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An immunoinformatic assay to design bio adjuvanted vaccine against infectious bursal disease virus

Mohammad Majid Ebrahimi[1,2], Shahla Shahsavandi[1*], Parviz Shayan[2], Hossein Goudarzi[1], Shahin Masoudi[1]

[1] Razi Vaccine & Serum Research Institute, P.O.Box 31975-148, Karaj, Iran

[2] Faculty of Veterinary Medicine, Tehran University, P.O.Box 14155-6453, Tehran, Iran

*Correspondence should be addressed to Shahla Shahsavandi, Razi Vaccine & Serum Research Institute, P.O.Box 31975-148, Karaj, Iran; Tell: +982634570038; Fax: +982634552194; Email: s.shahsavandi@rvsri.ac.ir.

ABSTRACT

Infectious bursal disease virus (IBDV) causes highly contagious and immunosuppressive disease in young chickens worldwide. The control of infectious bursal disease (IBD) depends mainly on vaccination and strict hygiene management of poultry farms, but the disease continues to pose an important threat to the commercial poultry industry. Recently, second-generation vaccines based on expression of VP2 in various vectors have been developed as new strategies for vaccination against IBD. A series of the vaccines were made using different adjuvant to examine their immunogenicity. In this study we explore the idea of using TLR7 as bio adjuvant to stimulate immune responses against IBDV. Eight conserved TLR7 motifs were found among Homo sapiens, Mus musculus, and Gallus gallus following alignment of the related sequences. Each of the TLR7 motif was fused to VP2 fragment and VP2/TLR7-1 to -8 constructs were designed. By using *in silico* analysis include physicochemical properties determination, protein structures prediction, antigenic site determination, and evaluation of model quality, one of the chimeric proteins was subjected to introduce as vaccine candidate. The results indicate that some TLR7 motifs can be potentially used as bio adjuvant for induction of immune responses against IBDV. It is necessary to determine the potential role of the peptide in induction of immunity against IBDV infection in chicken.

Key words: Infectious bursal disease virus, VP2 DNA vaccine, TLR7, Bio adjuvant

1. INTRODUCTION

Infectious bursal disease virus (IBDV) belonging to the *Birnaviridae* family resulted in immunosuppression and mortality of chickens 3 weeks of age and older. The virus targets the precursors of antibody-producing B cells in the bursal lymphoid follicles (1). The double-stranded RNA segments A and B encode five mature viral proteins; VP1-VP5. Segment B is monocistronic and encodes VP1, the RNA-dependent RNA polymerase, which is packaged inside the virion. The smaller open reading frame (ORF) of segment A encodes a nonstructural host membrane-associated protein (VP5) and the larger encodes VP4 polyprotein precursor. Structural units are derived from VP4 polyprotein precursor which is co- and posttranslationally self-cleaved to release three polypeptides: pVP2 (the capsid protein precursor), VP4 (the protease), and VP3 (a polypeptide with scaffolding activity among others). pVP2 is further processed at its C-terminal region to give the mature VP2 (2, 3). The VP2 mature protein or the major host-protective antigen is responsible for eliciting neutralizing antibodies (4). Serotype 1 of IBDV is pathogenic in chickens by targeting B lymphocytes maturation in the bursa of Fabricius lead to prolonged immunosuppression of chickens infected at an early age (1). Based on the virulence of IBDVs and the age of the infected chickens the disease might happen with clinical symptoms or more mortality (5, 6). The disease is prevented by administering attenuated or inactivated vaccines. Inactivated vaccines induce high antibody levels in breeders to protect younger chickens by maternal antibodies. The VP2 recombinant IBD vaccines have been used to induce both cellular and humoral immune responses without interfering with maternal antibodies, but the vaccines were not efficacious to protect chickens against the antigenic variant strains (1). Adjuvant should be interfered with low immunogenicity of VP2 protein to prolong the exposure time of antigen to the immune system. Molecular adjuvant is a new strategy for improving the immunogenicity of vaccines to prevent against viral infection (7). The Toll-like receptor (TLR) family of cell surface molecules is a highly conserved group of DNA

