] Some strains of H5 and H7 are Highly Pathogenic Avian Influenza (HPAI) occur mainly in birds, but they can also infect mammals, including humans. HPAI viruses are highly contagious and cause severe illness and high mortality rates, especially in domestic poultry.[13] The high mutability of the influenza virus hampers efforts focusing on the development of effective vaccines. This ongoing evolution of the virus necessitates further investigations into the development of anti-AIV vaccines.

With the advantage of various bio/immunoinformatic tools and genetic manipulation techniques, the identification of conserved and immunogenic epitopes, as well as the development of multiepitope vaccines is facilitated in a time and cost-effective manner, with lower safety concerns. Various software and databases are available to analyze genetic information of pathogens and identify potential vaccine candidates.[14] On the other hand, multiepitope or chimeric vaccines can be developed using various antigenic proteins from one or more species of pathogens. Furthermore, a multiepitope vaccine containing both T-cell and B-cell epitopes can significantly induce immune responses.[15] Adjuvants can be incorporated into the entire vaccine sequence to overcome low efficacy of subunit vaccines. Multi-epitope protein vaccines offer several significant advantages, including a favorable safety profile, decreased risk of allergenic reactions, cost-effective production, and the ability to be stored as freeze-dried dosage forms that do not require refrigeration.[16] According to the significant socioeconomic impact of IBV, NDV and influenza on the poultry industry and lack of effective vaccines for these diseases, in the present work extensive bioinformatics tools have been employed to design and evaluate a multiepitope vaccine targeting IBV, NDV and avian influenza.

Methods

Selection of antigenic proteins and retrieval of sequences

A multiepitope vaccine has been designed to protect chickens against IBV, NDV and avian influenza using antigenic proteins including hemagglutinin (HA) of h5 and h7 subtypes of influenza, fusion (F) protein and hemagglutinin-neuraminidase (HN) from NDV and spike (S) and nucleocapsid (N) from IBV. The protein sequences of these proteins were retrieved from the National Center for Biotechnology Information (NCBI) with the following accession numbers: YP_308669.1 for hemagglutinin (HA) of H5, YP_009118475.1 for hemagglutinin (HA) of H7, NP_040838.1 for N, NP_040831.1 for S, YP_009513197.1 for F and YP_009513198.1 for HN. Furthermore, the sequence of avian beta-defensin 1 (NP_990324.1) protein has been retrieved to be utilized as an adjuvant in the construction of the vaccine.

Prediction of epitopes with MHC-I binding potential

The sequence of the selected antigenic proteins were submitted to the Immune Epitope Database and Analysis Resource (IEDB) and NetMHC 4 servers to predict MHC-I binding epitopes. NetMHC 4 employs artificial neural network (ANN) algorithms as the sequence alignment method.[17] On the other hand, IEDB employs various strategies including, stabilized matrix method (SMM), ANN, and combinatorial peptide libraries (CombLib), to predict MHC binding peptides across a protein sequence.[18] Default settings were used for all predictions. Although no avian MHC alleles have been designated in the servers, previous studies have demonstrated that human alleles including HLA*B 41:04, HLA*B 40:06 and HLA*B 41:03 are highly similar to chicken MHC-I alleles and can be used for immunoinformatic studies.[19,20]

Prediction of epitopes with MHC-II binding potential

To predict epitopes with MHC-II binding potential,

the NetMHCIIpan 4 and IEDB servers were employed. MHC-II binding peptides are involved in the activation of CD4+ T-cells. The nine residue regions interacting to MHC binding cleft is determined by the NetMHCIIpan algorithms to estimate MHC-II binding potential of peptides quantitatively.[21] On the other hand, IEDB employs a consensus approach including NN-align, SMM-align, CombLib, and Sturniolo, to predict MHC-II binding peptides.[18] Human MHC-II alleles, including DRB1:1366, DRB1:1482, DRB1:1445, and DRB1:1310 have been reported as the best substitutes for chicken MHC-II alleles and were selected for peptide prediction.[19,20]

Prediction of linear B-cells epitopes

Linear epitopes of B-cells were predicted using the BepiPred 2 server. A random forest algorithm is trained in BepiPred 2 using epitopes retrieved from a database of antibody-antigen protein structures.[22]

Selection and evaluation of antigenic epitopes

To design the final vaccine construct, two MHC-I and two MHC-II antigenic epitopes were selected from each protein. Results of the various epitope prediction tools were consolidated, and high-ranking epitopes were selected. Epitopes that identified across multiple servers were prioritized. Since there are experimentally determined epitopes for HA5 and HA7 from the influenza virus in the Influenza Research Database (IRD), in the case of HA5 and HA7 the IRD database has been employed to select one MHC-1 and one MHC-II epitopes and the results of the prediction studies have been employed to select another MHC-1 and MHC-II epitopes.

Furthermore, AllergenFP,[23] and AllerTop,[24] servers were employed to evaluate allergenicity of the epitopes. AllergenFP develops a descriptor-based fingerprint approach using physicochemical properties of the protein/peptide to predict allergenicity of a peptide/protein. Its accuracy was reported about 88%.[23] AllerTop predicts allergenicity of molecules using alignment free strategies.[24] Only epitopes that were predicted as non-allergen by at least one of these servers, were selected for further consideration.

VaxiJen v2.0 was employed to evaluate the antigenicity of the epitopes. Antigenicity of peptide/proteins is predicted based on the principal chemical properties instead of sequence alignment in the VaxiJen v2.0 server resulting accuracy between 70 to 89%.[25] The vaccines inducing IFN-γ can improve development of immune response. The IFNepitope server predicts potential of INF-γ induction with maximum accuracy of 81%. Support vector machine (SVM) was selected as approach and IFN-gamma versus random was selected as model in IFNepitope server to predict IFN- γ inducing potential of the epitopes.[26]

The last criteria, which were considered in the evaluation and selection of epitopes, was epitope conservancy. Since the goal of the present study is development of a multiepitope vaccine against three avian viral diseases with high genome mutability, selection of conserve epitopes is essential. To do so, a set of homologous sequences of HA 5 and 7, F, HN, S and N proteins were retrieved from the BLASTp tool in NCBI and conservancy of the potential epitopes were evaluated using IEDB.[27]

Preparing the final vaccine construct

Appropriate linkers were employed to connect the selected epitopes constructing the final vaccine sequence. Furthermore, the sequence of avian beta-defensin 1 were inserted into the N-terminal part of the vaccine as an adjuvant improving the immunogenicity. The solubility of the overexpressed protein of the vaccine in Escherichia coli were evaluated using Solpro server. Solpro predicts solubility of proteins according to an SVM based model with overall accuracy of 74%.[28] ProtParam tool were employed to estimate the physicochemical properties of the vaccine.[29] Furthermore, antigenicity and allergenicity of the vaccine was also evaluated using ANTIGENpro, VaxiJen v2.0, AllergenFP and AllerTop servers. ANTIGENpro employes various machine-learning algorithms along with a final SVM classifier to develop powerful alignment-free strategy predicting antigenicity of peptides and proteins.[30]

