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Designing a multi-epitope vaccine for cross-protection against *Shigella* spp: An immunoinformatics and structural vaccinology study

Mokhtar Nosrati[a], Abbas Hajizade[b,*], Shahram Nazarian[b], Jafar Amani[c], Amir Namvar Vansofla[b], Yousof Tarverdizadeh[b]

[a] Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan, Iran
[b] Biology Research Center, Faculty of Basic Sciences, Imam Hossein University, Tehran, Iran
[c] Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

Shigellosis is a severe diarrheal disease with high mortality and morbidity rate. Until now, there is no approved vaccine against the disease. Therefore, the present study was planned to design a novel multi-epitope vaccine against *Shigella* spp., the causative agents of the disease based on the immunoinformatic tools. For this end, firstly seven conserved antigens of the bacteria, including IpaA, IpaB, IpaC, IpaD, OmpC, OmpF and VirG were selected. Then, linear B-cell epitope mapping of these proteins was carried out and top-ranked and shared epitopes were selected based on antigenicity, allergenicity, stability, toxicity and physicochemical properties for further analysis. In next step, B-cell derived T-cell epitopes were determined and appropriate epitopes were selected for incorporation into the final construct. Moreover, the selected epitopes and two mucosal adjuvants including ctxB and LT-IIc were joined using appropriate linkers. The three dimensional structure of the final construct was modeled and evaluated in term of structural quality and presence of conformational B-cell epitopes. Furthermore, binding affinity of the proposed vaccine to MHC I and II molecules were evaluated through molecular docking method using Hex 8.0. as well as the stability of the vaccine-MHC complexes was monitored by molecular dynamics method using the NAMD graphical user interface embedded in visual molecular dynamics. Finally, to evaluate the immunogenicity of the designed protein, the protein was administered to BALB/c mice and the serum IgG was determined by ELISA. The results indicated that the proposed vaccine has high structural quality and binding affinity to both MHC I and II molecules. Moreover, molecular dynamics studies confirmed that the vaccine-MHC docked complexes were stable during simulation time. Animal study showed that the proposed protein is able to evoke mice's humoral immune response. In sum, the results suggested that the proposed candidate vaccine could be considered as a promising anti-shigellosis vaccine.

1. Introduction

Shigellosis is an intestinal infectious disease caused by the gram negative bacteria of *Shigella* spp. Despite the great efforts made in the field of prevention, diagnosis and treatment of shigellosis, the disease is still a significant public health problem, especially in developing countries. It is estimated that approximately 1.1 million deaths occur due to *Shigella* infection annually (Schroeder and Hilbi, 2008). The genus *Shigella* has 4 species or subgroups (A, B, C, and D) and 43 serotypes that among them *S. dysenteriae, S. flexneri, S. boydii and S. sonnei* are able to cause the disease in human (Niyogi, 2005). Symptoms of shigellosis typically start 1–2 days after exposure and include: diarrhea, abdominal pain, fever and tenesmus. Shigella transmission can occur through direct person-to-person contact or via the contaminated food and water (Bennish, 1991). Depending on the severity of the disease, patient's condition, age and gender, various strategies are used for the treatment of shigellosis, from antibiotic therapy for the severe cases to consumption of fluids and rest in moderate ones (Steffen, 1990). Although, antibiotic therapy may be useful for severe cases of shigellosis and can reduce the duration of symptoms, but there are increasing antibiotic resistance among Shigella spp. so that antibiotic treatment frequently fails (Murray et al., 2017). Due to wide spread antibiotic resistance as well as costly, time-consuming and low effectiveness of antibiotic therapy, many efforts have been done to introduce effective vaccine against *Shigella* spp. (Kaminski and Oaks, 2009; Levine et al., 2007; Hajizade et al., 2016). However, despite many efforts on the

* Corresponding author.
E-mail address: abbashajizade@gmail.com (A. Hajizade).

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development of effective vaccines against shigellosis, there is not any approved vaccine for the disease, yet. Indeed, most introduced *Shigella* candidate vaccines are not able to protect against all species (Mani et al., 2016).

Developing an efficient vaccine against an infectious disease needs some basic considerations about life cycle and important virulence factors of the causing agent. Essential virulence factors for *Shigella* invasion and intracellular survival are encoded by the large virulence plasmid. Moreover, the proteins involved in type III secretion system (T3SA) have crucial roles in *Shigella* pathogenesis. In Shigella spp., T3SA is encoded by approximately 20 genes located in the mxi and spa operons on the virulence plasmid. This plasmid composed of a mosaic of around 100 genes and encoding important virulence factors for *Shigella* invasion to host cells (Celli and Finlay, 2002; Jiang et al., 2005). One 30-kb block of the plasmid containes several genes at the ipa/mxi-spa locus. This locus is responsible for encoding of Ipa proteins (IpaB, IpaC and IpaD) which are essential factors for epithelial entry of the pathogen by macropinocytosis, activation of neutrophils and programmed macrophage death (Bartoleschi et al., 2002). The outer membrane proteins (OMP) are another group of *Shigella* spp. virulence factors which can elicit immunological responses and, for this reason, currently are considered as a component of cell-free vaccines against *Shigella* spp. (Pore et al., 2011; Guan et al., 2015). Among OMPs, OmpA, C and F have high conservancy between different species of *Shigella*, so these proteins are proper targets to be included in candidate vaccines against the bacteria. The VirG (IcsA) protein is another virulence factor that plays a key role in *Shigella pathogenesis*. This protein is an outer membrane protein which is required for recruitment of host actin filament (F-actin) by intracellularly motile bacteria (Wang et al., 2014).

Because of some downsides of the first generations of vaccines, such as low stability, the risk of regaining the pathogenesis potential, introducing many antigens to the immune system, etc. in the recent years many efforts have been focused on developing recombinant vaccines, which are safe, easily engineerable, and easily producible (Nascimento and Leite, 2012). Multi-epitope vaccines (MEV) are a class of recombinant vaccines which have attracted many interests because of their high specificity, safety, stability and low-cost production; though, poor immunogenicity is considered as a main weakness of this type of vaccines (Suhrbier, 1997). In order to overcome this drawback, the use of natural adjuvants, such as toll-like receptors (TLRs) ligands, diphtheria toxin (DT), cholera toxin B-subunit (ctxB), and heat-labile enterotoxin B subunit (LTB) are associate with these vaccines (Nezafat et al., 2017; Bolhassani et al., 2017).

Laboratory evaluation of the efficiency of the proposed candidate epitopes is an expensive, tedious and laborious work. Hence, currently computational vaccine design has attracted enormous interest. Immunoinformatics or computational immunology includes the application of computational approaches to study the immunological processes and design of algorithms for finding B- and T-cell epitope mapping. Immunoinformatics design of multi-epitope vaccines has become the standard method for vaccine discovery due to more specificity, cost-effectiveness, potential and easy means of vaccine development against infectious diseases (Patronov and Doytchinova, 2013; De Groot et al., 2002).