molecules in both the invertebrate and vertebrate lineages (8, 9). Among the TLR receptors which involve in virus recognition, activation of TLR7 leads to the production of inflammatory cytokines such as IFN I, IL-6, IL-12, TNFα, up-regulation of co-stimulatory molecules (CD40, CD80, CD86), MHC molecules and chemokine receptors (10, 11). Structurally TLR7 contains a ligand binding, leucine-rich extracellular domain, a transmembrane region, and a conserved TIR domain, which transduces perceived signals and induces expression of immune responsive genes (12). Therefore, the importance of TLR7 function is evident by its role in the immune system. In our recent project, we use TLR7 to stimulate immune responses against IBD infection using bioinformatic and experimental strategies. Here we presented *in silico* analysis to determine the effect of of TLR7 as bio adjuvant on the immunogenicity of IBDV DNA vaccine. Understanding the interaction will enable us to defined interpretation of immune induction against the major immunosuppressive disease.

2. MATERIALS AND METHODS

2.1. VP2 infectious bursal disease virus

During previous studyseveral IBDV strains were isolated and characterized. The full length of VP2 encoding region of an imIBDV was determined and compared to VP2 nucleotide and deduced amino acid full length sequences deposited in NCBI GenBank (13). All of the sequences were aligned using ClustalW program with default parameters and their phylogenetic relationships determined by the minimum evolution (ME) analysis with 1,000 bootstrap replication (Tamura et al., 2007, available in MEGA4.

2.2. TLR7 sequence data collections

Datasets of TLR7 peptide sequences from Homo sapiens, Mus musculus, and Gallus gallus downloaded and aligned and eight conserved motifs in these sequences were determined (Table 1).

Table 1. Physicochemical properties of the infectious bursal disease VP2/TLR7 chimera peptides

TLR7 motif	Sequence	Length	Position
1	LLSLEANNIFS	11	Lucine-rich repeat
2	LRLHSNSLQ	9	Lucine-rich repeat
3	VLDLGTNFIKIA	11	Lucine-rich repeat
4	LSFLKCLNLSGN	12	Lucine-rich repeat
5	YLDFSNNR	8	Lucine-rich repeat
6	FNLCLEERDWLPGQPV	16	TIR domain
7	NLSQSIQLSKKT	12	TIR domain
8	DVIILIFLEK	10	TIR domain

2.3. VP2/TLR7 constructs design and characterization

To design a single peptide construction the C-terminus of VP2 fragment were fused to each of TLR7 motif using repeat of hydrophobic amino acid linkers (EAAAK). To increase the efficiency of translational initiation the kozak sequence was introduced. The bioinformatics analyses were ran on the three VP2/TLR7 constructs. The physicochemical properties, hydrophobicity, hydrophilicity, surface accessibility and electrostatic potential of the VP2/TLR7 constructs were identified using Prot-Param. These peptides sequences were inverted to nucleotide sequences and were consistent to *E.coli* practical codons by GeneScript. Then codon adaptaion index (CAI) score and the average GC content were estimated.

2.4. Proteins structures prediction

To aid alignment correction and loop modeling, secondary structures of VP2/TLR7 chimera proteins were predicted by using PSIPRED tool (http://bioinf.cs.ucl.ac.uk/psipred). Protein structure and three dimensional (3D) models of the chimeric constructs were predicted by Phyre (http://www.sbg.bio.ic.ac.uk/phyre2/html/). The 3D model is visualized in different representation patterns by Swiss-Pdb Viewer.

2.5. Homology modeling, model quality and validation

The 3D models were constructed from the sequence alignment between the constructs and the template proteins using SWISSMODEL with parameters of energy minimization value. The energy minimization was computed with the GROMOS96 implantation of the software. In order to assess the reliability of the modeled structure of VP2/TLR7, the root mean square deviation (RMSD) was calculated by superimposing it on the template structure using a 3D structural superposition. The phi (Φ) and psi (ψ) torsion angles were calculated using RAMPAGE for backbone conformation of the modeled structure. Finally, the quality of the consistency between the template and the modeled VP2/TLR7 was evaluated using validated by ProSA which gives the overall model quality based on the Cα positions.

2.6. Prediction of post-translational modifications

To evaluate the correct folding of the VP2/TLR7 peptides, N-glycosylation of NXS/T amino acids sequences (where X is any amino acids except P) was determined at Center for Biological Sequence Analysis.