3-Dimential structure modeling

The 3-dimential (3D) structure of the vaccine was modeled using the I-Tasser server. The server employs a four-step process to model the tertiary structure of proteins. At first, multiple alignment approaches are employed to retrieve proteins with similar sequence to the query protein from protein data bank (PDB) to be used as template. In the second step, the protein structure is assembled based on the template protein structures. Furthermore, ab initio approaches are involved in the modeling when they are needed such as tails and loops. The modeling process is followed by clustering of structure decoys to select the models and refinement of the models by molecular dynamics simulation (FG-MD) or ModRefiner. At last, COACH approach is employed to annotate the structure and to complete the modeling process. I-Tasser developes five 3D models for the query protein and calculates a confidence score (Cscore) for each model. Discovery studio 2020 is employed to visualize the structures.

Refinement and validation of the 3D model

Three models demonstrating higher C-score were selected and were further refined by GalaxyRefine and DeepRefiner. GalaxyRefine employs molecular dynamic simulations and repacking of the side-chains of the protein to relax the whole protein structure.[31] The deep learning approach of the DeepRefiner was extensively evaluated in the Critical Assessment of Techniques for Protein Structure Prediction (CASP) experiments and have ranked as second refinement server in CASP13 and 14. The refinement procedure was done with all default settings.[32]

All refined models were assessed using Ramachandran plot, Z-score of ProSA-web and ERRAT value. The

Z-score is calculated via estimation of the interaction energy between each residue and the rest of the protein in ProSA-web. The calculated Z-score is plotted on a graph demonstrating Z-scores of protein 3D structures deposited in the PDB. The Ramachandran plot and ERRAT value have been retrieved from SAVE server. The phi-psi torsion angles are employed to group residues to allowed and disallowed regions in Ramachandran plot.[33] Interactions of non-bonded atoms in a series of high-resolution crystallography structures are compared with the query model to develop ERRAT value which is also accessible in SAVE server.[34]

Prediction of discontinuous B-cell epitopes

In the present study, ElliPro was employed to predict discontinuous epitopes of B-cells. ElliPro employes Thornton's method in combination with a residue-clustering algorithm to predict discontinuous epitopes of B-cells from a protein's 3D structure.[35]

The vaccine-TLRs docking

Immunogenic potential of a vaccine can be improved by interaction with toll like receptors (TLR).[36] Protein–protein interactions can be investigated through several reliable servers using Protein–protein docking. Possible interactions of the vaccine with the TLR-2 and TLR-5 of chicken were investigated using ClusPro,[37-39] PatchDock,[40] and HawkDock,[41-43] servers. To do so, the PDB file of the best-refined model of the vaccine was considered as ligand, while PDB files of the 3D models of TLR-2 and TLR-5 were considered as protein. The 3D models of TLR-2 and TLR-5 were retrieved from Uniprot database with the IDs Q9DD78 and Q4ZJ82 respectively.

All docking parameters in different servers were set to default. However, to refine the outputs from PatchDock, FireDock was employed as recommended by the server. The complexs demonstrating the lowest free binding energy, calculated by HawkDock server, were selected and were further investigated.

Normal mode analysis studies

To investigate the structural dynamics of the docked vaccine-TLR complexes, normal mode analysis (NMA) studies have been done using the iMODS (https://imods.iqfr.csic.es/). iMODS is a fast and trustworthy server employing NMA of torsional coordinates of a protein complex to assess its collective motions.[44-46] It calculates various structural dynamics factors including the values of deformability, B-factor and Eigen values, which provide insights into the flexibility and stability of the protein complex. B-factor plots represent non-rigidity of a protein complex by estimation of its flexibility and deformability. The Eigen score is dependent on the energy, which is needed to deform the protein structure. Structures with the lower Eigen scores are less stable and their atomic coordinates are easily deformed.

Optimization of codons and in silico cloning

Sequence manipulation suite (https://www.bioinformatics.org/sms2/rev_trans.html) reverse-translated the amino acid sequence of the vaccine to generate the gene sequence of the vaccine. GC content, codon adaptation index (CAI) and codon frequency and distribution (CFD) of the gene sequence have been estimated using GenScript server. Furthermore, the N and C-terminals of the sequence were modified by addition of restriction enzymes NcoI and XhoI sites to facilitate using pET-28a vector.

Results

Selection of final epitopes

MHC-I and MHC-II binding epitopes of proteins including hemagglutinin of H5 and H7 subtypes of avian influenza, spike and nucleocapsid of IBV and fusion and hemagglutinin-neuraminidase of NDV disease were predicted using various approaches. Potential linear epitopes of B-cells were also predicted using BepiPred tool. To select appropriate epitopes for final construction of the multiepitope vaccine following criteria were considered. First, high-ranked epitopes demonstrating overlap across multiple servers were selected. All selected epitopes have been specified as antigen in VaxiJen server and have been categorized as probable non-allergen in at least one of the AllergenFP or AllerTop servers. The epitopes with higher levels of conservancy and potential of INF- γ stimulation were also prioritized. Two MHC-I and two MHC-II binding epitopes were selected from every antigenic proteins except HA of H5 and H7 of influenza. Since there are a list of experimentally determined epitopes for HA of H5 and H7 in IRD, two epitopes were selected from the present studies results and additional two epitopes were selected from IRD for each of HA5 and HA7 proteins. Table 1 is illustrated the list of the final selected epitopes and their properties.

Preparing and evaluating the final vaccine sequence

In the final multiepitope vaccine construct, the avian beta-defensin 1 is connected as an adjuvant to the selected epitopes using EAAAK linker. The MHC-I epitopes are connected by AAY linkers, while the MHC-II epitopes are connected with GPGPG linkers. Figure 1 illustrates the sequence of the final vaccine construct, which is the multiepitope vaccine consisting of 479 amino acids.

The physicochemical properties of the vaccine were estimated using the ProtParam server and are illustrated in Table 2. The vaccine has molecular weight (MW) of 51590.65 and grand average of hydropathicity (GRAVY) of -0.258. The instability index of 27.51indicates that the vaccine structure is stable and the theoretical isoelectric pH of the vaccine is estimated to be 9.28. Furthermore, the vaccine protein structure was specified as soluble with a probability of 0.718274 after overexpression in E. coli in the Solpro server.

Table 1. The selected epitopes and their properties.

