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Towards the first multi-epitope recombinant vaccine against Crimean-Congo hemorrhagic fever virus: A computer-aided vaccine design approach

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ABSTRACT

Crimean-Congo hemorrhagic fever (CCHF) is considered one of the major public health concerns with case fatality rates of up to 80%. Currently, there is no effective approved vaccine for CCHF. In this study, we used a computer-aided vaccine design approach to develop the first multi-epitope recombinant vaccine for CCHF. For this purpose, linear B-cell and T-cell binding epitopes from two structural glycoproteins of CCHF virus including Gc and Gn were predicted. The epitopes were further studied regarding their antigenicity, allergenicity, hydrophobicity, stability, toxicity and population coverage. A total number of seven epitopes including five T-cell and two B-cell epitopes were screened for the final vaccine construct. Final vaccine construct composed of 382 amino acid residues which were organized in four domains including linear B-cell, T-cell epitopes and cholera toxin B-subunit (CTxB) along with heat labile enterotoxin IIc B subunit (LT-IIc) as adjuvants. All the segments were joined using appropriate linkers. The physicochemical properties as well as the presence of IFN-γ inducing epitopes in the proposed vaccine, was also checked to determining the vaccine stability, solubility and its ability to induce cell-mediated immune responses. The 3D structure of proposed vaccine was subjected to the prediction of computational B-cell epitopes and molecular docking studies with MHC-I and II molecules. Furthermore, molecular dynamics stimulations were performed to study the vaccine-MHCs complexes stability during stimulation time. The results suggest that our proposed vaccine was stable, well soluble in water and potentially antigenic. Results also demonstrated that the vaccine can induce both humoral and cell-mediated immune responses and could serve as a promising anti-CCHF vaccine candidate.

1. Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a wide spread zoonotic viral disease with case fatality rates of up to 80% caused by CCHF virus (CCHFV) [1]. Recently, there is a drastic increase of CCHFV infection around the world, especially in Eastern Mediterranean region [2].

CCHFV is an enveloped single-stranded, ambisense sense RNA virus with a diameter of 90–100 nm belonging to the *Nairovirus* genus of the *Bunyaviridae* family, which includes five genera, and over 350 virus species. The virus genome organized in three segments including small (S), a medium (M), and a large (L) segment which encoding the nucleoprotein (N), glycoproteins (Gn and Gc), and the RNA-dependent RNA polymerase (RdRp) (L), respectively [3–5].

Unfortunately, until now there is no licensed vaccine or approved targeted therapies for prevention or treatment of CCHF, although supportive care and Ribavirin can help a lot to treat the disease. Despite high fatality rate, widespread distribution and lack of treatment and vaccine for CCHF, but there are limit studies about the disease [1,6]. Therefore, development of an effective vaccine against CCHFV is necessary. Generally, vaccine development is a complex, lengthy and accurate process that can be accelerated with detailed understanding of the molecular mechanism of diseases [7].

Designing an effective vaccine requires an adequate understanding of the structure and function of the immune system as well as the target pathogen. Generally, to initiate a specific immune response to an invasive pathogen, the immune system must be able to recognize infectious agent. After recognition, inducing adaptive immune responses needs antigen processing and presentation through Antigen Presenting Cells (APCs). Based on the origin of antigen there are two different pathways for antigen processing including cytosolic (for tumor and viral antigens) and endocytic (for exogenous antigens). Finally, the fragmented antigens expressed on the surface of APCs through specific glycoproteins named MHCs molecules. There are two different types of MHC molecules including MHC-I (MHC-Class I) and MHC-II (MHC-Class II). Simultaneous interactions between the MHC, processed antigen and T-Cell Receptor (TCR) trigger the cellular and humoral responses.

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Understanding of these processes can be very helpful for designing an effective vaccine [8,9]. Nowadays, due to the rapid development of molecular immunology and determination of the molecular mechanisms of pathogenesis, much research is aimed on vaccine design against different lethal diseases.

Recently, some efforts have been made to introduce a novel vaccine against the disease. In this regard, Garrison et al developed a DNA vaccine expressing the M-segment glycoprotein precursor gene of CCHFV with the ability to provoke strong humoral immune responses and prevention against CCHF and lethal in the studied animal model [10]. In another report, Dowall et al developed a recombinant candidate vaccine expressing the CCHFV nucleoprotein which was immunogenic but fails to create protection against the disease [11]. Kortekaas and colleague introduced a subunit vaccine with the ability to inducing high levels of humoral responses, but no protection was observed in mice after CCHFV challenge infection [12].

Generally, vaccines can be considered as effective immunity inducer against pathogens as well as an active way to control infectious diseases. Due to the wide variety and extensive genetic variation among different CCHFV serotypes, conventional vaccine platforms such as live-attenuated and inactivated vaccine cannot protect persons against different CCHFV strains. Furthermore, there are some drawbacks associated with conventional vaccines including weak immunity, multi-dose administration, allergenic potential and low safety. Consequently currently, new vaccine platforms such as Subunit Vaccines, DNA vaccine, and multi-epitope vaccine (MEV) are highly regarded [13]. Among them, due to high specificity, safety and low-cost production of MEVs these vaccines have attracted attention. Commonly, MEVs are engineered based on dominant and conserved B- and T-cell epitopes from desired antigens without potential IgE-epitopes. Accordingly, an effective MEV can provide an effective immunization against different serotypes of a pathogen. Despite mentioned advantages of MEVs, poor immunogenicity is considered as a major drawback to development of MEVs [14–16]. To overcome the mentioned blind spot use of some natural protein-based adjuvants such as diphtheria toxin (DT), cholera toxin B-subunit (CTxB), heat-labile enterotoxin B subunit *(*LTB*)* and toll-like receptors (TLRs) ligands in final construct of proposed vaccines is suggested [17–19]. In the study, CTxB and LT-IIc were selected as protein adjuvants. CTxB is a 124 amino acids, nontoxic, homopantameric, commercially available and membrane-binding subunit of cholera toxin that could increase homoral and mocusal immunity response. Furthermore, CTxB receptor is widely expressed in the plasma membrane of immune cells; therefor the protein is highly regarded as suitable natural adjuvant [20]. LT-IIc is a member of the type II subfamily of LTBs that composed by 121 amino acid residues. Moreover, LT-IIc is one of the best-studied type II LTB which has been shown to enhance antigen-specific $CD8^+$ T cell immune responses when co-administered with a model antigen [21].