2.7. Potential antigenic sites prediction

The amino acid sequences were predicted for linear B-cell epitopes using Immune Epitope Database (IEDB) server. The antigenic sites in the chimeric models were determined using Kolaskar and Tongaonkar antigenicity prediction method with about 75% accuracy. Solvent accessible scale for delineating hydrophobic and hydrophilic characteristics of the chimera protein sequences was predicted using Vadar. Prediction of T-cell epitopes in the protein sequences was performed based on

integrating the peptide major histocompatibility (MHC) class I binding, proteasomal C terminal cleavage and transporters associated with antigen processing efficiency by using the NetCTL tool in the server and SYFPEITHI.

3. RESULTS AND DISCUSSION

3.1. Chimeric VP2/TLR7 physicochemical properties

Each of the TLR7 motif 1 to 8 was selected and fused to VP2 peptide sequence named VP2/TLR7-1 to VP2/TLR7-8. The physicochemical properties of the constructs are shown in Table 2. Based on the predicted instability index, the chimera peptides are stable with range values of 26.51-29.62. The aliphatic index (AI) of a protein is the relative volume occupied by the aliphatic side chains (A, V, I and L). The index is involved as contributor to the increase thermal stability of globular proteins. The high AI computed for VP2/TLR7-8 protein (97.89) indicates the protein can be stable within a wide range of temperature compared with other peptides. The physicochemical properties were similar except that the GRAVY index for the motifs located at TIR domain (TLR7-6 to TLR7-8). The index is sum of amino acid hydropathy values divided by the number of residues in the sequence. At very low GRAVY index a protein is very reactive in water. The hydrophilic regions exposed on the surface possibly be more antigenic and contribute to pathogenicity. By optimizing codons to proposed constructs, CAI score achieved 1 and the average GC content was > 50 percent for these models.

Table 2. Physicochemical properties of the infectious bursal disease VP2/TLR7 chimera peptides

Chimera peptide	Physicochemical properties						
	MW (kDa)	Negatively charged residue (Asp+Glu)	Positively charged residue (Arg+Lys)	Theoretical pI	Instability index	Aliphatic Index	GRAVY*
VP2/TLR7-1	50463.3	40	32	5.15	29.07	96.06	0.078
VP2/TLR7-2	50310.1	39	33	5.48	29.32	95.42	0.050
VP2/TLR7-3	50546.5	40	33	5.25	26.51	96.46	0.089
VP2/TLR7-4	50551.4	39	33	5.37	27.74	95.64	0.076
VP2/TLR7-5	50271.0	40	33	5.25	27.68	93.95	0.038
VP2/TLR7-6	50688.5	41	32	5.07	29.62	94.99	-0.053
VP2/TLR7-7	50118.9	38	33	5.50	28.53	95.18	-0.045
VP2/TLR7-8	49974.9	40	32	5.14	28.37	97.89	-0.102

* Grand Average of Hydropathicity is calculated by adding the hydropathy value for each residue and dividing by the length of the sequence

3.2. Protein structures prediction

Analysis of the chimeric protein secondary structure indicated that VP2/TLR7-1 to -7 constructs had the same alpha helix and coil structures except VP2/TLR7-8 which showed higher β-sheet (Figure 1). An additional β-sheet structure was formed at the C-terminus of the construct due to adding the DVIILIFLEK motif. The 3D structures were modeled and evaluated for choosing the best model.