Virus	Protein	Epitope	HLA-I (Netmhc4-IEDB)	HLA-II (RANKPEP/IEDB)	Linear B Cell	AllergenFP	AllerTOP	Vaxijen	IFN-γ stimulation	Conservancy
AIV	Hemagglutinin 5 YP_308669.1	REEISGVKL	+	-	+	-	-	+	-	99%
		EFFWTILKPNDAINF	-	+	-	-	-	+	+	94%
		WTILKPSDTINFESN [b]	N/A	N/A	N/A	-	-	+	+	N/A
		TIGECPKYV [b]	N/A	N/A	N/A	+	+	-	-	N/A
	Hemagglutinin 7 YP_009118475.1	AEEDGTGCF	+	-	-	+	-	+	-	100%
		KCPRYVKQRSLLLAT	-	+	+	+	-	+	+	87%
		RESGG [b]	N/A	N/A	N/A	+	-	N/A	-	N/A
		RESGGIDKEAMGFTYSGIRTNGATSACRRSGSSFYAEMKWLLS [b]	N/A	N/A	N/A	-	-	-	N/A[a]	N/A
IBV	Nucleocapsid NP_040838.1	SDEERNNAQL	+	-	-	-	+	+	+	94%
		EERNNAQLEF	+	-	-	-	-	+	-	95%
		TAMLNLVPSSHACLF	-	+	-	-	+	+	+	90%
		GNFRWDFIPLNRGRS	-	+	+	-	-	+	+	83%
	Spike NP_040831.1	REYNGLLVL	+	-	-	-	+	+	+	98%
		QEGFRSTSL	+	-	-	-	-	+	+	98%
		QFYIKITNGTRRFRR	+	+	+	-	-	+	-	97%
		SFLSSFVYKESNFMY	+	+	-	-	-	+	+	100%
		GEFDATYQKNI	+	-	+	-	+	+	+	89%
NDV	Fusion YP_009513197.1	RELDCIKIT	+	+	-	-	-	+	-	99%
		AAGIVVTGDKAVNVY	-	+	-	-	+	+	+	94%
		KVNVRLTSTSALITY	+	+	-	-	+	+	+	94%
	Hemagglutinin-neuraminidase YP_009513198.1	TEDKVTSLL	+	-	-	+	-	+	-	97%
		HEKDLDTTV	+	-	-	-	-	+	-	92%
		YPLIFHRNHTLRGVF	-	+	+	-	+	+	-	91%
		YQIRMAKSSYKPGRF	-	+	+	-	+	+	+	97%

[a] More than 30 residue
[b] Epitopes selected from IRD

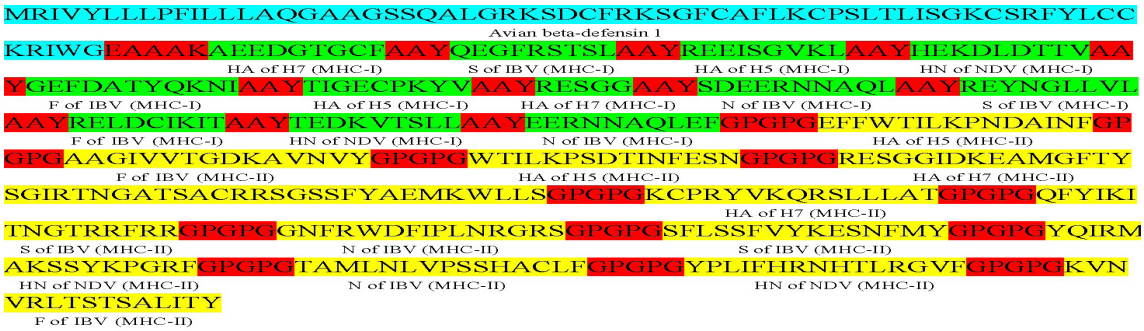

Figure 1. The final sequence of the vaccine construct. Linkers are highlighted in red.

Evaluation of the immunological characteristics of the vaccine

In addition to the selected epitopes, the ANTIGENpro and VaxiJen v2.0 servers also identified the entire vaccine construct as an antigen. The antigenicity of the vaccine was estimated to be 0.5423 in VaxiJen while it considers 0.4 as threshold. The probability of antigenicity was estimated to be 0.834482 in ANTIGENpro. Both the AllergenFP and AllerTop servers have classified the vaccine construct as a probable non-allergen (Table 2).

Evaluation of the modeled 3D structure of the vaccine

The 3D structure of the vaccine construct was automatically modeled by the I-Tasser server and three models with higher C-scores were further refined using GalaxyRefine and DeepRefiner servers. The refined models were evaluated based on the Ramachandran plot, Z-score, and ERRAT value to determine the best 3D model of the vaccine (Figure 2).

The selected 3D model has demonstrated a Z-score of 3.89, which falls within the range of Z-scores of experimentally determined 3D structures retrieved from PDB (Figure 3A). Figure 3B illustrates the Ramachandran plot developed by the ProtPram server from the 3D model of the vaccine. Only 3.7 percent of the amino acids are represented in the disallowed region. The ERRAT value of the modeled protein is 81.42 (Figure 3C). According to various studies, ERRAT values greater than 50 indicate an acceptable 3D model for a protein.[47,48] Overall, the selected 3D model meets the requirements of a satisfactory protein model and can be employed for further studies.

Table 2. Evaluated characteristics of the vaccine.

Physicochemical characteristics	Result
Predicted solubility / Solpro	Soluble with probability 0.718274
Molecular weight	51590.65
Instability index	27.51/stable
Gravy	-0.258
Aliphatic index	68.35
Theoretical pI	9.28
No. of amino acids	479
Total no. of negatively charged residues (Asp + Glu)	37
Total no. of positively charged residues (Arg + Lys)	54
Allergenicity	
AllergenFP	PROBABLE NON-ALLERGEN
AllerTop	PROBABLE NON-ALLERGEN
Antigenicity	
ANTIGENpro	0.834482/antigen
VaxiJen	0.54/ antigen

Identification of discontinuous B-cell epitopes

ElliPro has identified six discontinuous B-cell epitopes within the 3D structure of the vaccine, demonstrating the potential of the vaccine to stimulate humoral immune responses. The amino acid numbers and the 3D positions of the epitopes are illustrated in the Table 3 and Figure 4 respectively.

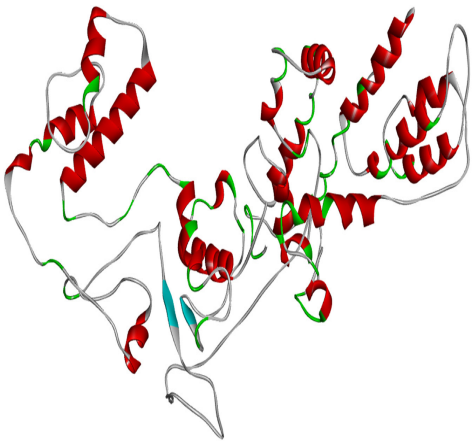

Figure 2. The best tertiary structure of the vaccine was modeled by I-Tasser and was refined by GalaxyRefine.