The present study was conducted to design a novel multi-epitope vaccine against *Shigella* spp. based on immunoinformatics tools. For this end, firstly linear B-cell epitope mapping of seven conserved protein virulence factors including IpaA, IpaB, IpaC, IpaD, Omp C, OmpF and VirG was carried out and top-ranked and share epitopes were selected for more analysis. In the next step, B-cell derived T-cell epitopes were determined and appropriate epitopes were selected to be included in the final construct. Finally, selected epitopes and two mucosal adjuvants including ctxB and LT-IIc were joined using appropriate linkers and evaluated in term of structural quality and binding affinity to MHC I and II molecules.

Table 1
Selected antigens for incorporating into the candidate vaccine.

Protein	Specie	Accession number
IpaA	*Shigella flexneri*	P18010
	Shigella dysenteriae	B3 × 7C6
	Shigella sonnei	Q3YTQ5
	Shigella boydii	B2TSX4
IpaB	*Shigella flexneri*	Q8KHS6
	Shigella dysenteriae	Q8KHV4
	Shigella sonnei	Q8KXR7
	Shigella boydii	Q8KHR0
IpaC	*Shigella flexneri*	P18012
	Shigella dysenteriae	Q03946
	Shigella sonnei	A0A0I0E452
	Shigella boydii	B2TSX6
IpaD	*Shigella flexneri*	P18013
	Shigella dysenteriae	Q03947
	Shigella sonnei	Q3YTQ4
	Shigella boydii	B2TSX5
OmpA	*Shigella flexneri*	I6D072
	Shigella dysenteriae	A0A1S9K6I7
	Shigella sonnei	A0A0I1ING4
	Shigella boydii	A0A1S9JG60
OmpC	*Shigella flexneri*	I6CLP4
	Shigella dysenteriae	I6G3S0
	Shigella sonnei	A0A0I0YH91
	Shigella boydii	I6EP79
OmpF	*Shigella flexneri*	I6D1G6
	Shigella dysenteriae	B3 × 4T1
	Shigella sonnei	A0A0I2AZS2
	Shigella boydii	I6DCM4
VirG (IcsA)	*Shigella flexneri*	Q8KXH8
	Shigella dysenteriae	Q8KXH1
	Shigella sonnei	Q8KXG7
	Shigella boydii	Q8KHF1
ctxB	*Vibrio cholerae*	Q57193
LT-IIc	*Escherichia coli*	H6W8F2

2. Methodology

2.1. Retrieval amino acid sequences

Amino acid sequences of the most important virulence factors from different species of *Shigella*, including IpaA, IpaB, IpaC, IpaD, OmpA, OmpC, OmpF and VirG were retrieved from Uniprot database at (http://www.uniprot.org/), which are presented in Table 1.

2.2. Determination of conservancy and antigenic region

In order to determine conserved regions of the proteins among *Shigella* spp., multiple sequence alignment method was performed using Clustal Omega at (https://www.ebi.ac.uk/Tools/msa/clustalo/). Furthermore, antigenic regions of the proteins were predicted using PAP server at (http://imed.med.ucm.es/Tools/antigenic.pl). This server predicts the antigenic areas of a protein based on the occurrence of amino acid residues in experimentally known segmental epitopes.

2.3. Linear B-cell epitope prediction

Linear B cell epitope prediction was carried out using BCPREDS server at (http://ailab.ist.psu.edu/bcpred/predict.html) with fixed length epitope prediction method and specificity of 80%. The server predicts linear B-cell epitopes by using SVM combined with subsequence kernel (SSK) attitude with an accuracy of 74.57%. Furthermore, for cross-validation of the predicted epitopes, the proteins were checked through BepiPred-2.0 at (http://www.cbs.dtu.dk/services/BepiPred/), SVMTrip at (http://sysbio.unl.edu/SVMTriP/

Table 2
Used bioinformatics tools for linear B-cell screening.

Tool	Short description	References
VaxiJen	A web based tool for prediction antigenicity of a protein	[54]
AlgPred	Predictor tool for determination of allergenicity of a protein	[55]
Toxin pred	Prediction of the toxic regions in a protein sequence	[56]
PepCalc	A online tool for prediction of protein solubility from its sequence	[57]
IEDB	A Immunoinformatics tool for prediction of the population coverage of a epitope	[58]

prediction.php) and LBtope at (http://crdd.osdd.net/raghava/lbtope). Finally, high-ranked and shared B-cell epitopes were selected for further study.

2.4. B-cell epitope selection

In order to design a cross-protective vaccine, the high-ranked and shared B-cell epitopes which were located in the conserved and antigenic regions were further evaluated in term of antigenicity, allergenicity, toxicity and solubility using a set of bioinformatics tools that presented in Table 2. Finally, the epitopes with appropriate properties were selected for more study.

2.5. T-cell epitopes prediction and epitope selection

The screened B-cell epitopes were further investigated to identify T-cell epitopes. The MHC-I restricted epitopes were predicted using Propred1 at (http://crdd.osdd.net/raghava/propred1/) with defaults parameters for 47 MHC-I alleles. Moreover, the MHC-II restricted epitopes were predicted using Propred server at (http://osddlinux.osdd.net/tmp/propred/) with 3% threshold for 51 MHC-II alleles. Subsequently, effective T-cell epitopes were selected based on the ability to bind to most alleles of both MHC-I and MHC-II, highest antigenicity in VaxiJen, highest score in VirulentPred and least IC50 in MHCpred. Finally, the B-cell epitopes containing effective T-cell epitopes were selected for designing the candidate vaccine.

2.6. Vaccine design and physicochemical properties

The screened epitopes based on the results of the previous sections were selected to design a chimeric structure as a multi-epitope candidate vaccine. For this aim, the screened B-cell epitopes, containing T-cell epitopes, were joined by KK linker. The linker (KK) is a common spacer in antigen engineering researches and an important proteasome for MHC-II-restricted antigen processing as well as simultaneously improved proteasome processing. In the next step, final candidate vaccine was built trough fusing two mucosal adjuvant including ctxB at the C-termius and heat labile enterotoxin IIc B subunit (LT-IIc) at N-terminus to the merged epitopes using GPGPGPG linker. The linker can be considered as independent flexible units and do not affect the function of the individual domains to which they attach. Furthermore, amino acid composition and some physicochemical properties of constructed protein including molecular weight, iso-electric point, net charge at pH 7, estimated solubility in water, estimated half-life in the mammalian reticulocytes, instability index and water solubility were determined using Protparam at (http://web.expasy.org/cgi-bin/protparam/protparam) and Pepcalc at (http://pepcalc.com/). Moreover, due to vital role of solvent accessibility of the epitopes for inducing of immune responses; this parameter was evaluated for the final selected linear B-cell epitopes using SABLE server at (https://sable.cchmc.org/) with wApproximator predictor type. In the used tool; solvent accessibility of each amino acid residues is presented with a number ranges from 0 to 9; with 0 corresponding to fully buried and 9 corresponding to fully exposed residue respectively.

2.7. Secondary and 3D structure prediction

Secondary structure of the constructed protein was predicted by Prabi server at (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_gor4.pl) with GOR4 secondary structure prediction method. Also 3D structure of the chimeric protein was generated using the Swiss model server at: (https://swissmodel.expasy.org) followed by optimization with Discovery Studio (Accelrys) software.

2.8. Model assessment

The geometry quality of constructed protein was validated using Ramachandran analysis at (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) and Z-score of ProSA server at (https://prosa.services.came.sbg.ac.at/prosa.php) as well as ERRAT quality factor (http://services.mbi.ucla.edu/ERRAT/).