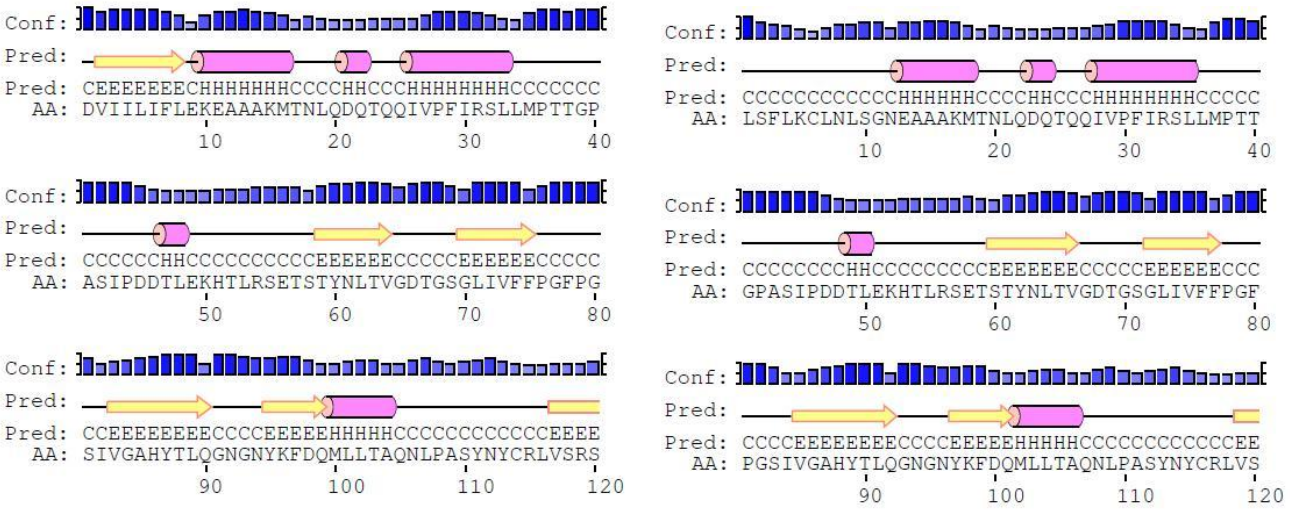

Figure 1. PSIPRED graphical result of secondary structure prediction of VP2/TLR7 chimera peptides, left motif 8 and right motif 4. Additional β-sheet structure was shown in VP2/TLR7-8 construct compared to the others constructs that showed the same secondary structure

3.3. Homology modeling, model quality evaluation

Homology modeling was used to determine the 3D structure of VP2/TLR7 peptides. SWISSMODEL search with default parameters was performed to find suitable templates for homology modeling. The constructs was modeled with 99.7% identity between query and template protein sequence (PDB entry 1wcd.1.A) by the single highest scoring template. To select the best chimera protein model, the generated 3D VP2/TLR7 models were compared together. The models were qualifies by model geometry RMSD, energy minimization, and the Ramachandran plot. The similar RMSD values of 3.26, 3.42 and 3.47 were estimated for these constructs. The predicted VP2/TLR7 models were subjected to energy

minimization by implementation of the Swiss-PDB viewer ranged from -18313.07 for VP2/TLR7-1 to -19253.953 kcal/mol for VP2/TLR7-8. Analysis of the results was indicated that the constructs had acceptable stability. The predicted structure was further validated for its reliability and structural quality based on the Ramachandran plot. The plots for the three VP2/TLR7 peptides were generated to assess the quality of the structures built using homology modeling. The plots showed φ and ψ torsion angles for all residues in these models and clustered around secondary structure regions that define the backbone conformation. Results of RAMPAGE showed 92.2% residues of the VP2/TLR7 constructs are located in the favored region, 5.3% in the allowed and only 2.5% in the outlier regions (Figure 2).

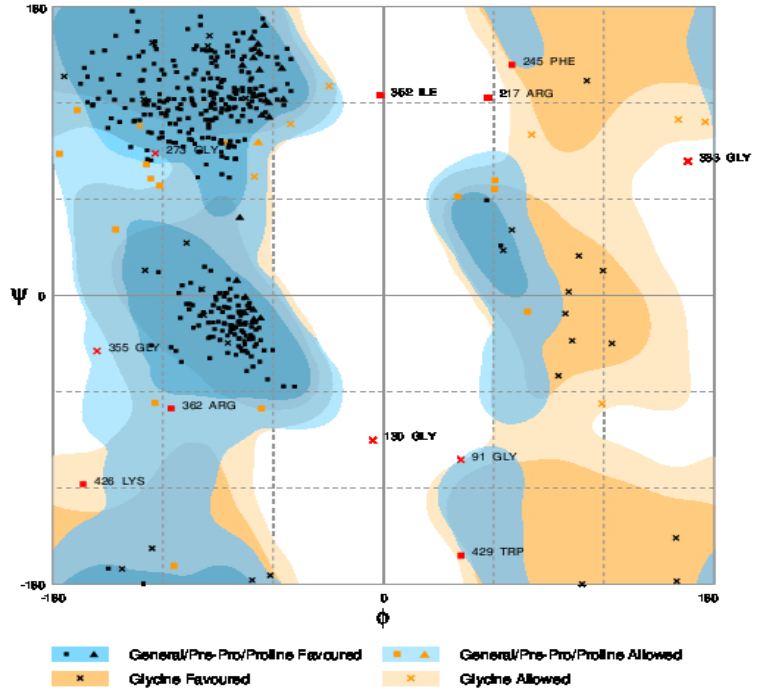

Figure 2. Evaluation of the VP2/TLR7 model quality by Ramachandran plot. 92.2% residues of all VP2/TLR7 constructs are located in the favored region, 5.3% in the allowed and 2.5% in the outlier regions. The φ and ψ torsion angles of amino acid residues in the proteins were reasonably accurate