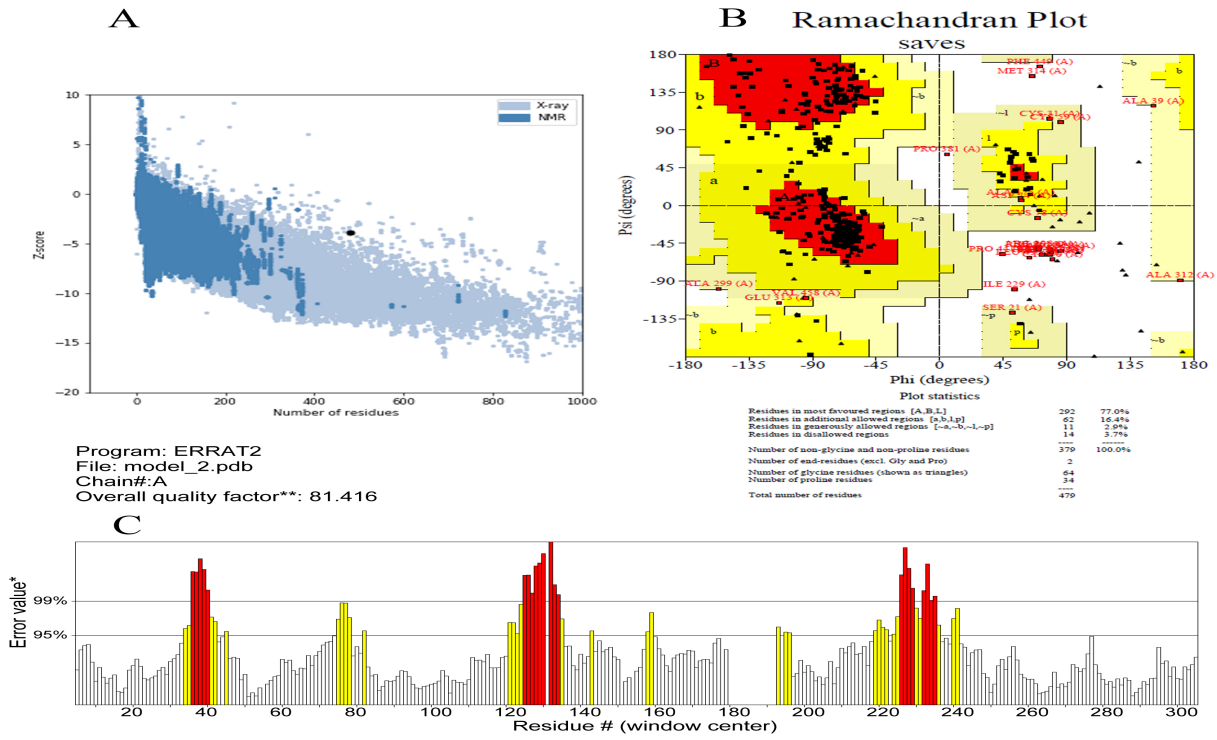

Figure 3. Evaluation of the 3D model of the vaccine. The ProSA-web Z-score plot of the vaccine is illustrated in part A. The large black dote is illustrating Z-score of the model which is in the range of Z-scores for PDB retrieved experimentally 3D structures. The Ramachandran plot (part B) is demonstrating 3.7% of the amino acids in the disallowed region. ERRAT chart of the vaccine demonstrates ERRAT value of 81.416 (part C).

Prediction of possible interactions between the vaccine and TLRs

Protein-protein docking is a reliable approach for investigating possible proteins interactions. The 3D model of the vaccine construct was docked into chicken TLR2 (Q9DD78) and TLR5 (Q4ZJ82) as receptor using ClusPro, HawkDock and PatchDock servers. The ligand-receptor complexes were subjected to calculation of free binding energy using the MM-GBSA approach in the HawkDock server (Table 4). The complexes developed by ClusPro were selected for further investigation because they have demonstrated the lowest free binding energy (Figures 5 and 6). Possible interactions between the vaccine and TLRs have been investigated using UCSF Chimera software. The H-bond analysis tool of Chimera has identified 33 hydrogen bonds (Figure 5b and 6b). Moreover, Chimera has demonstrated different hydrophobic and polar interactions between the vaccine's residues and TLR2/TLR5 proteins, demonstrating the potential of the vaccine to induce immune responses through TLR-dependent mechanisms against influenza, IBV and NDV (Figures 5c and 6c).

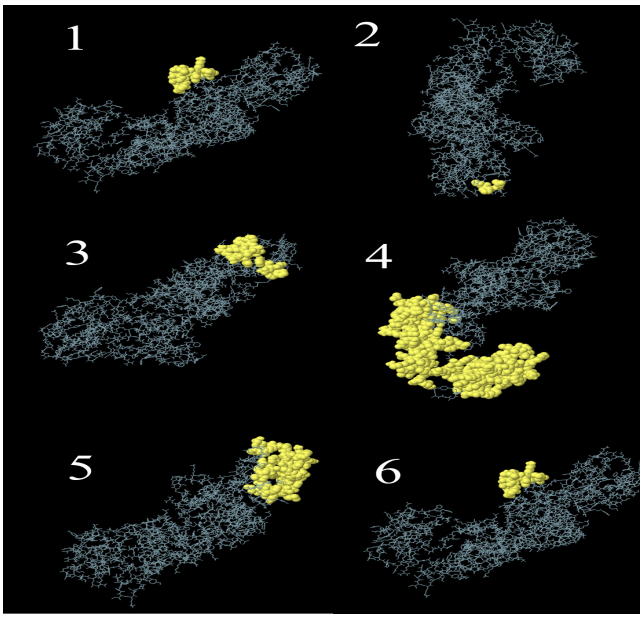

Figure 4. Discontinuous B-cell epitopes. The discontinuous epitopes of B-cells have been predicted using ElliPro server and are illustrated by yellow balls in the 3D model of the vaccine.

Table 4. Free binding energy of the docked complexes.

TLR	ClusPro	PatchDock	HawkDock
TLR2	-153.80	-96.54	-44.74
TLR5	-183.33	-22.63	-47.39

Table 3. Conformational B-Cell Epitopes.