2.9. Conformational B-cell epitope prediction

The conformational B-cell epitopes in the vaccine model were predicted using ElliPro server at (http://tools.iedb.org/ellipro/) with following epitope prediction parameters: minimum score 0.5 and maximum distance 7 (angstrom).

2.10. Molecular docking study

In order to validate the binding affinity of the vaccine to HLA-I and HLA-II molecules, molecular docking was performed between the proposed candidate vaccine (which composed by nine linear B-cell epitopes containing T-cell epitopes fused with ctxB and LT-IIc) and the most common binding alleles in the human population, including HLA-B51:01 with PDB entry of 1E27, and HLA-DRB1_01:01 with PDB entry of 1AQD. Protein-protein molecular docking study is a key tool in structural molecular biology to understand protein-protein interactions for the rational drug and vaccine design. Molecular docking studies were carried out using HEX 6.0 software. The selected parameters for docking study were FFT Mode – 3D fast life, Distance Range – 40, Twist range – 360, Correlation type – Shape only, Grid Dimension – 0.6, Receptor range – 180 and Ligand Range – 180. Finally the obtained complexes from molecular docking were further analyzed for determination of the interactions type and active residues using Protein Interactions Calculator (PIC) at (http://pic.mbu.iisc.ernet.in/).

2.11. Molecular dynamics (MD) simulation

Molecular dynamic is a computational approach which is used to describe properties of the molecules' behavior, ligand-receptor interactions, solvation of molecules, stability of molecules and fluctuations as well as conformational changes of molecules. In the present study, MD simulation was performed on the molecular docking outputs as a validation approach. For this, the stability of achieved complexes between the vaccine (epitopes-adjuvants) with HLA alleles was investigated using NAMD graphical interface module incorporated visual molecular dynamics (VMD) during 10 ns. The protein structure file (PSF) of the complexes was built using automatic PSF generator module in VMD. MD simulations were done under NPT equivalent conditions at

1 bar and 300 K and using accessing PSF and PDB files, DCD trajectory files were generated by NAMD. Finally, the MD simulations results were analyzed based on root mean square deviation (RMSD) and Radius of gyration (Rg).

2.12. Revers translation, codon optimization and in silico cloning

Toward clone and express the proposed candidate vaccine in a suitable expression vector, the amino acid sequence of vaccine model was backtranslated into nucleotide sequences using Gene infinity server at (http://www.geneinfinity.org/sms/sms_backtranslation.html) based on codon usage table of *Escherichia coli* K12. The optimized DNA was further evaluated in term of Codon Adaptation Index (CAI), GC content and Codon Frequency Distribution (CFD) using GenScript Tool at (https://www.genscript.com/tools/rare-codon-analysis). These parameters have key roles in optimized protein expression in the host. In latest step, restriction sites in the DNA were mapped using NEBcutter V2.0 at (http://nc2.neb.com/NEBcutter2/) then appropriate restriction sites were added to 5′ and 3′−OH of the optimized DNA.

2.13. Administeration of the designed protein to mice

In order to evaluate the immunogenicity of the designed protein, the gene was cloned in pET 32 (+), expressed in *E. coli* BL21 (DE3) expression system, purified by Ni-NTA column, and administered to BALB/c mice through subcutaneous rout. For immunization study, 12 female BALB/c mice (18–20 g) were devided into two groups: the test group which received the chimeric protein in combination with Freunds' adjuvant; and the control group which received PBS in combination of Freunds' adjuvant. Immunization was done in a 3-dose regimen by administration of 10 μg of the protein and blooding was performed one week after the second and third administrations. To perform ELISA, the same method as Hajizade et al. was exploited (Hajizade et al., 2018).

3. Results

3.1. Determination of conservancy and antigenic region

The results of multiple sequence alignments between different virulence factors of *Shigella* spp. confirmed the high level of conservancy between all the proteins among studied strains. Furthermore, the results showed that there are 21, 8, 10, 11, 11, 13 and 11 antigenic regions in IpaA, IpaB, IpaC, IpaD, OmpA, OmpC, OmpF and VirG amino acid sequences respectively.

3.2. Linear B-cell epitope prediction

B-cell epitope prediction is one of the important stages in epitope-based vaccine design. Hence, *in silico* identification of linear B-cell epitopes was performed using four servers to obtain conserve and high ranked epitopes. The identified linear B-cell epitopes which had high rank in BCpred and three other predictors are listed in Table 3. Based on the results a total of 22 linear epitopes of the proteins were predicted with 100% conservancy level among the *Shigella* spp. The results also showed that least and most epitopes were identified for IpaD and VirG, respectively.

3.3. B-cell epitope selection

In addition to high conservancy and possibility, an epitopes should have appropriate properties in term of antigenicity, allergenicity, solubility, and toxicity to be considered as a suitable epitope for vaccine design, hence, the predicted B-cell epitopes from previous section were evaluated in terms of mentioned parameters using Vaxijen, Algpred, PepCalc and Toxin pred, respectively (Table 4). The obtained results from VaxiJen showed that VirG and OmpA derived epitopes had most and least antigenicity. Prediction of potential allergenicity of the epitopes revealed that three epitopes from IpaA and IpaB have allergenic potential. Furthermore, investigation of toxicity potential of the predicted epitopes showed that two epitopes from OmpC and OmpF have cytotoxicity potential. Moreover, evaluation of solubility of the epitopes indicated that most of them have good solubility in water, especially the high ranked epitopes in term of antigenicity.

After evaluation of antigenicity, allergenicity, solubility, and toxicity of the predicted linear B-cell epitopes a total of 10 epitopes were selected for further studies (Table 5). The selected epitopes included two epitopes from IpaC and VirG but of the other proteins, only one epitope was selected.

Table 3
Results of linear B-cell epitope prediction. Most and least epitopes ware predicted in VirG and IpaD amino acid sequences.

Protein	Epitope sequence	Start position
IpaA	DGLPTPPEDMPDGGPTPGAN	272
	TTPPLHPEGVTSSNDNSSDT	428
	VYDNSYHENPENDAQSPTSQ	319
IpaB	TANNNINTTNAHSTSNILIP	46
	TNKITAWKSQQQARQQKNLE	99
	TRLSELAPDSPEKKKLRREE	163
IpaC	KQTQSSSETQKSQNYQQIAA	19
	AGNISTSGGRYASALEEEQ	289
IpaD	EELKEKYKDKPLYPANNTVS	200
OmpA	GAGAFGGYQVNPYVGFEMGY	57
	GWSQYHDTGFINNNGPTHEN	35
	ARGMGESNPVTGNTCDNVKQ	297
OmpC	PEFGGDTYGSDNFMQQRGNG	130
	QNGDGVGGSITYDYEGFGIG	193
	TDQLTGYGQWEYQIQGNTSE	68
OmpF	PEFGGDTYGSDNFMQQRGNG	130
	QNGDGVGGSITYDYEGFGIG	193
	TDQLTGYGQWEYQIQGNTSE	68
VirG	PEFGGDTAYSDDFFVGRVGG	138
	SKGNGENSYGGNGDMTYARL	46
	LGKNERDTARRSNGDGVGGS	180
	GETRNATPITNKFTNTSGFA	254

3.4. T-cell epitopes prediction and epitope selection

Due to the importance of T-cell epitopes in stimulating the immune system against microbial agents, the screened B-cell epitopes were subjected to determine MHC-I and MHC-II restricted epitopes. The results of T-cell epitope prediction are shown in Table 6. The results showed that most of the predicted T-cell epitopes, especially IpaA-, IpaB- and IpaC-derived epitopes have appropriate properties. Results also indicated that the IpaD-derived T-cell epitope has not suitable properties in terms of antigenicity and virulence score, so the epitope was rejected for being included in the final construct. Hence, a total of 9 linear B-cell epitopes containing potent T-cell epitopes were selected as final epitopes for vaccine design.