The protein adjuvants can increase the level of mean antibody titers, the generation of immune memory (especially T cell memory) and seroconversion rates in populations. The adjuvants also can reduce amounts of antigen in vaccine formulation as well as permit immunization with fewer doses of vaccine [22,23]. These biological activities can be achieved through induce of multiple chemokines and cytokines in bone marrow-derived dendritic cells (BMDCs) including keratinocyte-derived chemokine (KC), eotaxin, IL-27, IFN-γ, Th2-associated cytokine IL-5 and Th1-type cytokines IL-12. Furthermore, activating Toll-like receptors (TLRs) signaling pathway, inducing pro-inflammation cytokines (such as IL-6, IL-10, and IL-12), promotion of the infiltration of antigen-presenting cells (APC) and induction of antigen-specific $CD8^+$ T cells are other mechanisms of action of the protein adjuvants associated biological activities [24–26]. Furthermore, presence of linear and conformational B- and T-cell epitopes in protein adjuvant can improve immune responses against main antigens.

Experimental determination of dominant epitopes and evaluation of potential vaccines efficacy, are tedious and costly. Therefore, recently computational methods have highly considered as alternative or complementary approaches for epitopes determination and vaccine design. Computer-aided vaccine design, which also called computational immunology, can provide an integrated pipeline for mapping potential B- and T-cell epitopes, allergenic sites in studied antigens and prediction of HLA-epitope affinity. Thus, the approach could reduce the time and cost required for vaccine design [27–29]. In recent decade, computational design of epitope-based vaccine have been used for vaccine development against many infectious disease and even cancer. For this purpose, recently many bioinformatics tools have been developed that facilitates the development of epitope-based vaccines.

Bioinformatics and computational tools can provide converting of large-scale immunological data such as antigen presentation and processing, antigen-antibody interactions and antigen determination to obtain expressive interpretations. Therefore, currently vaccine design process especially epitope based vaccine development is facilitated along with introducing applied bioinformatics tools such as epitope mapping (software/web service), protein modeling software and protein-protein/ligand interaction analysis software [30].

Although not much time has passed since the introduction of the first report about computational vaccine design, in the recent years, a lot of progress has been made in this regard. Subsequently, numerous vaccines were developed based on computational approaches that include efficient vaccine against *Toxoplasma gondii* [31], *Rickettsia prowazekii* [32], *Streptococcus pneumoniae* [33], *Leishmania infantum* [34], *Chlamydia pneumoniae* [35], *Brucella abortus* [36], *Staphylococcus aureus* [15], *Escherichia coli* [37], *Vibrio cholera* [38], *Human immunodeficiency virus-1* [39], *Hepatitis C* virus [40] and many others. In many empirical studies, the efficacy of computationally designed vaccines is approved [34,39,41,42].

There is a common procedure to in silico vaccine design, which is used in most of the previous studies. The procedure, includes antigen selection, epitope prediction, vaccine engineering and vaccine evaluation. The results from the common protocol may be limited by some drawbacks such as inappropriate physicochemical properties of predicted epitopes or final construct, toxic epitopes, instability of final vaccine and unable to effective expression of designed vaccine in a desired host [43,44]. Therefore, in the present study, we used a special multi-steps procedure to decrease mentioned obstacles. In our protocol linear B cell epitopes were selected based on their antigenicity, allergenicity, toxicity, and water solubility as decisive parameters. Similarly, antigenicity, hydrophobicity, population coverage and allergenicity were considered as determining parameters to T-cell epitope prediction. The screened epitopes were then merged to each other as well as to two natural adjuvants using appropriate linkers for organization of final vaccine construct. Furthermore, the efficacy and stability of the vaccine were evaluated by a set of bioinformatics approaches including molecular docking, molecular dynamics and in silico cloning.

2. Material and methods

The procedures used for the multi-epitope vaccine development are depicted in Fig. 1.

2.1. Primary data collection

As the first stage of our study, the reference amino acid sequences of CCHFV proteins including nucleoprotein, glycoprotein precursor, and RNA-dependent RNA polymerase were retrieved from NCBI (https://www.ncbi.nlm.nih.gov) in FASTA format with accession numbers of NP_950237.1, NP_950235.1 and ACM78472.1 respectively. In addition, the primary amino acid sequence of CTxB and LT-IIc were obtained from Uniprot (http://www.uniprot.org) with accession entry of Q57193 and H6W8F2 respectively. Also, the three-dimension structure of MHC-I and II were retrieved from Protein Data Bank (https://www.rcsb.org/) in PDB format with PDB entry of 1E27 and 1AQD