No	Residue	Number of residues	Score
A1	A:S147, A:G148, A:G149, A:A150, A:A151, A:Y152	6	0.91
A2	A:I372, A:P373, A:L374	3	0.857
A3	A:R333, A:S334, A:L335, A:L336, A:L337, A:A338, A:T339, A:G340, A:P341, A:G342, A:P343, A:G344, A:Q345, A:F367, A:W369, A:D370, A:F371	17	0.746
A4	A:S50, A:G51, A:K52, A:C53, A:S54, A:R55, A:F56, A:Y57, A:C60, A:Y82, A:Q83, A:E84, A:G85, A:F86, A:R87, A:S88, A:T89, A:S90, A:L91, A:A92, A:A93, A:Y94, A:R95, A:E97, A:I98, A:S99, A:G100, A:V101, A:K102, A:L103, A:A104, A:A105, A:Y106, A:H107, A:E108, A:K109, A:D110, A:L111, A:D112, A:T113, A:T114, A:V115, A:A116, A:A117, A:Y118, A:G119, A:E120, A:F121, A:D122, A:A123, A:T124, A:Y125, A:Q126, A:K127, A:N128, A:I129, A:A130, A:A131, A:Y132, A:T133, A:I134, A:G135, A:E136, A:C137, A:P138, A:K139, A:Y140, A:V141, A:A142, A:A143, A:Y144, A:R145, A:E146, A:S153, A:D154, A:E155, A:E156, A:R157, A:N158, A:N159, A:A160, A:Q161, A:L162, A:A163, A:A164, A:Y165, A:R166, A:E167, A:N169, A:G170, A:L171, A:L172, A:V173, A:L174, A:A175, A:A176, A:Y177, A:R178, A:E179, A:L180, A:D181, A:C182, A:I183, A:K184, A:I185, A:T186, A:A187, A:A188, A:Y189, A:T190, A:E191, A:Y201, A:E202, A:E203, A:R204, A:N205, A:N206, A:A207, A:Q208, A:L209, A:E210, A:F211, A:G212, A:P213, A:G214, A:P215, A:G216, A:E217, A:F218, A:F219, A:W220, A:T221, A:I222, A:L223, A:K224, A:P225, A:N226, A:D227, A:A228	139	0.743
A5	A:F346, A:I348, A:K349, A:F357, A:G365, A:R368, A:N375, A:R376, A:G377, A:R378, A:S379, A:G380, A:P381, A:G382, A:P383, A:G384, A:S385, A:F386, A:L387, A:S388, A:S389, A:F390, A:V391, A:Y392, A:E394, A:S395, A:N396, A:F397, A:M398, A:Y399, A:G400, A:P401, A:G402, A:P403, A:G404, A:Y405, A:Q406, A:I407, A:R408, A:M409, A:A410, A:K411, A:S412, A:S413, A:Y414, A:K415, A:P416, A:G417, A:R418, A:F419, A:G420, A:P421	52	0.727
A6	A:G252, A:P253, A:G254, A:P255, A:G256, A:W257, A:T258, A:I259, A:L260, A:K261, A:P262, A:D264, A:T265	13	0.568

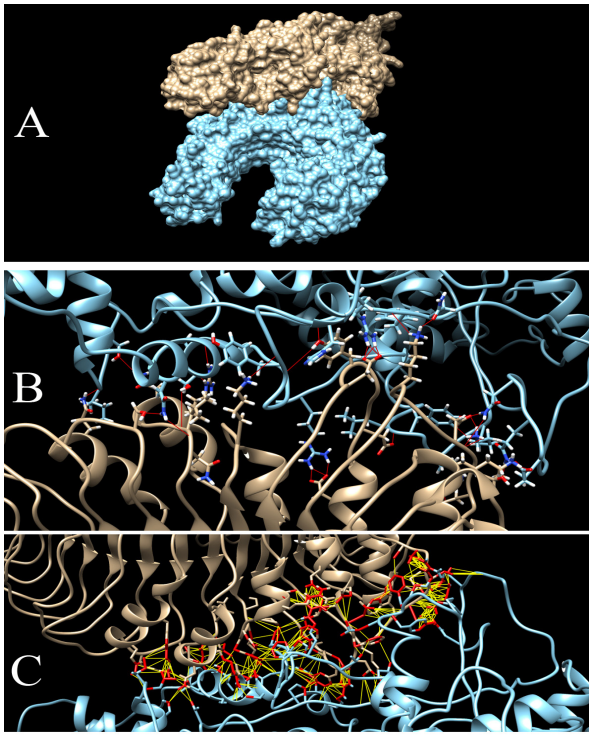

Figure 5. The vaccine-TLR-2 docking. The complex of the vaccine (blue) and TLR-2 (chocolate) are illustrated in part A. The complex of the vaccine and TLR-2 arex stabilized by formation of several H-bonds (red sticks) and hydrophobic interactions (yellow sticks) (part B and C).

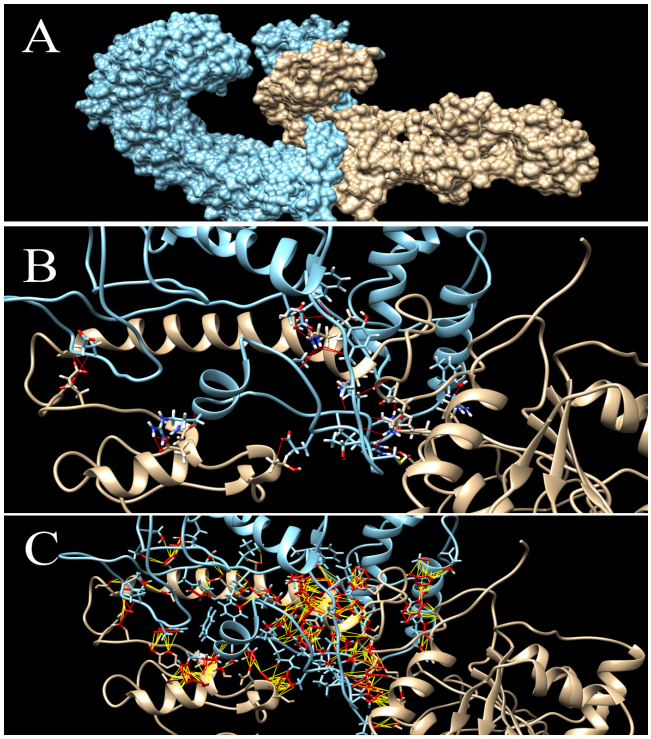

Figure 6. The vaccine-TLR-5 docking. The complex of the vaccine (blue) and TLR-5 (chocolate) is illustrated in part A. The complex of the vaccine and TLR-5 is stabilized by formation of several H-bonds (red sticks) and hydrophobic interactions (yellow sticks) (part B and C).