3.5. Vaccine design and physicochemical properties

The proposed vaccine including 9 linear B-cell epitopes and two mucosal adjuvants, which is depicted using the Illustrator for Biological Sequences (IBS) Ver 1.0 server, is shown in Fig. 1. The vaccine model is composed of 455 amino acids and molecular weight of 49.2 kDa. The theoretical pI, net charge and extinction coefficient of the protein were 9.44, 18.3 and 46,090 $M^{-1} cm^{-1}$, respectively. Furthermore, half-life was appraised to be 30 h in mammalian reticulocytes, more than 20 h in yeast and more than 10 h in *E. coli*. The vaccine model was predicted as

Table 4
Results of top-ranked linear B-cell epitopes screening.

Protein	Epitope sequence	VaxiJen score	Allergenicity	Toxicity	Solubility
IpaA	DGLPTPPEDMPDGGPTPGAN	0.51	No	No	Good
	TTPPLHPEGVTSSNDNSSDT	1.16	Yes	No	Good
	VYDNSYHENPENDAQSPTSQ	0.60	Yes	No	Good
IpaB	TANNNINTTNAHSTSNILIP	0.94	No	No	Poor
	TNKITAWKSQQQARQQKNLE	0.49	No	No	Good
	TRLSELAPDSPEKKKLRREE	0.72	Yes	No	Good
IpaC	KQTQSSSETQKSQNYQQIAA	1.31	No	No	Good
	AGNISTSGGRYASALEEEEQ	0.90	No	No	Good
IpaD	EELKEKYKDKPLYPANNTVS	0.44	No	No	Good
OmpA	GAGAFGGYQVNPYVGFEMGY	0.35	No	No	Poor
	GWSQYHDTGFINNNGPTHEN	−0.02	No	No	Poor
	ARGMGESNPVTGNTCDNVKQ	1.16	No	No	Good
OmpC	PEFGGDTYGSDNFMQQRGNG	1.16	No	No	Good
	QNGDGVGGSITYDYEGFGIG	1.74	No	No	Poor
	TDQLTGYGQWEYQIQGNTSE	0.87	No	Yes	Poor
OmpF	PEFGGDTYGSDNFMQQRGNG	1.16	No	No	Good
	QNGDGVGGSITYDYEGFGIG	1.74	No	No	Poor
	TDQLTGYGQWEYQIQGNTSE	0.87	No	Yes	Poor
VirG	PEFGGDTAYSDDFFVGRVGG	0.38	No	No	Good
	SKGNGENSYGGNGDMTYARL	2.03	No	No	Good
	LGKNERDTARRSNGDGVGGS	2.46	No	No	Good
	GETRNATPITNKFTNTSGFA	0.30	No	No	Good

a stable and water soluble protein with instability index of 34.39. The results of relative solvent accessibility of the selected epitopes in the proposed vaccine are summarized in Table 7. The obtained results demonstrated that all evaluated epitopes have mean confidence score more than 4.5 that represented appropriate solvent accessibility of them. Comparison between the tested epitopes showed that the epitopes from VirG and OmpC have most and least scores and these results maybe reflect the role of selected epitopes in inducing immune responses.

3.6. Secondary and 3D structure prediction

The secondary structure of the vaccine model is illustrated in Fig. 2. The results indicated that amino acid sequence of the proposed vaccine is organized in 30.55% helix, 15.38% extended strand and 54.07% random coil. Three dimensional structure of the vaccine model was predicted by Swiss model server. The best predicted model was selected based on local quality plot and Z-score. Local quality plot shows the similarity of the predicted model with the native structure based on the location of residues in the predicted model sequence. Normally, residues presenting a score below 0.6 are considered to be of low quality. Z-score provides an approximation of the grade of nativeness and indicates model quality in comparison to high-resolution crystal structures. The Local quality plot and Z-score diagram of predicted model are depicted in Fig. 3. The results showed that most areas of the model structure have a score of over 0.6 that indicating good quality of the model. Furthermore, the Z-score of the model was estimated in the range of 1–2, which indicates appropriate nativeness of the predicted model.

3.7. Model assessment

The primary 3D model of the proposed vaccine was further assessed for determining geometry quality using Ramachandran plot, ProSA *z*-score and ERRAT quality factor. As displayed in Fig. 4a, Ramachandran plot of the vaccine model indicated that 99% and 1% of residues were located in favored and allowed regions without residues in outlier region. Furthermore, the Z-score of the model was -5.54, which presented our structure is within the range of scores naturally found for native proteins of comparable size (Fig. 4b). Moreover, the ERRAT quality score was 100%, which indicated there is no reject region in the model sequence (Fig. 4c). Hence, based on the results of Ramachandran plot, ProSA *z*-score and ERRAT quality factor, it can be concluded that 3D model of the proposed vaccine was quite good and can thus be applied as a reliable model for further assessments.

3.8. Conformational B-cell epitope prediction

Conformational B-cell epitopes have an essential role in immune response to antigens and it has been appeared that more than 90% of B-cell epitopes are discontinuous B-cell epitopes. Therefore, the presence of conformational B-cell epitopes in a chimeric vaccine can improve its effectiveness. Consequently, the 3D model of proposed vaccine was subjected to determine conformational B-cell epitopes using ElliPro server. The results of conformational B-cell epitope prediction are shown in Table 8. The results showed that the all predicted epitopes are located in C-terminus domain which was related to ctxB mucosal adjutant and for merged linear B-cell epitopes as well as LT-IIc domain no conformational epitope was predicted.

Table 5
Selected linear B-cell epitopes for the candidate vaccine design.

Protein	Epitope	Protein	Epitope
IpaA	DGLPTPPEDMPDGGPTPGAN	OmpA	ARGMGESNPVTGNTCDNVKQ
IpaB	TNKITAWKSQQQARQQKNLE	OmpC	PEFGGDTYGSDNFMQQRGNG
IpaC	KQTQSSSETQKSQNYQQIAA	VirG	SKGNGENSYGGNGDMTYARL
	AGNISTSGGRYASALEEEEQ		LGKNERDTARRSNGDGVGGS
IpaD	EELKEKYKDKPLYPANNTVS	OmpF	PEFGGDTYGSDNFMQQRGNG

Table 6
T-cell epitope prediction from the selected linear B-cell epitopes. The selected linear B-cell epitope from IpaD don't have appropriate T-cell epitope.