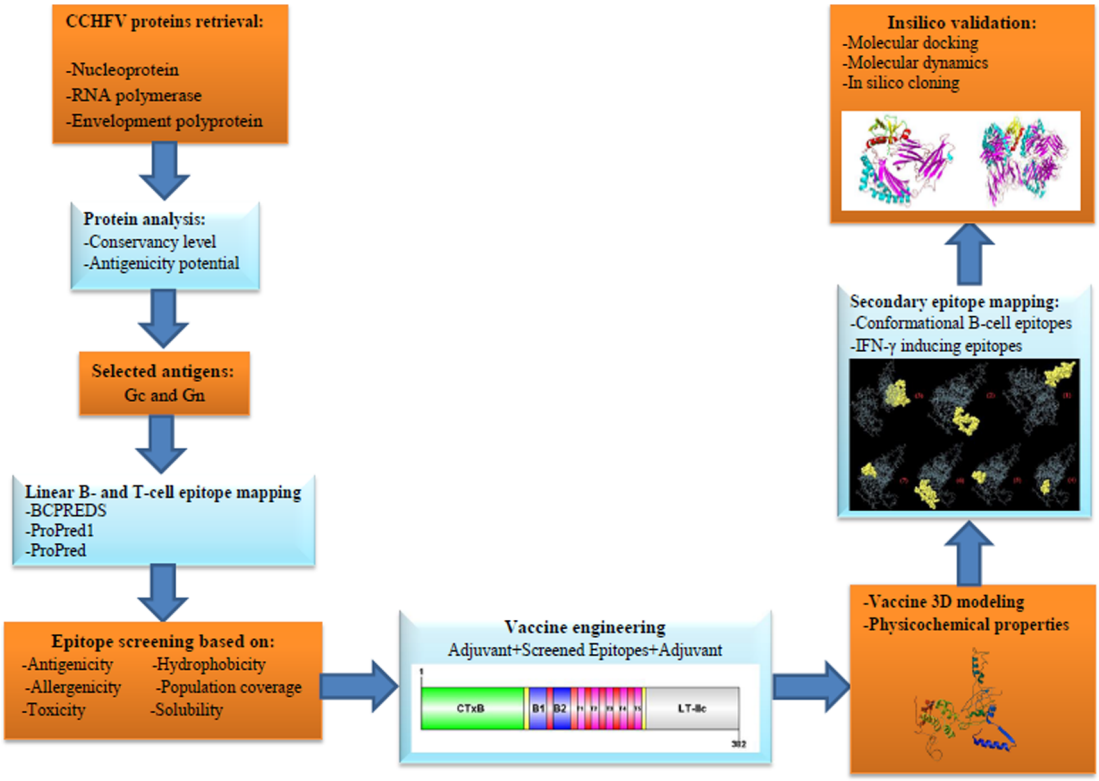

Fig. 1. Schematic presentation of the procedures used for multi-epitope vaccine development for CCHFV.

respectively.

2.2. Multiple sequence alignment and antigen selection

To determine conservancy level of the virus proteins between different CCHFV serotypes protein BLAST was performed (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, for determining the conserved region(s) in the protein sequences multiple sequence alignment was performed by Clustal Omega server (https://www.ebi.ac.uk/Tools/msa/clustalo). Additionally, antigenicity of the CCHFV proteins was evaluated using VaxiJen 2.0 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) and Predicting Antigenic Peptides (http://imed.med.ucm.es/Tools/antigenic.pl). Finally, most conserved and antigenic protein was selected for further analysis.

2.3. Antigen analysis and input preparation

The selected antigen was further analyzed to determine antigenic regions, conserved domain(s), sequence features and physicochemical properties. For this purposes, a set of online tools including Predicting Antigenic Peptides (http://imed.med.ucm.es/Tools/antigenic.pl), NCBI's conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), Uniprot (http://www.uniprot.org) and ProtParam (https://web.expasy.org/protparam/) were used.

2.4. T-cell epitope prediction and selection

The MHC-I restricted epitopes were predicted using ProPred-1 server (http://crdd.osdd.net/raghava/propred1/index.html) with default parameters. The server uses special matrices for 47 MHC-I alleles. Similarly, the MHC-II restricted epitopes were predicted through ProPred server (http://crdd.osdd.net/raghava/propred/) with a threshold of 5%. The server uses special matrices for 51 HLA-DR alleles that cover more than 90% of MHC-II molecules expressed on antigen presenting cells. Furthermore, the predicted epitopes were screened based on antigenicity, Hydrophobicity, allergenicity and population coverage using a set of online tools including VaxiJen, peptide2 (https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php), AllerTOP (http://www.pharmfac.net/allertop/) and IEDB analysis resource (http://tools.iedb.org/population/) respectively. Finally, the epitope(s) with appropriate properties were selected for the vaccine construct.

2.5. Linear B-cell epitope prediction and screening

The linear B-cell epitope prediction was carried out using BCPREDS server at (http://ailab.ist.psu.edu/bcpred/predict.html) with fixed length epitope (20 amino acids) 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 predicted epitopes were checked through BepiPred-2.0 at (http://www.cbs.dtu.dk/services/BepiPred/), SVMTrip at (http://sysbio.unl.edu/SVMTriP/prediction.php) and ABCpred at (http://crdd.osdd.net/raghava/abcpred/ABC_submission.html). Later, high-ranked and shared B-cell epitopes were selected for further study. Furthermore, the B-cell epitopes were further evaluated in term of antigenicity, allergenicity, toxicity, and solubility using VaxiJen, AlgPred (http://crdd.osdd.net/raghava/algpred/submission.html), Toxin pred (http://crdd.osdd.net/raghava/toxinpred/) and PepCalc (https://pepcalc.com/) online servers respectively. Finally, the predicted linear B-cell epitopes with appropriate properties were selected for locating in the vaccine structure.

2.6. Vaccine engineering and physicochemical properties

The determined appropriate B- and T-cell epitopes were organized in the final vaccine construct. For this purpose, the screened B- and T-cell epitopes were merged using GPGPGPG amino acid linker in a random pattern. The joined epitopes were considered as a multi-epitope

segment or core of the proposed vaccine. The final construct of the proposed vaccine was prepared after merging the multi-epitope part with two natural adjuvants including CTxB and LT-IIc at N and C terminal using EAAAK linker respectively. Furthermore, amino acid composition and some physicochemical properties of the proposed vaccine including molecular weight, iso-electric point, net charge at pH 7, estimated solubility in water, estimated half-life in the mammalian reticulocytes and instability index were determined using Protparam (http://web.expasy.org/cgi-bin/protparam/protparam), Pepcalc (http://pepcalc.com/),

2.7. Secondary and tertiary structure prediction

The secondary structure of the proposed vaccine was predicted using Prabi server at (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html) through GOR4 secondary structure prediction method. The method uses all possible pair frequencies within a window of 17 amino acid residues with a mean accuracy of 64.4%. In addition, the three-dimension structure of the vaccine was generated using I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). I-TASSER is a ranked approach to protein structure and function prediction that relies on the level of similarity between the input and available template structures in PDB.

2.8. Model refinement and quality assessment

To reduce possible structural mistakes in the predicted tertiary structure of the vaccine, refinement of the predicted model was done using 3Drefine server (http://sysbio.rnet.missouri.edu/3Drefine/). Additionally, the geometry quality of the vaccine was validated based on Ramachandran plot using RAPAGE server (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) also ERRAT quality factor (http://services.mbi.ucla.edu/ERRAT/).

2.9. Conformational B-cell and IFN-γ inducing epitopes 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 seven (angstrom). Furthermore, IFN-γ inducing epitopes in the amino acid sequence of the vaccine were predicted using IFNepitope server (http://crdd.osdd.net/raghava/ifnepitope/scan.php) with motif and SVM hybrid prediction approach.

2.10. Molecular docking study

To validate the binding affinity of the vaccine to MHC-I and MHC-II molecules, molecular docking was performed between the proposed candidate vaccine and HLA-A0201 with PDB entry of 4UQ3, and HLA-DRB1_01:01 with PDB entry of 1AQD. Molecular docking studies were carried out using Patchdock server (https://bioinfo3d.cs.tau.ac.il/PatchDock/) with default complex type and clustering RMSD of 4 Å. The server uses object recognition and image segmentation techniques in its algorithm.