The overall quality of these models was validated for structure prediction and modeling to recognize errors in 3D structures of proteins. The ProSA Z-scores were obtained -7.8 for these constructs and -7.21 for the template's 3D structure (Figure 3).

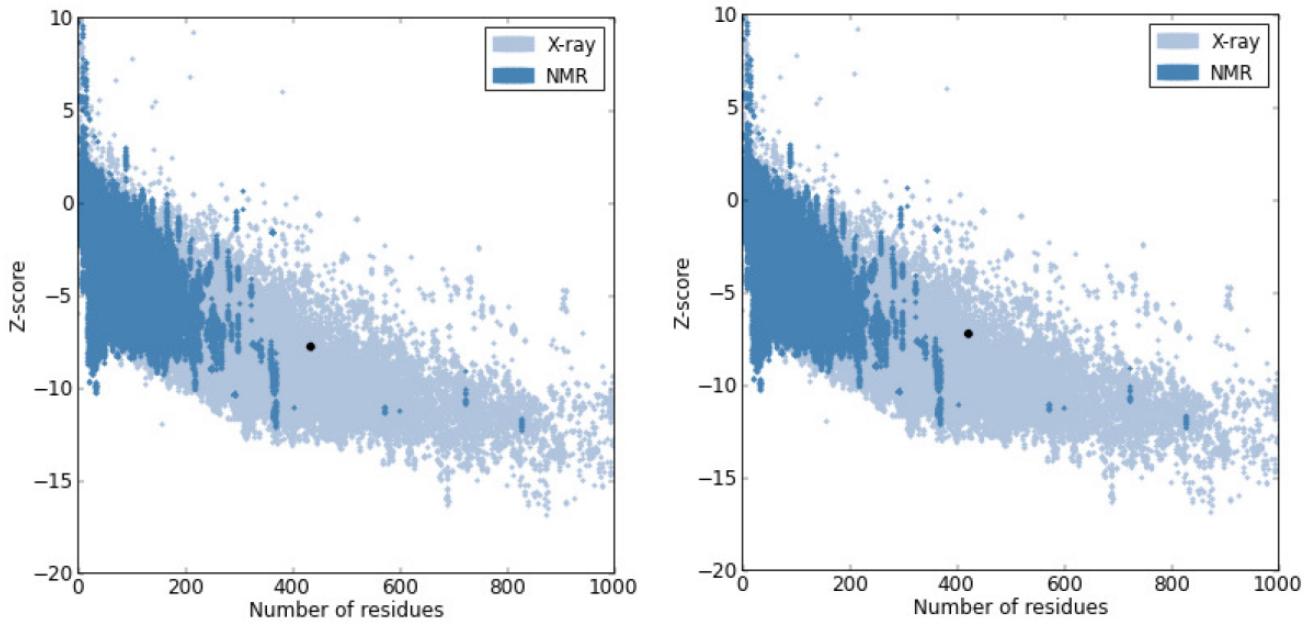

Figure 3. Z-score were obtained -7.8 for the VP2/TLR7 proteins (right) and -7.21 for the template's 3D structure (left) by ProSA model quality

3.4. Prediction of post-translational modifications

Three N-glycosylation sites at positions 61 (NLTV), 136 (NGTI), and 411 (NYTK) were determined in all of the VP2/TLR7 chimera proteins which were similar to the VP2 peptide of IBD viruses. One additional N-glycosylation site at position 1 (NLSQ) was only found in VP2/TLR7-7.

3.5. Antigenicity evaluation

The ultimate results of the potential B cell epitopes of these peptides with antigenic propensity score were shown in Table 3. In contract to TLR7 motifs located in lucine-rich repeat domain, an additional potentiated B cell epitope was predicted for motifs in TIR domain.