Protein	Origin Epitope	T-cell epitope	Antigenicity	VirulentPred	IC50
IpaA	DGLPTPPEDMPDGGPTPGAN	MPDGGPTPG	1.24	1.06	106
IpaB	TNKITAWKSQQQARQQKNLE	WKSQQQARQ	1.61	1.06	7.35
IpaC	KQTQSSSETQKSQNYQQIAA	KQTQSSSET	1.94	1.06	24.32
	AGNISTSGGRYASALEEEEQ	ISTSGGRYA	1.96	1.07	65.46
IpaD	EELKEKYKDKPLYPANNTVS	LYPANNTVS	−0.04	1.05	34.83
OmpA	ARGMGESNPVTGNTCDNVKQ	VTGNTCDNV	0.91	1.06	17.91
OmpC	PEFGGDTYGSDNFMQQRGNG	YGSDNFMQQ	0.62	1.06	13.8
VirG	SKGNGENSYGGNGDMTYARL	YGGNGDMTY	2.67	1.06	127.06
	LGKNERDTARRSNGDGVGGS	LGKNERDTA	1.36	1.08	93.97
OmpF	PEFGGDTYGSDNFMQQRGNG	YGSDNFMQQ	0.62	1.06	13.18

Fig. 1. Schematic representation of the vaccine model structure. LT-llc and ctxB are mucosal adjuvants and E1-E9 are screened linear B-cell epitope. The yellow and black areas indicate the GPGPGPG and KK linkers, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 7
Results of conformational B-cell epitope prediction. Seven epitopes were predicted in the proposed vaccine amino acid sequence.

Epitope sequence	origin	Position in the final construct	Confidence level of S.A*
DGLPTPPEDMPDGGPTPGAN	IpaA	**129-149**	**4.5**
TNKITAWKSQQQARQQKNLE	IpaB	**152-172**	**5.5**
KQTQSSSETQKSQNYQQIAA	IpaC	**175-195**	**5**
AGNISTSGGRYASALEEEEQ	IpaC	**196-216**	**6.1**
EELKEKYKDKPLYPANNTVS	IpaD	**217-237**	**6**
ARGMGESNPVTGNTCDNVKQ	OmpA	**238-258**	**5.5**
PEFGGDTYGSDNFMQQRGNG	OmpC	**259-279**	**4.4**
SKGNGENSYGGNGDMTYARL	VirG	**280-300**	**7.5**
LGKNERDTARRSNGDGVGGS	VirG	**301-321**	**7**
PEFGGDTYGSDNFMQQRGNG	OmpF	**322-342**	**6.1**

3.9. Molecular docking study

The binding affinity of the proposed vaccine to HLA-I and HLA-II was evaluated by protein-protein molecular docking analysis using Hex software. If some information is known about the binding sites of target proteins (here is our proposed vaccine), it is often worthwhile to limit the range of the docking search to exclude from consideration relative orientations that don't involve the binding epitope(s). However, in this study due to de novo design of the vaccine, binding site was not defined and a blind molecular docking was performed. Furthermore, in protein-protein molecular docking studies performed by Hex, the center of each protein's binding epitope is initially located on the intermolecular axis; therefore, binding affinity of the vaccine to HLA alleles was interpreted by binding energies of predicted binding sites. The results revealed that the vaccine has strong interactions to both HLA-I and HLA-II (Fig. 5a, b) with total free energies of -523.86 and -681.28 kJ/mol, respectively. Furthermore, the analysis of occurred interactions between the obtained vaccine-HLA complexes indicated that the hydrophobic interactions are dominant forces in the vaccine-HLA interaction. Results also showed that the number of occurred hydrogen bonds between vaccine-HLA-II is significantly more than vaccine-HLA-I.

3.10. MD simulation

In order to determine the stability of the vaccine-HLA complexes these structures were subjected to molecular dynamics simulation. The study of proteins backbone RMSD changes with respect to the initial conformation can be considered as an analytical tool for study of protein conformation and structure stability as well as the stability of the protein-protein complexes. As depicted in Fig. 6a and b, the backbone RMSD of both vaccine-HLA-I and vaccine-HLA-II was increased progressively until 2 ns and then the system almost reached to the steady state. Indeed, vaccine and HLA molecules structures were changed to get the best conformation relative to each other during the MD simulation. Furthermore, the compactness of vaccine-HLA complexes was also investigated by monitoring radius of gyration of the complexes during MD simulation. The results showed that Rg values of the both vaccine-HLA complexes reduced significantly after 2 and 3 ns respectively (Fig. 7a, b), which indicated that these complexes gained a more dense and stable structures.

3.11. Revers translation, codon optimization and in silico cloning

The amino acid sequence of the proposed candidate vaccine was back translated into nucleotide sequence and then codon optimized. Furthermore, the key properties of the optimized gene (coding sequence of the vaccine) which have an essential role in achieving a high level of protein expression were also predicted. A CAI value of the optimized gene was 1, which considered ideal for expression in the desired expression organism. Moreover, the average GC content of the gene is 54.99% which is in optimal percentage range (30–70%), as well as CFD of the gene was 0% in *E. coli.* The obtained results indicate that the optimized gene is appropriate for cloning and expression. Finally, for cloning the gene in *E.coli*, the recognition sites of *Sal*I and *Xho*I restriction enzymes were added to 5′ and 3′ − OH of the optimized gene, respectively.

3.12. Mice IgG assay

The ability of the chimeric vaccine in stimulating the mice immune system was investigated through specific IgG assay (Fig. 8). As the figure shows, the chimeric protein was able to elicit the production of IgG antibody in treated mice ($P < 0.001$).

4. Discussion

Shigellosis is a severe and lethal diarrheal infection, responsible for more than1000,000 deaths per year, especially in developing countries (Von Seidlein et al., 2006). Because of the reduced effectiveness of antibiotic therapy, currently many efforts have been done to introduce effective vaccine against shigellosis (Coster et al., 1999; Wu et al., 2016; Pahil et al., 2017). However, no licensed vaccine is available yet.

Due to time and cost consuming of common vaccine development approaches, recently computational vaccinology is highly considered for vaccine design against different diseases, especially for infectious diseases (Nosrati et al., 2017). Identification of protective antigens as well as B- and T-cell epitope mapping with a high degree of accuracy