2.11. Molecular dynamics (MD) simulation of ligand-receptor complex

Molecular dynamic is a computational approach, which is used to describe properties of the molecules' behavior, ligand-receptor interactions, solvation, stability and fluctuations as well as conformational changes of molecules. In the present study, MD simulation was performed on the vaccine-MHC model complexes using NAMD graphical interface module incorporated visual molecular dynamics (VMD) during 15 ns stimulation. The protein structure file (PSF) of the complexes was built using automatic PSF generator module in VMD. The MD simulations were done under NPT equivalent conditions at 1 bar and 300 K and using accessing PSF and PDB files. Afterward, 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

To clone and express the vaccine in a suitable expression vector, the amino acid sequence of the vaccine model was back translated into nucleotide sequence using Gene infinity server (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 Rare Codon Analysis Tool at (https://www.genscript.com/tools/rare-codon-analysis). These parameters have key roles in optimized protein expression in the host expression system. 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.

3. Results

3.1. Multiple sequence alignment and antigen selection

To designing an effective multi-epitope vaccine for cross protection against CCHFV, firstly all protein encoded by the virus were studied for determining more conserved protein between different serotypes of the virus. For this end, protein BLAST was done. The results of protein BLAST for CCHFV proteins are summarized in Table 1. The results showed that RNA polymerase has most conservancy level between different CCHFV serotypes. Furthermore, average antigenicity and VaxiJen score of the proteins were determined (Table1). The results demonstrated that Glycoprotein precursor has the highest antigenicity followed by RNA polymerase and Nucleoprotein respectively. Due to high antigenicity, good conservancy and higher exposure probability to immune system, Glycoprotein precursor was selected for further analysis and the vaccine design.

3.2. Antigen analysis and input preparation

The potential antigenic regions in the glycoprotein precursor sequence are illustrated in Fig. 2. The results showed that there are 72 antigenic determinants in the glycoproteins. Results also confirmed that six regions in the glycoprotein sequence including 414–469, 654–720, 798–841,

Table 1
Results of CCHFV proteins BLAST and antigenicity prediction. Glycoprotein precursor had highest average antigenicity (1.3096) and VaxiJen score (5207), while sequence identity of the glycoprotein between different serotypes of CCHFV was least value in compare to other studied antigens.

Protein	Average antigenicity	VaxiJen score	Minimum identity (%)	Maximum identity (%)
Nucleoprotein	1.0138	0.3330	92	99
Glycoprotein precursor	1.3096	0.5207	84	99
RNA polymerase	1.0324	0.4348	96	99

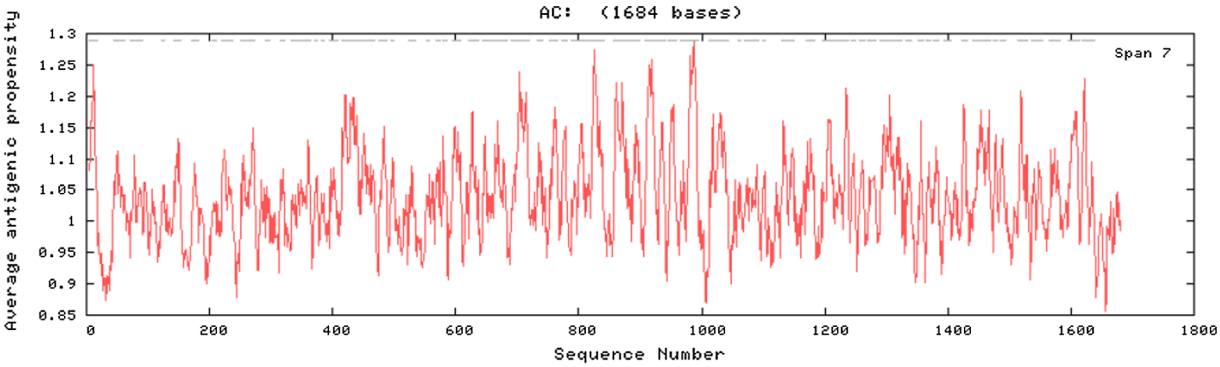

Fig. 2. Antigenicity plot of glycoprotein precursor from CCHFV. There are 72 antigenic determinants in the glycoproteins. Six regions including 414–469, 654–720, 798–841, 1012–1043, 1290–1325 and 1441–1470 were found to be high antigenic potential with average antigenic propensity more than 1.15.

Table 2
Molecular features of glycoprotein precursor from CCHFV. After processing of the precursor six structural and non-structural elements and a signal peptide are originated.

Feature key	Position(s)	Description	Sequence length
Short sequence	1–18	Signal peptide	18
Chain	19–1684	Envelopment polyprotein	1666
Chain	19–247	Mucin-like variable region	229
Chain	248–519	GP38	272
Chain	520–842	Glycoprotein N	323
Chain	843–1040	Non-Structural protein M	198
Chain	1041–1684	Glycoprotein C	644

1012–1043, 1290–1325 and 1441–1470 have high antigenic potential with average antigenic propensity more than 1.15. Results also showed that there are three conserved domains in the Glycoprotein including Nairovirus M polyprotein-like, Herpes virus major outer envelope glycoprotein (BLLF1) and Hantav rus glycoprotein G2 which were located in 241–884, 32–234 and 1177–1250 regions in the glycoprotein sequence respectively. Furthermore, the results demonstrated that after processing of the precursor six structural and non-structural features including the signal peptide, Mucin-like variable region, non-structural glycoprotein GP38, glycoprotein N, Non-Structural protein M and glycoprotein C originated (Table 2). Therefore, glycoprotein N and glycoprotein C were selected to further analysis due to their high antigenicity along with good conservancy level and more probabilities to expose the immune system. Finally, amino acid sequences of the glycoproteins were considered as inputs for more analysis and final vaccine construct.

3.3. T-cell epitope prediction and selection

In order to prediction of T-cell epitopes, we used a two-step special screening. Briefly, MHC-I and II restricted epitopes were predicted and ranked according to their score using ProPred1 and ProPred respectively. A total number of 44 and 47 MHC-I restricted epitopes were predicted for Gc and Gn respectively. The predicted MHC-I restricted epitopes were compared to MHC-II restricted epitopes to determine shared epitopes. In the next step, 12 and 13 share epitopes from Gc and Gn were selected for further screening respectively (Table 3). Finally, the epitopes with the ability to binds most MHC-I and II alleles along with high antigenicity, hydrophobicity, population coverage and non-allergenicity properties were considered as the final T-cell epitopes. After final screening, a total number of three and two T-cell epitopes were determined from Gc and Gn respectively (Table 4).