Table 3. Prediction of linear B- cell epitopes in VP2/TLR7 chimera peptides

Sequence	B-cell epitope
TLR7-1	QQIVPFIRSLLM
TLR7-2	QQIVPFIRSLLM
TLR7-3	IKIAEA+QQIVPFIRSLLM
TLR7-4	LKCLNL+QQIVPFIRSLLM
TLR7-5	QQIVPFIRSLLM
TLR7-6	PGQPVEAA+QQIVPFIRSLLM
TLR7-7	QSIQLS+QQIVPFIRSLLM
TLR7-8	IILIFLEKE+QQIVPFIRSLLM
VP2	QQIVPFIRSLLM

For VP2/TLR7-1, -2, and -5 constructs, the average antigenic propensities were calculated 0.892 as the same as VP2 protein. The predicted antigenic potentiated peptide of the construct was started by QQIVPFIRSLLMP sequence related to the VP2 protein but not TLR motif. The VP2/TLR7-3 and -4 showed the relative high average antigenic propensity of 0.91. The residue with average antigenic above 1.0 is potentially antigenic in Kolaskar and Tongaonkar (Figure 4). According to the algorithm the VP2/TLRs may not be elicit an antibody response, while the three motifs in TIR domain (TLR7-6, -7, -8) with higher average antigenic propensity 1.197 may potentiate immune response induction. Estimation of the solvent accessible scale was shown that the chimera peptides are hydrophobic and contains segments of low complexity and high-predicted flexibility. The vaccine-formulated adjuvant can be greatly modulated the T helper responses. NetCTL tool was used to identify the T cell epitope of VP2/TLR7 chimera peptides and no differences shown among the constructs.

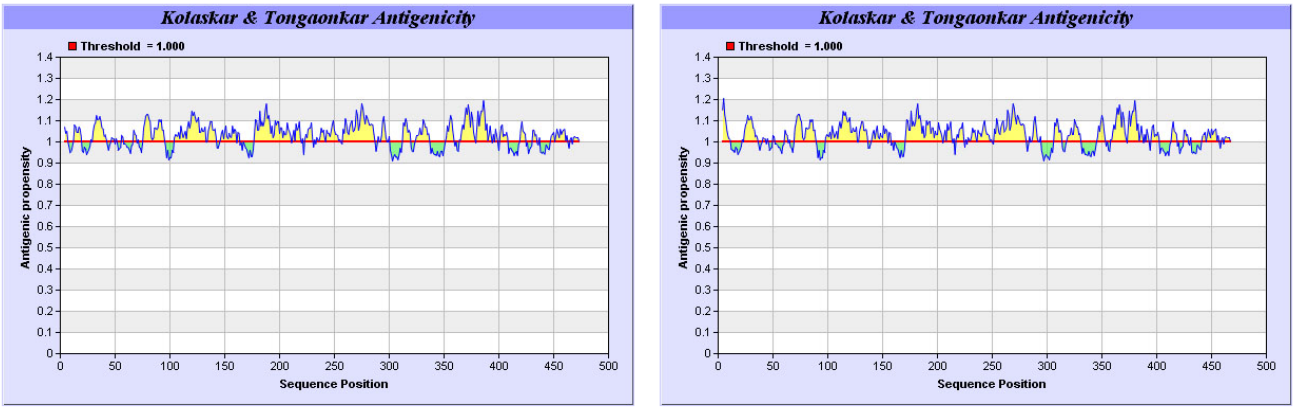

Figure 4. Antigenicity prediction plot of VP2/TLR7 chimera peptides using Kolaskar-Tongaonkar method. Regions with antigenic propensity scale more than 1 are predicted as antigenic regions. According to the algorithm the VP2/TLRs may not be elicit an antibody response, while The motifs in TIR domain (TLR7-6, -7, -8) showed higher average antigenic propensity (right) may potentiate immune response induction compared to the other constructs (left)

Vaccination is currently the most effective strategy to control of IBD. The inactivated and attenuated vaccines may associated with poor efficiency and emergence of new IBDV variants (4). Because of these limitations, developing new VP2 recombinant subunit vaccines using various prokaryotic and eukaryotic expression vectors (14-22) are targeted by vaccine researchers (23, 24). Since the previous studies on the development of a recombinant vaccine focus on expression strategy, their practical use still needs to be evaluated, particularly with regard to factors such as adjuvant, safety, efficacy, delivery route, and dosage. The present study was aimed at designing an intermediate IBDV vaccine which is modeled based on VP2 protein and TLR7 motif as bio adjuvant. The strategy involved three steps including *in silico* modeling analysis of the constructs to introduce an ideal vaccine, construction of the DNA VP2 vaccine and TLR7 molecular adjuvant, and evaluation of their impact on induction of immune responses against IBDV in chickens. For developing DNA VP2 vaccine we candidate an intermediate IBDV strain. Protection against the virus infection is highly dependent on the antigenic correlation between the circulating virus strains and the vaccine strain (25). Here the *in silico* analysis was discussed to support the probability of those