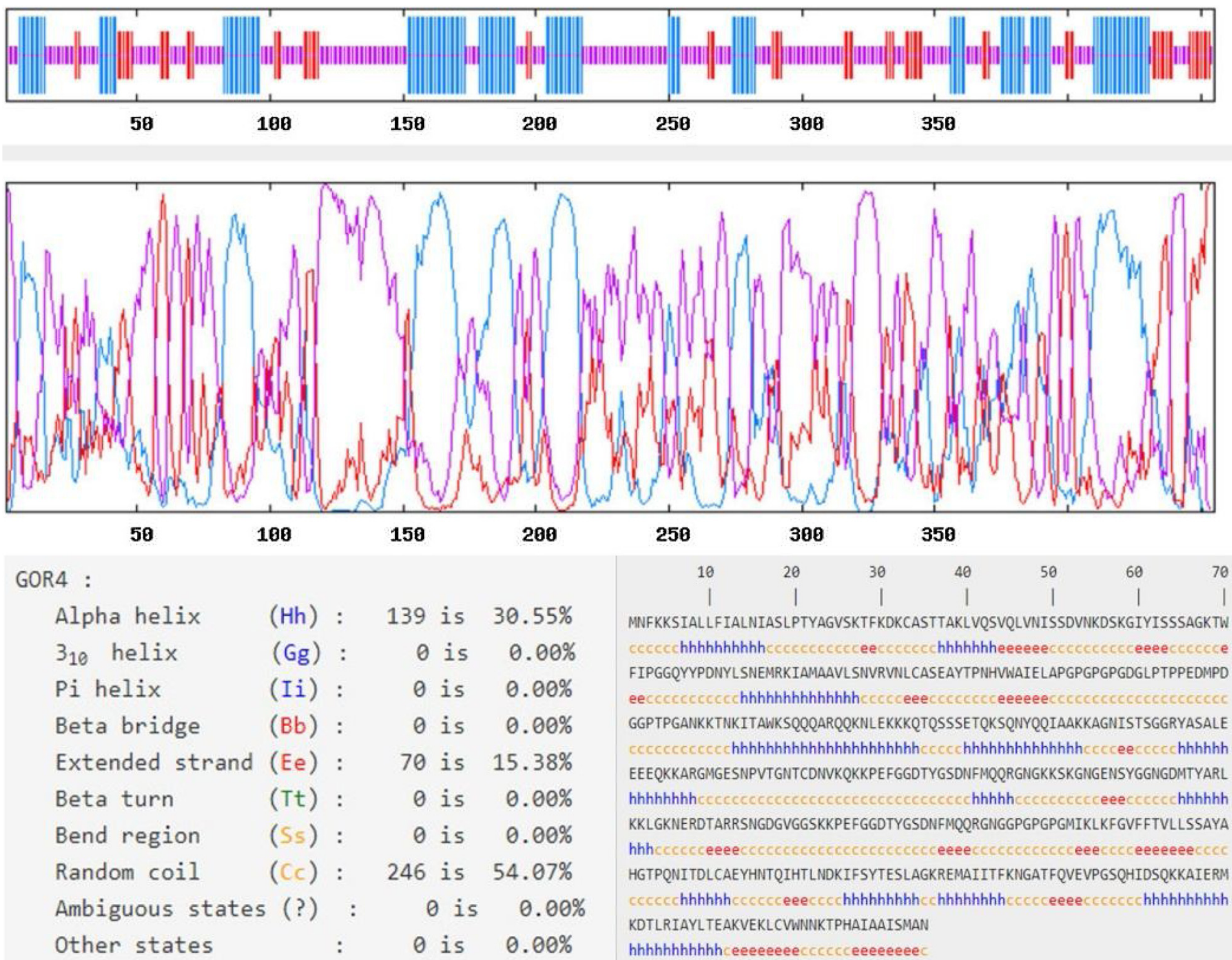

Fig. 2. Secondary structure of the proposed vaccine. Random coil and α-helix are dominant structures with 54 and 30.55% in the whole structure, respectively.

using immunoinformatics tools can promise to develop an effective and safe vaccine against diseases. Having advantages such as including several immunodominant epitopes in one molecule, exerting adjuvant effects and including conserved epitopes in multi-epitope vaccines, this area is considered as a high potential area for development of vaccines in recent years (Ahmad et al., 2016a, b).

Although the field of computational vaccinology is so young, however, numerous efficient vaccines, such as vaccines against *Rickettsia prowazekii* (Caro-Gomez et al., 2014) *, Streptococcus pneumoniae*, *Chlamydia pneumoniae* (Pourhajibagher and Bahador, 2016)*, Staphylococcus aureus* (Amani et al., 2014), Enterotoxigenic *Escherichia coli* (Mehla and Ramana, 2016) and many others have been developed based on immunoinformatics approaches.

Although, there are some previous reports about the design epitope-

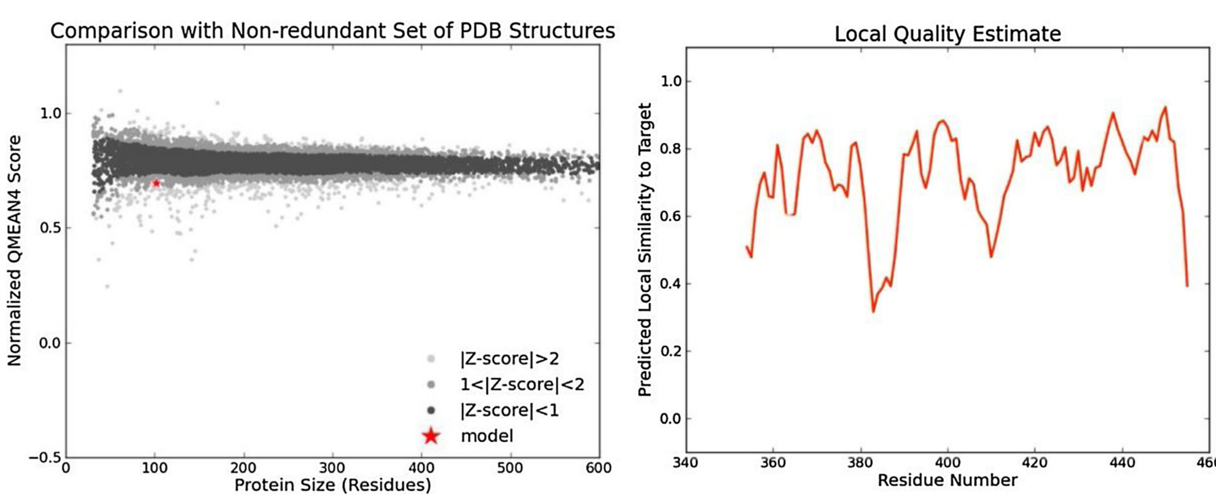

Fig. 3. Structural quality of the proposed vaccine. Z- score and local quality of the vaccine showed appropriate quality of the vaccine.

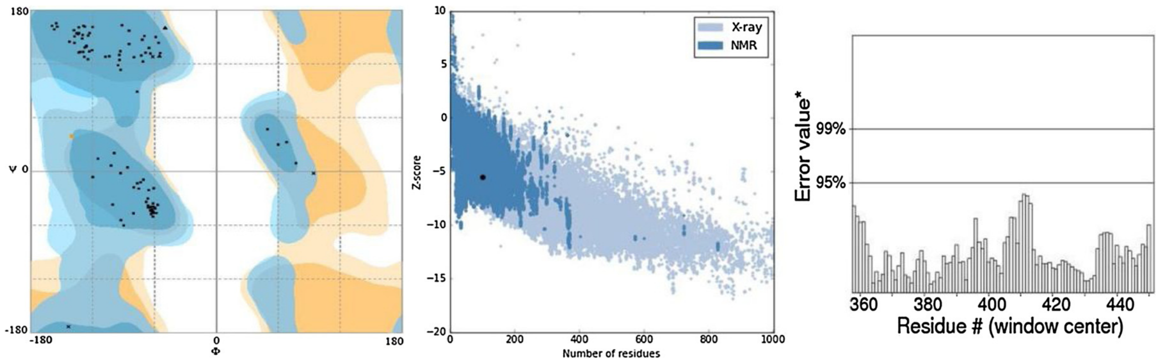

Fig. 4. The results of Structural quality of the proposed vaccine. Ramachandran plot (a), ProSA z-score (b) and ERRAT quality plot (c) indicated that the three dimensional structure of the vaccine has high quality.