3.4. Linear B-cell epitope prediction and screening

Prediction of linear B-cell epitopes from the antigens was performed through a cross-checking method. For this, firstly linear B-cell epitope form both Gc and Gn were predicted using BCPREDS. A total number of 11 and six epitopes were predicted from Gc and Gn respectively. Furthermore, to determine share epitopes and increasing the confidence, BCPREDS outputs were cross-checked using three different servers including ABCpred, SVMTrip and, BepiPred-2.0 (Table 5). Since in the mentioned servers linear B-cell epitope prediction is performed through different algorithms, sequence similarity and score of predicted epitopes were considered as decisive factors for screening share epitopes. Subsequently, the epitopes with a high score and sequence similarity in the used server were selected for final screening. Considering the mentioned conclusive factors, five epitopes were selected from the antigens. The screened epitopes were further evaluated regarding antigenicity, allergenicity, toxicity, solubility and hydrophilicity for defining final B-cell epitopes. The results showed that one epitope from each studied antigens (Table 6) has appropriate properties, so considered as final B-cell epitopes for the vaccine construct.

3.5. Vaccine engineering and physicochemical properties

Based on the immuno-informatics analysis finally, one epitope from each Gc and Gn were considered as linear B-cell epitopes. Furthermore, five epitopes from mentioned antigens were selected as T-cell epitopes. The final vaccine composed of 382 amino acids, which organized in four domains: CTxB and LT-IIc as adjuvants, Linear B-cell epitopes and T-cell epitopes, which were joined using appropriate linkers. A graphic diagram of the vaccine domain structures with linker's sites designed is depicted in Fig. 3. Furthermore, physicochemical properties and amino acid composition of the final construct were predicted using Protparam and Pepcalc. The results revealed that the vaccine protein was a 40.7 kDa stable soluble protein with pI 8.74, a net charge of 5.4 and estimated half-life was found to be 30 h. The Protein half-life is defined as the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. Therefore, more half-life can provide more time for our proposed chimeric protein to exposure to the immune system [45]. Final amino acid sequence and amino acid composition of the vaccine is depicted in Fig. 4.

3.6. Secondary and tertiary structure prediction

The secondary structure of the final vaccine construct was predicted using GOR IV method on the basis the amino acid sequence of the protein. The results showed that 29.06, 22.25 and 48.69% of total 382 amino acids were organized in alpha helix, extended strand and random coil respectively (Fig. 5). Furthermore, the primary 3D model of the proposed vaccine was predicted by I-TASSER online server. The ten best threading templates were selected (PDB entry: 3jc8Q, 5gaoE, 4l6t, 5jcsS, 1ltrF, 4btgA, 1ltrF, 5voxO, 1ltrA, and 5yfpH) through Local Meta-Threading-Server program to model our proposed vaccine. Finally, the top five models for the protein vaccine were suggested with C-score of

Table 3
Results of T-cell epitope prediction. The labled sequences with (*) are shared T-cell epitopes between MHC-I and II risterected epitopes. The share epitopes with high affinity to more MHC-I and MHC-II alleles were screened for further evaluation. A total number of 44 and 47 MHC-I restricted epitopes were predicted for Gc and Gn respectively which among them 12 and 13 shared epitopes were selected for further evaluation.

MHC-I restricted epitopes in Gc	MHC-I restricted epitopes in Gn	Number of MHC-I bonded alleles for predicted epitopes		Number of MHC-II bonded alleles for predicted epitopes	
		Gc	Gn	Gc	Gn
LSEPRNIQQ	TAEIHGDNY	1	1	0	0
FLFLAPFIL*	ILCKAIFYL*	5	4	46	22
FILLILFFM*	LNLERIPWV*	2	2	46	13
KLPPEIITL	YLLIIVGTL	2	7	0	41
KVNGHLIHK	YVITCILCK	2	3	0	50
MYSPVFEYL*	NYGGPGDKI	4	2	2	0
GLFKYRHLK	FLFWFSFGY*	2	6	0	38
ILFFMFGWR*	RLTSDGLAR	1	1	5	0
ESIMKLEER	ESTGVALKR	1	2	0	0
CVELTSQER	YVITCILCK*	2	3	0	50
VKWKVEYIK*	GKMAIYICR	1	1	3	0
FLAPFILLI*	LLRTETAEI*	2	1	25	48
ERLADRRIA	KRLKQYREL	1	6	0	0
RRTRGLFKY	CRQGYCLRI	3	2	0	0
GRSESIMKL	ARHVIQCPK	4	1	0	0
EDASESKLL	IPKGTGDIL	1	6	0	0
IHVDEPDEL*	CDTSCEIMI	2	2	11	0
NHASFVNLL	NHPKTTMAF	3	1	0	0
LQVYHIGNL*	QHFLKDNLI	1	2	26	7
KEWPHSRNW	QQHFLKDNL	1	3	0	0
LESVKSFFY*	QEGRGHVKL	1	2	13	0
EPDELTVHV	CEIMIPKGT	2	2	0	0
SGISCKVRI	IPLLGKMAI*	1	7	0	40
GAGEITVLV	IPWVVRKLL	1	8	0	0
QQKLPPEII	SATGKNCEI	1	3	0	0
WPSCTYTGV	FGYVITCIL*	4	6	0	36
FGWRILFCF*	FWFSFGYVI*	3	5	19	9
TEAIVCVEL	YLLIIVGTL*	2	7	0	37
GEITVLVEV	IPWVVRKLL	1	8	0	0
ILLILFFMF*	KTTMAFLFW	1	1	23	0
EPRNIQQKL	QEGRGHVKL	4	2	0	0
HPRIEEGFF	CETTPVNAI	3	2	0	0
EPRNIQQKL	RLGSELGCY	4	1	0	0
VFMGIFLFL*	ASRLTSDGL	3	1	13	0
GYRRIIEKL	HPKTTMAFL	4	6	0	0
NHASFVNLL	INRVRSFKL*	3	1	0	19
MPKTSLCFY	KRLKQYREL	4	6	0	0
KNLLNSTSL	HPKTTMAFL	2	6	0	0
ASFVNLLNI	YLLIIVGTL*	2	7	0	37
RGLFKYRHL	VPVKCRQGY*	3	4	0	10
–	ITICNGSTI*	–	2	–	9
–	LGCPKIPLL	–	2	–	0
–	EETELYLNL	–	4	–	0
–	NYGGPGDKI	–	2	–	0
–	SEEPSDDCI	–	3	–	0
–	IPWVVRKLL	–	8	–	0

−1.66, −0.56, −3.34, −3.62 and −2.42 respectively. The C-score is defined in the range of −5 to 2 and usually higher score indicate a model with higher confidence. Accordingly, the model with C-score of −0.56 was selected for further analysis.