TLR7 motifs can be potentiated for developing a vaccine capable of stimulating effective immune response against IBDV. As shown in table 2 the eight chimera constructs were slightly differing in physicochemical properties. Negative GRAVY and positive GRAVY values indicate hydrophilic and hydrophobic proteins. As the score became more negative, this meant that the protein was more hydrophilic. The residues in TIR domain (TLR7-6, -7, -8) had negative indices, which indicate the protein became more stable. The secondary structure prediction disclosed the presence of beta strand for VP2/TLR7-8 construct in compared to other constructs. However, there is no direct correlation between stability and percentage of the strand and α-helix secondary structure the highly β-sheet proteins frequently are problematic upon unfolding. The possible function of VP2/TLR7 was determined by subjecting the sequences to comparative protein structure modelling. SWISSMODEL was used for building the models and global energy minimization. The topological similarity of the peptide structures was assessed by RMSD. The calculated RMSD values for the predicted models were not different. It has been suggested that a model of an RMSD of around 4Å is useful for designing drug and for performing small ligand docking predictions (26). Because the fold of these models was expected to be correct, function of the proteins analyzed by PROCHECK for the evaluation of the Ramachandran plot quality. According to the plot for all of the constructs, residues in most favored regions, residues in allowed regions, and residues in disallowed regions were respectively, 92.2%, 5.3%, and 2.5%. The Ramachandran plot of φ and ψ torsional angles distribution in the models is developed using PROCHECK for checking non-glycin residues at the disallowed regions. The angles of amino acid residues in the proteins were reasonably accurate that ensure the geometrically acceptable quality of these models. ProSA-web was used to validate the model by analyzing the energy distribution in protein structure. Based on the Z-scores, these models are of good quality of structure. The ultimate results of the potential B cell epitopes of VP2/TLR7 peptides were shown in table 3. Similar B cell epitopes started with QQIVPFIRSLM were predicted for VP2 protein and VP2/TLR7-1, -2, and -5 constructs. While other motifs had an additional potentiated epitope indicate the possible function of the peptides to elicit immune response. Figure 5 shows the antigenic determinant plot for HA2/Mx1; x-axis shows amino acid position and y-axis shows antigenic propensity score. Average antigenic using Kolaskar and Tongaonkar for VP2/TLR7-6, -7, and -8 chimera proteins is higher than VP2 (1.197 vs 0.892); all residues above 1.0 are potentially antigenic in the algorithm. The antigenic index directly showed the TLR7 motifs located in TIR domain may induce immune responses. On the other hand, estimation of the solvent accessible scale was shown that the chimera peptide is hydrophobic and contains segments of low complexity and high-predicted flexibility. Cumulative results from the previous studies have shown that VP2 protein is promising candidate for design of vaccine construct aimed at providing broad-spectrum immunity to IBDV. Protective potential of the neutralizing antigen could be increased by optimization of their delivery and immunogenicity using adjuvant. It has been demonstrated that the efficacy of the vaccines can be modulated by co-delivery of various cytokine genes (27). Some of the components have been proven to effective in immunoregulator, e.g., interleukins (IL) which play central role in the regulation of immune and inflammatory responses to infections. IL-1, IL-2, IL-6, and IL-18 can significantly stimulate humoral immune response to IBDV (28, 29).

4. CONCLUSION

Our *in silico* data analysis revealed that TLR7 motifs are strong immunoregulator of the VP2 IBDV vaccine and enhance the specific antibody response especially the TLR7-6 that located in TIR domain defined for the IL-1 receptor family. To the knowledge, this is the first study in which the stimulatory effects of TLR on humoral and cellular immune responses of chickens. The immunoregulatory effects of the TLR7 motif on T and B cells *in vivo*, and its feasibility as an adjuvant for IBDV vaccine needs to be investigated in future study.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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