Normal mode analysis studies

NMA investigations can be employed to evaluate the flexibility and stability of the vaccine-TLR complexes. Various structural dynamics factors of the vaccine and TLR complexes were estimated using the iMODS server. As demonstrated in Figures 7A and D, the deformability of the complex is significantly reduced in the vaccine-TLR-2 complex compared to TLR-2 alone, suggesting the stabilization of the complex. Figures 8A and B demonstrate the deformability chart of TLR-5 and the vaccine-TLR-5 complex, indicating a slight enhancement in the deformability of the vaccine-TLR5 complex compared to TLR-5 alone. Furthermore, the B-factor of two complexes are represented in Figures 7B and 8B, demonstrating minor atomic movement of the structures. However, the vaccine-TLR-5 complex exhibits greater atomic distortions compared to the vaccine-TLR-2 complex. The eigenvalues of the vaccine-TLR-2 complex and TLR-2 were estimated to be 5.840585e-6 and 1.337318e-05 respectively, demonstrating deformation of the vaccine-TLR-2 complex with lower energy compared to TLR-2 (Figures 7C and F). The eigenvalues of the vaccine-TLR-5 complex and TLR-5 were estimated to be 2.998798e-06 and 5.604980e-07, respectively, demonstrating deformation of the vaccine-TLR-5 complex with higher energy compared to TLR-5 (Figures 8C and F). The variance analysis, which is inversely proportional to the eigenvalue, is demonstrated in Figures 7I and 8I for the vaccine-TLR2 and vaccine-TLR5 complexes respectively. Individual variances are displayed in purple shades, while cumulative variances are displayed in green shades. The correlated, uncorrelated, and anti-correlated motions between the residues of the vaccine-TLR2 and vaccine-TLR5 complexes were evaluated using covariance analysis (Figures 7H and 8H). Furthermore, the dark gray springs in the elastic network charts demonstrate the compactness and rigidity of the vaccine-TLR2 and vaccine-TLR-5 complexes (Figures 7G and 8G, respectively). Overall, it seems the vaccine-TLR complexes have stable and compact conformation with minor fluctuations.

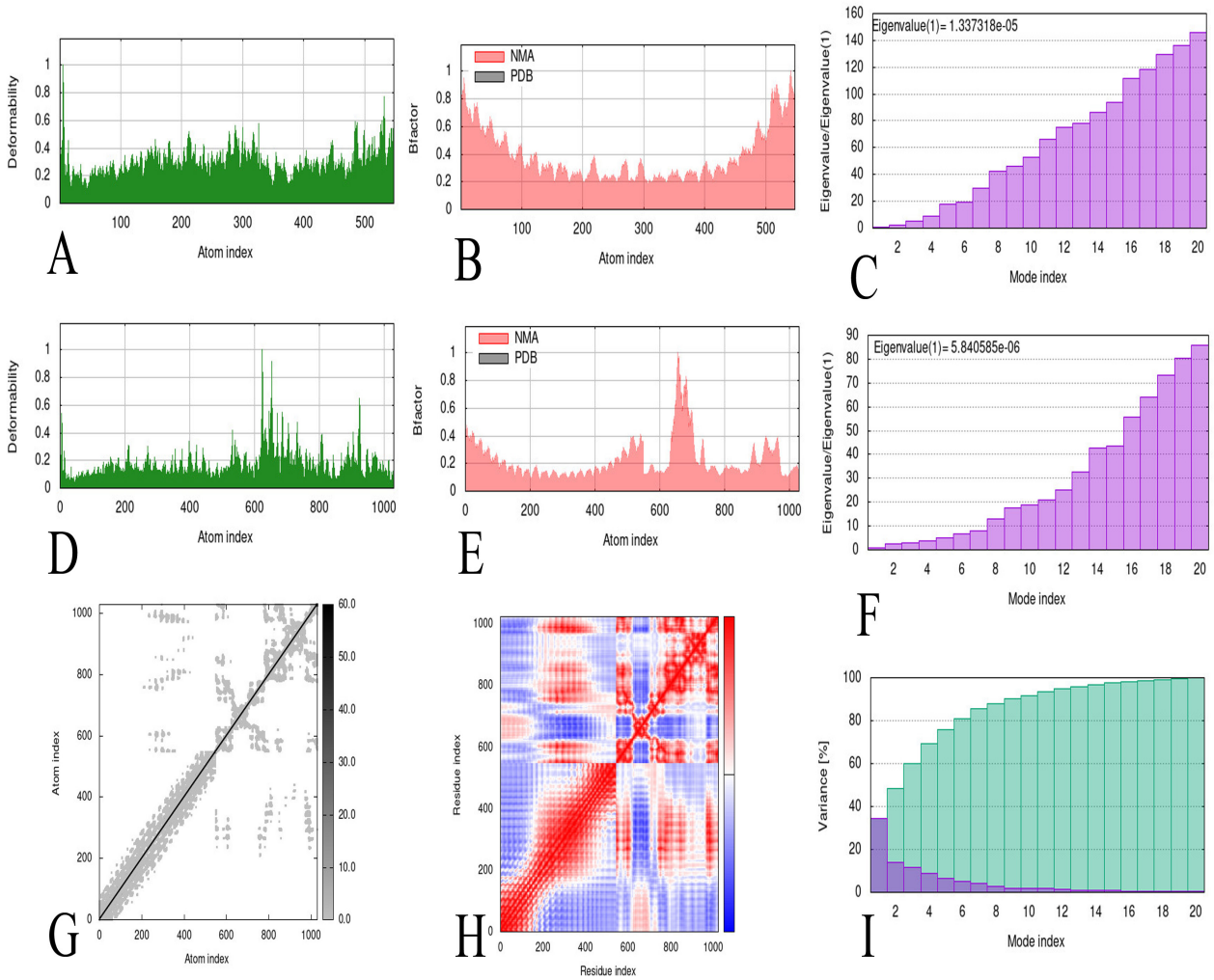

Figure 7. NMA analysis results. Parts A, B and C are illustrating deformability, B-factor values and eigenvalue of TLR-2 and parts D, E and F are demonstrating the same factor for the vaccine-TLR-2 complex respectively. The elastic network analysis, which defines the pairs of atoms connected by springs (G), the covariance that indicates coupling between pairs of residues (red), uncorrelated (white) or anti-correlated (blue) motions (H) and Variance (I) graphs of the vaccine-TLR-2 complex are also illustrated.