3.7. Model refinement and quality assessment

The selected model of the vaccine was then subjected to refinement processes by 3Drefine server. For this purpose, the whole protein structure refinement including secondary structure elements, loop regions and protein side-chains were refined. Selection of refined model in 3Drefine server is based on five factors including 3Drefine score, GDT-TS, GDT-HA, RMSD, RWplus and MolProbity which indicated potential energy (3Drefine score and RWplus), similarity score (GDT-TS and GDT-HA), division score and physical realism score respectively. Consequently, the refined model with appropriate properties was selected for further evaluations concerning mentioned factors (Table 7). Furthermore, the geometric quality of primary and refined models was evaluated by applying Ramachandran plot, ERRAT quality factor, and ProSA z-score. The results showed that in the raw model 68.7%, 22.1% and 9.2% of residues were located in favored, allowed, and outlier regions, respectively (Fig. 6-a) while, in the refined model, 73.4%, 18.9%, and 7.6% of residues were positioned in favored, allowed, and outlier regions, respectively (Fig. 6-b). Moreover, the quality factor and z-score of primary model were 71.5517 and −1.74 while after refinement mentioned parameters were determined 75.4098 and −2.02 that indicates improving in geometric quality of the primary model after refinement. The refinement and primary 3D model of proposed vaccine are illustrated in Fig. 5.

3.8. Conformational B-cell and IFN-γ inducing epitopes prediction

Due to the key role of conformational B-cell epitopes as well as IFN-γ inducing epitopes in adaptive and innate immune responses, the protein vaccine was subjected to prediction of the mentioned epitopes. IFN-γ is the signature cytokine of both the innate and adaptive immune systems with ability to provok antiviral immune responses and protection against reinfection. The release of IFN-γ is the important step of the Th1 response that induce the production of protective virus-neutralizing IgG and enhance the magnitude of antiviral cytotoxic T lymphocytes (CTLs) responses [46]. To prediction of conformational B-cell epitopes, the 3D structure of the proposed vaccine was used as an input for prediction of probable conformational B-cell epitopes via ElliPro. A total number of seven conformational B-cell epitopes were predicted from the vaccine, which included 207 residues out of 382 residues. Results also showed that most of the residues, which were located in the multi-epitope region in our proposed vaccine, were included in the predicted conformational B-cell epitopes (Table 8). The predicted conformational B-cell epitopes are depicted in Fig. 7. Furthermore, the amino acid sequence of the final proposed vaccine was applied for prediction of 15-mer IFN-γ inducing epitopes using MERCI method in IFNepitope server. The results showed that there were 40 positive IFN-γ inducing epitopes with a score of greater than or equal to one (Table 9). Results also revealed that the predicted epitopes were located in three regions including 20–62, 125–149 and 283–356, which were related to CTxB, multi-epitope region and LT-IIc respectively.

3.9. Molecular docking study

The binding affinity and interaction between the proposed vaccine and MHC-I and II molecules were evaluated using molecular docking via Patchdock server. PatchDock docking output is a list of candidate complexes including specified receptor and ligand molecule that ranked based on Geometric shape complementarity score, approximate interface area of the complex and Atomic contact energy (ACE). The results of molecular docking studies between our proposed vaccine and MHC molecules revealed that the proposed vaccine has high affinity to both MHC-I and II. The best-docked model for vaccine-MHC-I and vaccine-MHC-II complexes showed a docking score of 17,810 and 18,498 respectively. Furthermore, approximate interface areas of the mentioned complexes were found to be 3338.70 and 3749.50 Å correspondingly. Also, ACE of the selected top rank complexes were calculated as −266 and −419.48. The high ranked complexes between both MHC molecules and the vaccine were selected for further analysis through molecular dynamics.

3.10. MD simulation results

To validate the stability of the vaccine-MHC complexes molecular dynamics was performed. For this purpose, the high ranked complexes

Table 4
The results of final T-cell epitope screening. A total number of three and two T-cell epitopes (labeled with *) were screened from Gc and Gn respectively based on their antigenicity, hydrophobicity, allergenicity and population coverage. Generally, predicted epitopes from Gc have better properties than Gn derived ones.

Protein	T-cell epitopes	Antigenicity	Hydrophobicity (%)	Allergenicity	Population coverage (%)	Final decision
Gc	FLFLAPFIL	0.2586	100	No	29.10	–
	FILLILFFM	0.2343	100	No	10.06	–
	MYSPVFEYL	0.0270	55.56	No	11.21	–
	ILFFMFGWR	1.6738	77.78	Yes	71.04	–
	VKWKVEYIK	−0.2568	44.44	Yes	24.32	–
	FLAPFILLI	0.6746	100	No	76.69	*
	IHVDEPDEL	−0.0627	44.44	Yes	32.05	–
	LQVYHIGNL	1.0338	44.44	No	59.74	*
	LESVKSFFY	−0.1855	44.44	No	23.12	–
	FGWRILFCF	0.6046	66.67	No	89.12	*
	ILLILFFMF	0.3806	100	No	24.76	–
	VFMGIFLFL	−0.0320	88.89	No	13.12	–
Gn	ILCKAIFYL	−0.1887	66.67	No	62.54	–
	LNLERIPWV	1.3691	66.67	Yes	88.54	–
	FLFWFSFGY	1.3227	66.67	Yes	33.32	–
	YVITCILCK	−0.1778	44.44	No	19.54	–
	LLRTETAEI	0.0964	44.44	No	79.54	*
	IPLLGKMAI	0.3897	77.78	No	43.22	–
	FGYVITCIL	−0.0073	55.56	Yes	27.12	–
	FWFSFGYVI	1.5147	66.67	Yes	41.05	–
	YLLIIVGTL	0.6623	66.67	No	45.32	*
	INRVRSFKL	−0.3028	44.44	Yes	18.03	–
	YLLIIVGTL	−0.5074	66.67	No	36.12	–
	VPVKCRQGY	1.2904	33.33	No	35.32	–
	ITICNGSTI	−0.2864	33.33	No	10.04	–