Table 8
Results of conformational B-cell epitope prediction. Seven epitopes were predicted in the proposed vaccine amino acid sequence.

Number	Residues	Number of residues	Score
1	P354,Q355,N356,T358,D359	5	0.825
2	A362,E363,Y364	3	0.739
3	E381,E382,L383,A384,G385,K386,R387,E388,V404	9	0.736
4	P405,G406,S407,Q408,H409,I410,D411,S412,K414	9	0.712
5	M453,A454,N455	3	0.69
6	N373,D374,K375,I376,F394,K395,N396,G397,A398,R425,I426,Y428,L429,T430,E431,A432,K433,	17	0.612
7	H365,N366,N441,N442,K443,T444,P445	7	0.598

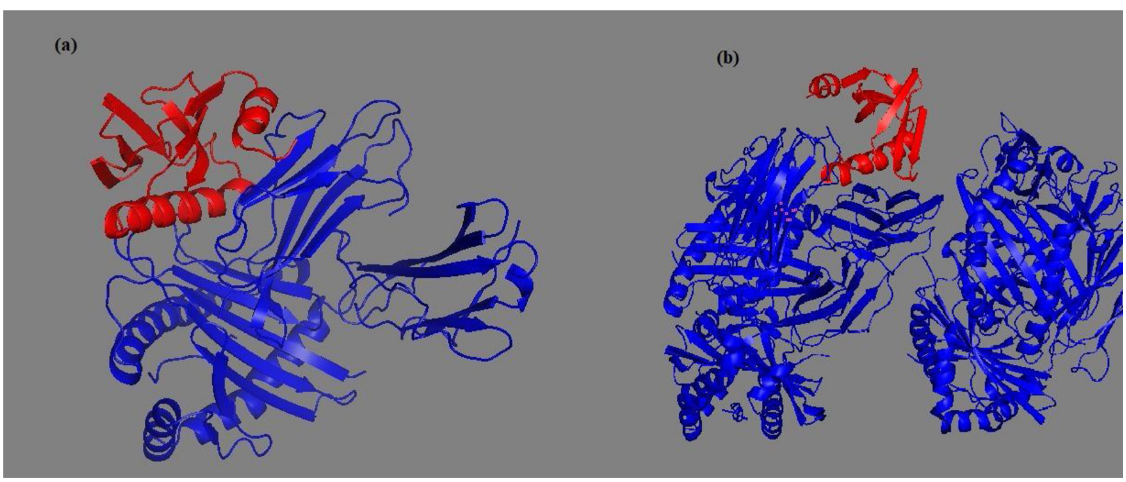

Fig. 5. Graphical representation of molecular docking studies between HLA-I-vaccine (a) and HLA-2-vaccine (b).

based vaccine against shigellosis, however, earlier proposed vaccines were designed against only a *Shigella* species. In the present study, we designed a multi-epitope vaccine candidate for cross-protection against four *Shigella* species, including *S. dysenteriae, S. flexneri, S. boydii and S. sonnei* using immunoinformatics and structural vaccinology strategies. As shown in Fig. 1, the candidate vaccine is composed of nine linear B-cell epitopes containing T-cell epitopes and two mucosal adjuvants, LT-IIc and ctxB which are joined to N- and C-terminal of the resulted protein by proper linkers.

In recent years, some efforts have been done to develop effective subunit, peptide and epitope-based vaccines against shigellosis, which are mainly, based on well-studied and characterized virulence factors such as Shiga toxin, outer membrane proteins, fimbrial proteins, membrane transporters and type III secretion system effector proteins. In this regard, Basser et al introduced a multi-epitope vaccine against *Shigella sonnei* which composed of putative B- and T-cell epitopes from outer membrane porin protein (Baseer et al., 2017). Jarzab et al. reported that epitope derived from Outer Membrane Protein C may serve as a component of a vaccine designed against *Shigella flexneri* 3a infections (Jarząb et al., 2013). Pahil et al. assessed five peptides which were conserved among multiple serotypes of Shigella spp. and reported that these peptides can be considered as immunogenic components for vaccine development against Shigellosis (Pahil et al., 2017). Sharma et al predicted some B- and T-cell epitopes of six OMPs of *Shigella flexneri* 2a as effective epitope for vaccine development against Shigellosis. In our proposed vaccine we selected some conserved protein virulence factors among *Shigella* spp., including four Ipa proteins, three OMPs and VirG for vaccine design (Sharma et al., 2016).

B- and T-cell epitope mapping of the defined protective antigens is a crucial step in multi-epitope vaccine development. Hence, in the present study, T-cell epitopes as well as B-cell epitopes of selected virulence factors were defined using a set of immunoinformatics tools. Consequently, nine high-ranked linear B-cell epitopes which contained T-cell epitopes were selected based on antigenicity, allergenicity, toxicity and solubility. After this, screened epitopes were incorporated into our vaccine construct. Similar to other enteropathogenic bacteria,

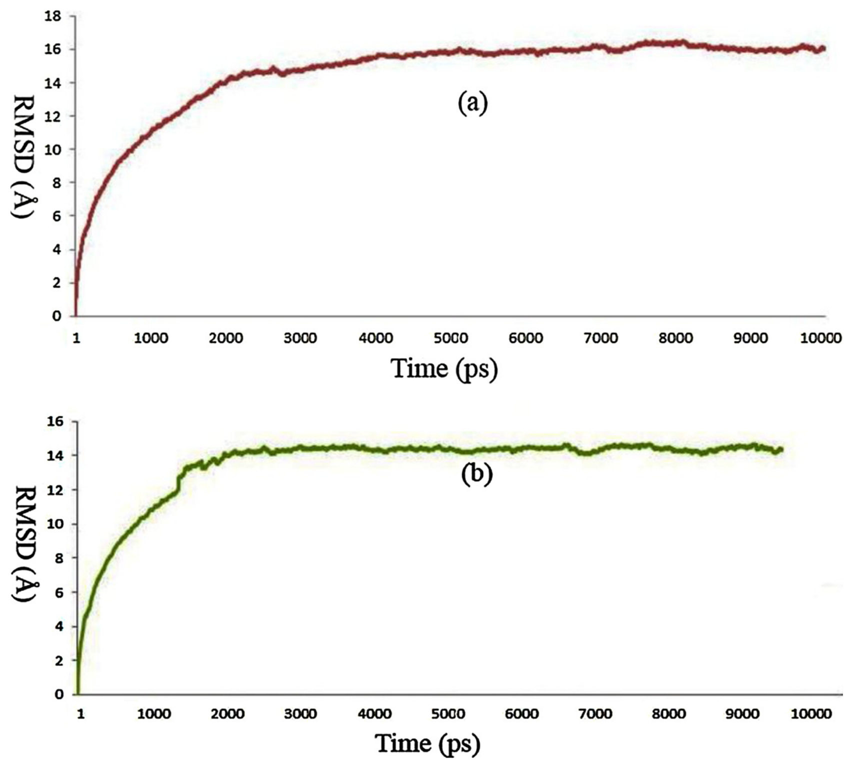

Fig. 6. RMSD plots of the vaccine-MHCI (a) and vaccine-MHC II backbones (b).