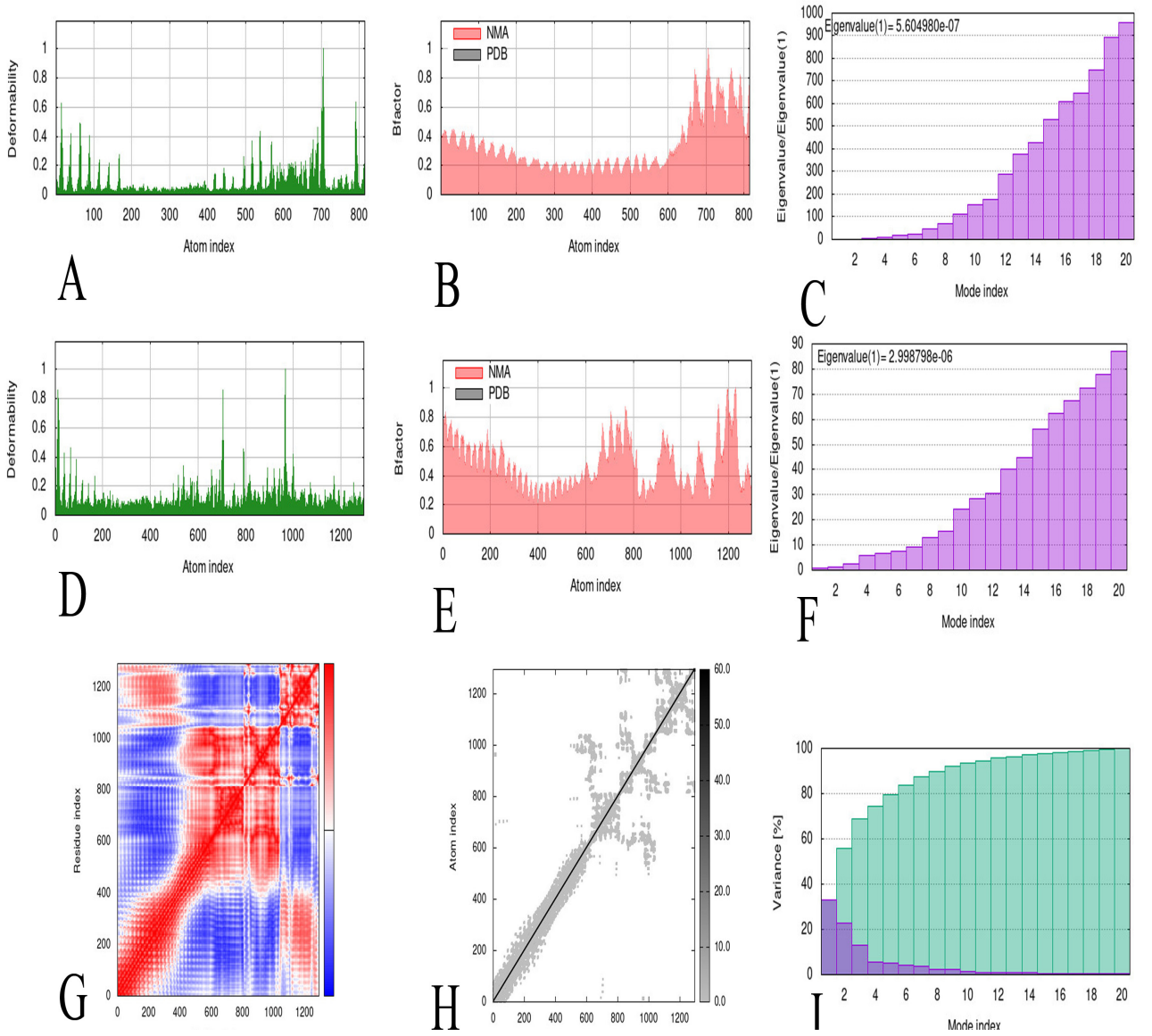

Figure 8. NMA analysis results. Parts A, B and C are illustrating deformability, B-factor values and eigenvalue of TLR-5 and parts D, E and F are demonstrating the same factor for the vaccine-TLR-5 complex. The elastic network analysis, which defines the pairs of atoms connected by springs (G), covariance that indicates coupling between pairs of residues (red), uncorrelated (white) or anti-correlated (blue) motions (H) and Variance (I) graphs of the vaccine-TLR-5 complex are also illustrated.

Optimization of the gene sequence of the vaccine

The sequence manipulation suit has reversely translated the amino acid sequence of the vaccine into the nucleotide sequence. The CAI of the vaccine was estimated to be one demonstrating ideal sequence for expression of the protein in E. coli as the host (Figure 9A). The appropriate range of GC content according to Genscript is 30-60%. The designed vaccine has a GC content of 60.70% (Figure 9B). Furthermore, the frequency and distribution of codons for every amino acid are appropriately selected with a CFD of 100 % (Figure 9C). Overall, the evaluation of the vaccine sequence has shown that the sequence can be effectively translated in E. coli as a host. Furthermore, the N and C terminal of the sequence were modified with NcoI and XhoI restriction sites, and the sequence was inserted into the pET-28a vector (Figure 9D).

Discussion

The poultry industry plays a significant role in the food supply and the economic performance of various countries around the world. The severity and highly infectious nature of IBV, NDV and influenza viruses lead to significant economic losses in poultry industry, including meat-type and egg-laying birds, threatening the food supply for humans. Since no approved medicines have been developed to effectively control IBV, NDV and influenza infections, vaccination seems the only defense protecting the birds against these diseases. High levels of mutation and diversity among different variants of IBV, NDV and influenza viruses have decreased the effectiveness of currently available conventional vaccines. This situation necessitates further investigations to develop new generation of vaccines. Here, a multiepitope vaccine

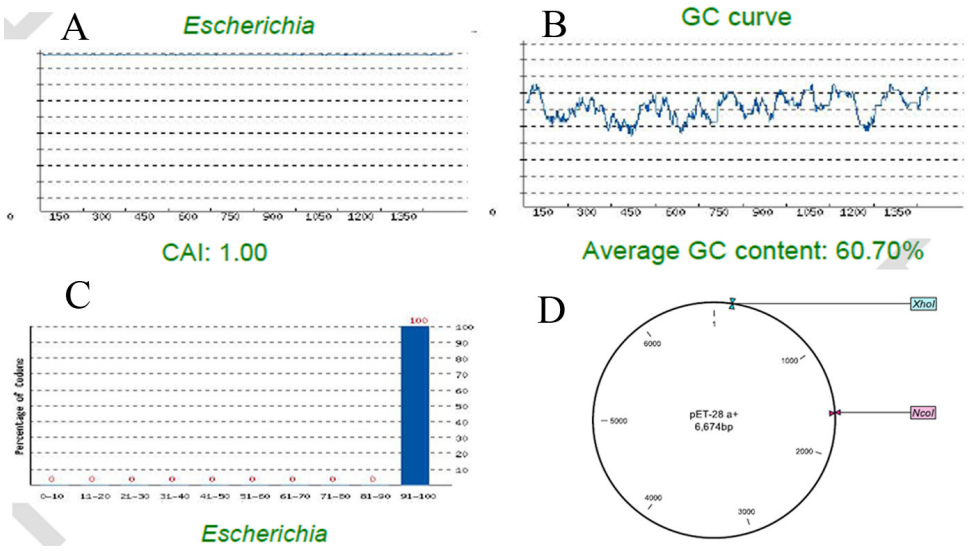

Figure 9. Optimization of gene sequence of the vaccine and in-silico cloning. The gene sequence of the vaccine have demonstrated CAI of 1 (A), GC content of 60.70% (B) and CFD of 100 (C) facilitating appropriate transcription and translation of the vaccine in E. coli. Part D illustrates vector map of the vaccine in pET-28a vectors.