formed by PatchDock were subjected to MD simulation. As the first step, energy values, pressure, temperature, volume and density of the system were checked at the equilibration steps and the end of the simulation. The results revealed that MD simulation steps were performed appropriately. The backbone RMSD of the vaccine-MHC complexes are depicted in Fig. 8. The results demonstrated that in both studied complexes after a gradual increase in backbone RMSD until the 6th ns the systems almost reached steady state. In fact, the protein vaccine and MHC molecules try to get the best conformation relative to each other during the MD simulation. Radius of gyration is an important indicator of protein compactness and stability. Therefore, the R_g values of the vaccine-MHC alleles were monitored during MD stimulations (Fig. 9). The results showed that the R_g values of both the vaccine-MHC-I and MHC-II complexes decreased significantly after three and four ns respectively, which indicated the complexes gained more compactness and stability during MD simulations. In other words, this trend revealed suitable interactions between our proposed vaccine and MHC alleles.

Table 6
The final screening of selected linear B-cell epitopes from Gc and Gn. One linear B-cell epitope from each antigen was selected for final vaccine construct. The selected epitopes were found to be good water soluble, high antigenic (antigenicity score more than 0.8) and without allergenicity and toxicity potential.

Epitope	Antigenicity	Allergenicity	Toxicity	Solubility
DELTVHVKSDDPDVVAASSS*	0.8272	No	No	Good
KLQSCTHGVPGDLQVYHIGN	0.2226	Yes	No	Poor
LIHKIEPHFNTSWMSWDGCD	0.2253	No	No	Poor
GLARHVIQCPKRKEKVEETE	0.5288	Yes	No	Good
VDQRLGSELGCYTINRVRSF*	0.8924	No	No	Good

Table 5
Results of linear B-cell epitope prediction using three different servers. The score and sequence similarity were considered as determining factors for comparison of the server outputs and epitope selection. A total number of three and two epitopes from Gc and Gn were screened for final evaluation respectively. Selected epitopes are labeled with (*).

Protein	Epitope	Score				Sequence similarity (%)		
		BCPREDS	ABCpred	SVMTrip	BepiPred-2.0	ABCpred	SVMTrip	BepiPred-2.0
Gc	DELTVHVKSDDPDVVAASSS*	0.998	0.85	0.516	High	85	60	60
	GDRQVGEWPKATCTGDCPER	0.996	0.69	–	High	70	–	90
	EETGYRRIIEKLNNKKGKNK	0.994	0.74	0.386	Low	90	25	45
	IEHKGTIIGKQNSTCTAKAS	0.978	0.79	0.238	High	95	20	50
	KLQSCTHGVPGDLQVYHIGN*	0.957	0.69	0.528	High	100	85	55
	NMGDWPSCTYTGVTQHNHAS	0.935	0.74	–	Low	60	–	50
	RNWRCNPTWCWGVGTGCTCC	0.915	0.76	–	Low	70	–	25
	LIHKIEPHFNTSWMSWDGCD*	0.915	0.68	0.295	High	90	20	100
	APWGAINVQSTYKPTVSTAN	0.901	0.87	0.238	Intermediate	90	20	50
	SCSEEDTKKCVNTKLEQPQS	0.849	0.70	–	High	65	–	100
	QKLPPEIITLHPRIEEGFFD	0.835	0.87	–	High	90	–	95
Gn	IHGDNYGGPGDKITICNGST	0.999	0.90	–	Intermediate	75	–	55
	GLARHVIQCPKRKEKVEETE*	0.995	0.86	0.603	High	100	60	100
	RLKQYRELKPQTCTICETTP	0.993	0.60	–	Intermediate	65	–	45
	PKGTGDILVDCSGGQQHFLK	0.959	0.74	–	Intermediate	80	–	55
	IDLGCPKIPLLGKMAIYICR	0.908	0.85	–	Low	85	–	30
	VDQRLGSELGCYTINRVRSF*	0.841	0.65	0.533	High	95	50	85

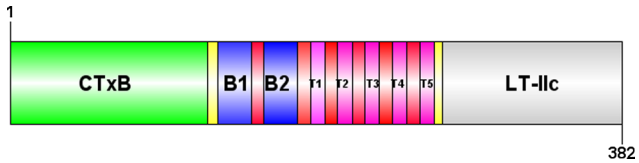

Fig. 3. Graphical presentation of the purposed vaccine for CCHF. The vaccine is organized in four different domains including CTxB and LT-IIc as adjuvants, Linear B-cell epitopes (blue regions) and T-cell epitopes (pink regions). The used linker for merging mentioned domains were GPGPGPG (red regions) and EAAAK (yellow regions). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.11. Revers translation, codon optimization and in silico cloning

To evaluate the cloning and expression of the vaccine within the expression vector in silico cloning was performed through Gene infinity server. For this, the amino acid sequence of the vaccine was reverse translated according to the codon usage of *E. coli* expression system. Furthermore, the designed gene was evaluated in term of GC-content, CAI and CFD to determine codon usage frequency and codon usage distribution using GenScript rare codon analysis tool. The results showed that GC-content, CAI and CFD of the improved sequence were found to be 57.46%, 1 and 100% that were satisfactory. Furthermore, after determination of restriction sites within the optimized DNA, XhoI and NdeI restriction sites were created in 5′ and 3′-OH of the DNA respectively, followed by adding six histidine residues on both ends.

4. Discussion

Due to error-prone nature of CCHFV polymerase, high infectious rate and lack of animal model, the development of a vaccine against CCHFV is very challenging. However, recently, with the development of bioinformatics and computational methods, these limitations are diminished. In this regard, using computational method, epitope mapping, design of recombinant proteins and the evaluation of physicochemical properties as well as candidate vaccines efficacy can be available [40,47]. Therefore, this study was planned to design an efficient multi-epitope recombinant vaccine against CCHFV using a special multi-step bioinformatics approach.

Based on our best knowledge there is only one study about computational design epitope-based vaccine for CCHF. Oany et al identified a highly conserved epitope in RNA-dependent RNA polymerase of CCHFV and introduced it as a potential epitope-based vaccine for CCHFV [48]. In Oany et al study degree of conservancy was considered as determining factor while along with conservancy some other factors such as immunogenicity level, allergenicity, population coverage and physicochemical properties of an epitope must be considered for epitope-based vaccine design. The mentioned parameters were considered in the present study. Furthermore, in the mentioned study envelope glycoprotein of CCHFV was also evaluated for determination of conserved epitopes. The results were contrary to our study, because high variation in the envelope glycoprotein was observed, while this variation is focused in the N terminal mucin-like domain, which is not part of mature glycoprotein [49].