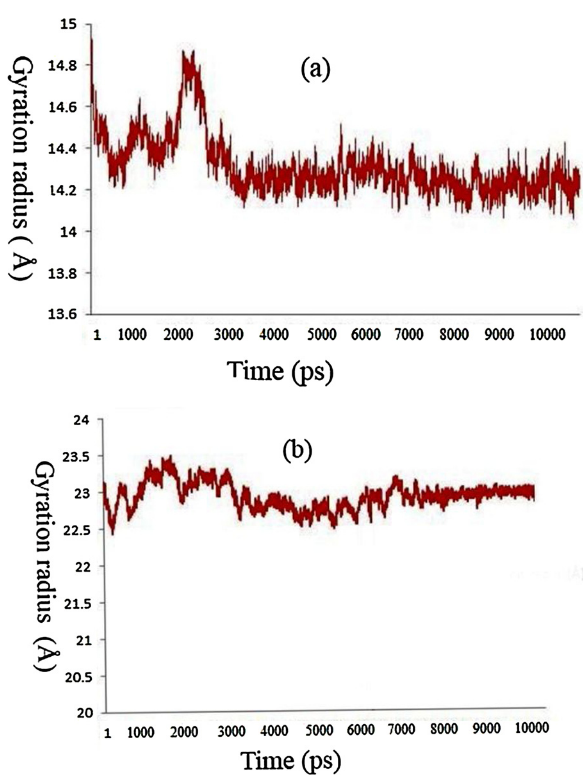

Fig. 7. Graph displaying the changes in Gyration radius of vaccine-MHC-II (a) and vaccine-MHC-I (B) complexes.

Shigella is spread at the human mucosal surface. Therefore, the mucosal routes (oral or intranasal) are desirable for the enterance of such infections. Due to the low immunogenicity of proteins, especially epitope-based vaccine, when administered mucosally, employment of robust mucosal protein adjuvant can notably increase their immunogenicity

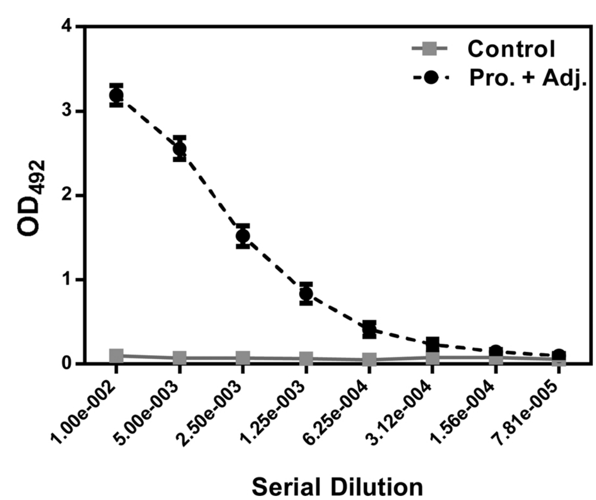

Fig. 8. Serum IgG response following the third immunization. Chimeric protein was coated in the wells and goat HRP-conjugated anti-mouse IgG was used as the secondary antibody.

(Nezafat et al., 2017). Therefore, in the proposed candidate vaccine we used two mucosal adjuvants, including LT-IIc and ctxB to improve the immunogenicity of the vaccine. These natural adjuvants were respectively added to N- and C-terminals of the candidate vaccine using appropriate linkers. The mentioned adjuvants are able to stimulate secretion of serum antigen specific immunoglobulins, especially IgG and IgA, as well as mucosal specific immunoglobulin, ie. secretory IgA (sIgA), therefore, they may improve the effectiveness of the vaccine. Furthermore, the selected epitopes were merged using KK linkers which prevents the formation of neo-epitope from epitope linkage sites as well as the linker is able to target the principal enzyme for MHC-II antigen presentation (Yano et al., 2005). Due to of linkers for joining the different parts of the vaccine, final construct the use was evaluated in term of structural and physicochemical properties as well as immunogenicity using different bioinformatics tools. The results revealed that the proposed vaccine is stable, water soluble, highly antigenic and non-allergenic.

From the practical point of view, to reach an optimum-level protein expression in *E. coli*, some important parameters, such as CAI, CFD, and GC content of the gene should be optimized. The results confirmed that our proposed gene could be expressed efficiently in *E.coli* host.

Three-dimension structure of a protein has key role in discontinuous (conformational) B cell-epitope identification and protein-protein and protein-small molecules interaction (Livingston et al., 2002). Therefore, 3D structure of designed vaccine was modeled using Swiss model server. Structure quality and stability of the predicted model were evaluated using Ramachandran plot, ProSA *z*-score, and ERRAT quality factor. The results indicated that the predicted model has high structure quality.

It is estimated that more than 90% of B-cell epitopes are conformational. These epitopes have a key role in immunity via stimulation of antibody production (Mirza et al., 2016). Hence, conformational epitope prediction was performed using ElliPro server. As shown in Table 7, seven conformational epitopes were predicted for the proposed vaccine which indicates the vaccine can elicit a strong humoral immunity against *Shigella* spp.

The ability of an antigen to binds to both classes of MHC molecules is essential for antigen processing and presentation by immune cells (Wieczorek et al., 2017; Mahdavi and Moreau, 2016). Therefore, binding affinity of the vaccine to MHC-II and I was evaluated using molecular docking method. The results indicated that the vaccine has high affinity to both MHC molecules, especially to MHC-II. Stability of the vaccine-MHC complexes was evaluated using MD method (Hospital et al., 2015). The obtained results indicated that the studied complexes have appropriate stability in tested time.

This study is first report on multi-epitope vaccine for cross-protection against *Shigella* spp. and obtained promising results for the development of a novel vaccine for prevent Shigellosis. On the other hand, though bioinformatics approaches have some advantages such as time and cost effectiveness, but due to some limitations such as database restrictions, low accuracy, considering interactions in ideal conditions and failure to simulate complex environments, such as blood (Mao et al., 2015; Rhee, 2005), in vitro and in vivo evaluations, it is required to confirm or reject the results. Hence, experimental studies are needed to confirm the effectiveness of the proposed vaccine.

Mice IgG assay showed that the proposed chimeric protein ia able to elicit the humoral immunity of the treated mice efficiently. It has not escaped our notice that more tests are needed to evaluate the protective effect of the chimeric protein in animal models; however, it demands a separate study.

5. Conclusion

The present study was planned to design a novel multi-epitope vaccine for cross-protection against *Shigella* spp. by using immunoinformatics tools and approaches. For this purpose, B- and T-cell epitope mapping of some main and previously-approved immunogens of the bacteria, including IpaA, IpaB, IpaC, IpaD, OmpC, OmpF and VirG was performed. Subsequently, epitope screening was performed based on antigenicity, allergenicity, conservancy and physicochemical properties. The nine top ranked and share epitopes and two mucosal adjuvants (ctxB and LT-IIc) were selected to be incorporated in the final construct. Finally, structural quality, binding affinity to MHC-I and II and vaccine-MHC complexes stability were evaluated. The obtained results indicated that proposed vaccine can elicit humoral and cellular immunity and is a proper candidate for *in vitro* and *in vivo* studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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