against IBV, NDV and influenza viruses has been designed using extensive bioinformatics tools. Epitopes that bind to MHC-I and MHC-II have been identified from antigenic proteins, including HA of H5 and H7 subtypes of influenza, spike (S) and nucleocapsid (N) from IBV and fusion (F) protein and hemagglutinin-neuraminidase (HN) proteins from NDV. The multiepitope vaccine is constructed by joining eight epitopes for each disease along with avian beta-defensin 1 sequence as an adjuvant. All of the selected epitopes have been specified as antigen in the VaxiJen server and were predicted to be probable non-allergen. Additionally, half of the selected epitopes have shown the potential to induce IFN-γ. Furthermore, 17 epitopes have shown at least 90 % conservancy and three epitopes have shown 80% conservancy, demonstrating the effectiveness of the vaccine against various strains of IBV, NDV and influenza virus. The vaccine construct has been identified as an antigen and non-allergen in various servers and the vaccine has been specified as soluble and stable after overexpression in E. coli (Table 2). Furthermore, the vaccine can be effectively translated in E.coli according to its appropriate CAI (Codon Adaptation Index), CFD (Codon Frequency Distribution), and GC content factors. The Z-score of ProSA-web, ERRAT value, and the Ramachandran plot of the vaccine's 3D structure meet the criteria for an acceptable protein model (Figure 3). Protein-protein docking is a reliable technique to investigate possible interactions between two proteins. Analysis of the results of the vaccine-TLRs docking has revealed formation of stable complexes between the vaccine and TLRs, demonstrating potential of the vaccine to induce immune responses through TLR pathways (Figures 4B, 4C). ElliPro server has identified six discontinuous B-cell epitopes in the 3D model of the vaccine. B-cell epitopes are involved in the development of the humoral immune response (Figure 4).

Avian flu outbreaks, especially those caused by highly pathogenic H5 and H7 subtypes, are significant threat poultry industries, wild birds and humans. HA is a trimeric surface glycoprotein facilitating the entry of the virus to the host cells. Influenza viruses have 11 viral proteins but antibody epitopes have been reported in five viral proteins, with a significant number located in the HA protein.[49] Furthermore, only HA and NA as the two major surface glycoproteins, are exposed on the surface of the virus.[50] Therefore, the HA protein of H5 and H7 subtypes is considered an appropriate antigenic protein for vaccine development. In the present study, eight antigenic and non-allergenic peptides have been selected from the HA of H5 and H7 subtypes to construct the vaccine. Four peptides were retrieved from the IRD with demonstrated antigenic effect and four peptides were selected based on the results of immunoinformatic tools. Three peptides have demonstrated a conservancy of over 90% and three peptides have shown INF-gamma inducing potential.

Newcastle disease is highly significant avian disease affecting a wide range of bird species worldwide. Despite the considerable socioeconomic impact of NDV on poultry industries, current vaccines and drug therapies have been unable to effectively eliminate NDV outbreaks. The F and HN proteins of NDV have been recognized as independent neutralization and protective antigens.[51] The HN protein is involved in the entry of the virus into its host cell through neuraminidase activity and recognition of receptors containing sialic acid. It also activates the F protein. Fusion of the virion envelope with the cellular plasma membrane is mediated by the F protein. Since the HN and F proteins are located on the surface of NDV and have demonstrated antigenic effects, they have been employed to design the multiepitope vaccine in the present

study. Among the eight epitopes selected from the F and HN proteins, seven peptides have demonstrated over 90% conservancy, and four peptides have demonstrated IFN-gamma inducing potential.

IBV is a coronavirus causing respiratory disease in chickens and can lead to extensive economic losses in the poultry industry. The spike and nucleocapsid proteins were selected to design the vaccine according to their conservancy and immunogenic properties. Among the eight immunogenic peptides selected from the spike and nucleocapsid proteins, seven peptides have demonstrated conservancy of over 90%. Furthermore, six peptides have demonstrated IFN-gamma inducing potential, which shows effectiveness of the vaccine against IBV infection.

The development of multiepitope vaccines is greatly facilitated with the advantage of immunoinformatic tools. Identification of epitopes that can induce B-cell and T-cell dependent immunity and combination of several epitopes in a vaccine construct, offer several advantages over traditional vaccines.[14] Immunodominant epitopes from different antigenic proteins of a pathogen in a multiepitope vaccine can develop immune responses against various antigenic proteins improving the efficacy of the vaccine. Furthermore, the selection of relevant epitopes can lead to immune responses that are more specific and prevent non-protective responses, immune evasion, or unwanted side effects, such as autoimmunity. Moreover, combinations of immunodominant epitopes from multiple pathogens represents a great opportunity to induce immune responses against multiple pathogens with a single vaccine.[52] These advantages have encouraged development of multiepitope vaccines using immunoinformatic tools against infectious pathogens and cancerous diseases in recent years.[53-55] Since various peptide and protein adjuvants have been introduced, the immunogenicity of multiepitope vaccines can be improved by joining the epitope sequences to an adjuvant sequence by appropriate linkers. In the present study the sequence of avian beta-defensin 1 have been joined to the N terminal of the multiepitope vaccine using EAAAK linker. Avian beta-defensin 1 contains 65 amino acid demonstrating antibacterial and immunomodulatory effects.[56] The development of the 3D structure of the adjuvant is facilitated by using rigid linkers including EAAAK to connect the sequence of the adjuvant to the epitopes in the vaccine. Furthermore, interaction of adjuvants with their target is not affected by epitopes due to the rigid linker. GPGPG and AAY linkers have also been selected to connect the epitopes recognized by MHC-II and MHC-I respectively.[57]

Conclusion

In conclusion, extensive immunoinformatic tools have been employed to design a multiepitope vaccine targeting IBV, NDV and avian influenza as three important infectious disease affecting poultry and wild birds. The antigenic proteins of the viruses were selected according to the literature and were subjected to various immunoinformatic tools to identify the immunodominant and non-allergenic peptides. The immunization potency of the vaccine is improved by addition of avian beta-defensin 1 as adjuvant. Although the results of this bioinformatics study require experimental validation, the evaluation of the vaccine's immunological and physicochemical characteristics indicates its potential to induce immune responses against IBV, NDV and avian influenza diseases. New generation of vaccines can be developed time and cost effectively with the advantage of immunoinformatic tools especially in the currently incurable diseases. The combination of immunodominant epitopes of antigenic proteins from multiple diseases presents a promising strategy to induce immune responses against two or more diseases by one vaccine.

Author Contributions

Morteza Ghandadi: Conceptualization, Methodology, Investigation, Writing - Original Draft.

Conflict of Interest

The author has no relevant financial or non-financial interests to disclose.

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