To develop an efficient vaccine for CCHF we used both immunoinformatics and structural vaccinology strategies. In recent years, the strategies are used to design many vaccines. In this study, we made some modification in the common method. In conventional method, after selecting the desired antigen, an epitope mapping is performed and some evaluation such as affinity to MHCs, physicochemical properties and mRNA prediction [27,30]. However, in our study along with the mentioned steps multi-step epitope screenings besides more in silico validations are performed.

As illustrated in Fig. 2 our proposed multi-epitope vaccine is composed of two natural adjuvants (CTxB and LT-IIc), two B-cell epitopes and five T-cell epitopes, which were merged to each other by appropriate linkers. In our vaccine construct, after analysis of all CCHFV virulence factors, Gc and Gn glycoproteins were selected as main antigens and the sources of final B- and T-cell epitopes due to its high level of conservancy (up to 84%) and antigenicity.

Generally, viral glycoproteins play a vital role in the catalysis of virus fusion to host cells as well as assist in the invasion [50,51]. Consequently, recently, they are considered as crucial components for vaccine development against viral infections such as *HIV-1* [52], *HCV* [53], *Ebola* virus [51], *human coronavirus* [54], *Dengue* virus [55] and many others.

Determination of immunogenic B- and T-cell epitopes is a crucial step in epitope-based vaccine design. T-cells have the key role in antibody production through polarization to Th2 and antigen presenting during viral infections [56,57]. Accordingly, dominant T-cell epitopes of both Gc and Gn were predicted with the help of a multi-step screening procedure. To screening T-cell epitopes, firstly MHC-I restricted T-cell epitopes were predicted. In the next step, the predicted

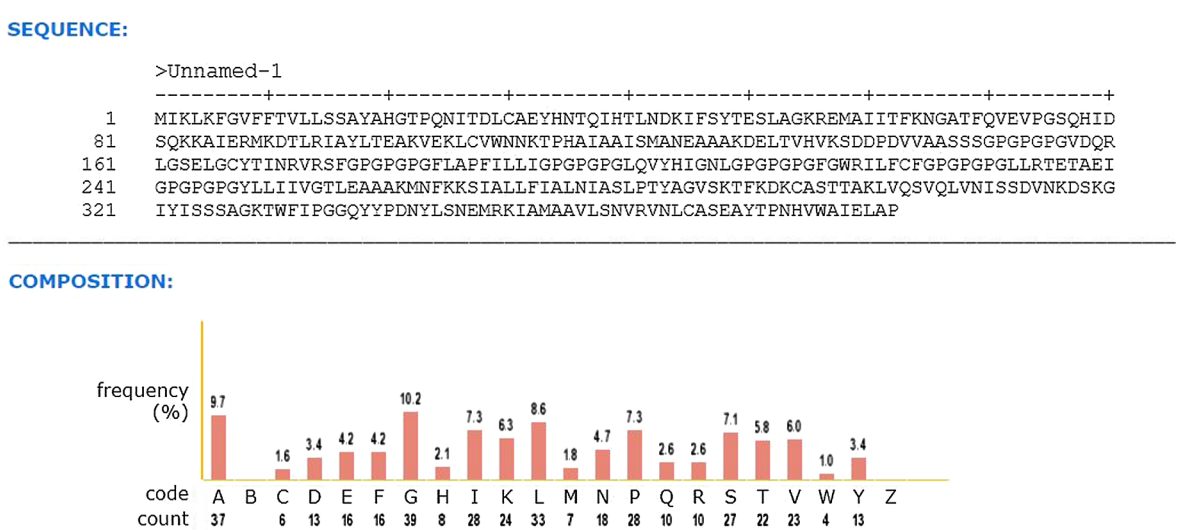

Fig. 4. Amino acid sequence and composition of final vaccine construct. The Amino acid sequence and composition can affect the protein 2D and 3D structures as well as its function. The results reflected that the protein is composed by 382 amino acids. Glycine and Tryptophan are the most and the least residues in the final vaccine construct.

```
        10        20        30        40        50        60        70
         |         |         |         |         |         |         |
MIKLKFGVFFTVLLSSAYAHGTPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAIITFKNGATFQ
ccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccc
VEVPGSQHIDSQKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMANEAAAKDELTVHVKSDD
ccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccc
PDVVAASSSGPGPGPGVDQRLGSELGCYTINRVRSFGPGPGPGFLAPFILLIGPGPGPGLQVYHIGNLGP
ccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccc
GPGPGFGWRILFCFGPGPGPGLLRTETAEIGPGPGPGYLLIIVGTLEAAAKMNFKKSIALLFIALNIASL
ccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccc
PTYAGVSKTFKDKCASTTAKLVQSVQLVNISSDVNKDSKGIYISSSAGKTWFIPGGQYYPDNYLSNEMRK
ccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccccc
IAMAAVLSNVRVNLCASEAYTPNHVWAIELAP
hhhhhhhhccccccccccccccccccccccc
```

```
GOR4 :
   Alpha helix      (Hh) :   111 is  29.06%
   3₁₀ helix        (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    85 is  22.25%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :   186 is  48.69%
   Ambiguous states (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

Fig. 5. Secondary structure of the final vaccine construct predicted by GOR4 method. Most amino acid residues of the vaccine were organized in random coil (48.69%) followed by alpha helix (29.06) and extended strand (22.25%) respectively.

Table 7
Results of the model refinement. The low 3Drefine, MolProbity and RWplus scores indicate better quality model. On the contrary, higher GDT-TS, RMSD and GDT-HA scores indicate conservative refinement and higher quality. Based on the mentioned parameters, model No.1 was selected as the best-refined model.

Model	3Drefine score	GDT-TS	GDT-HA	RMSD	RWplus	MolProbity
1	29,866	0.9993	0.9666	0.376	−53,727.135	3.461
2	30,223.2	1	0.9758	0.351	−53,610.179	3.413
3	30,879	1	0.9863	0.318	−53,450.421	3.390
4	31,941	1	0.9935	0.275	−53,327.261	3.381
5	35,185	1	0.9993	0.203	−53,166.064	3.339

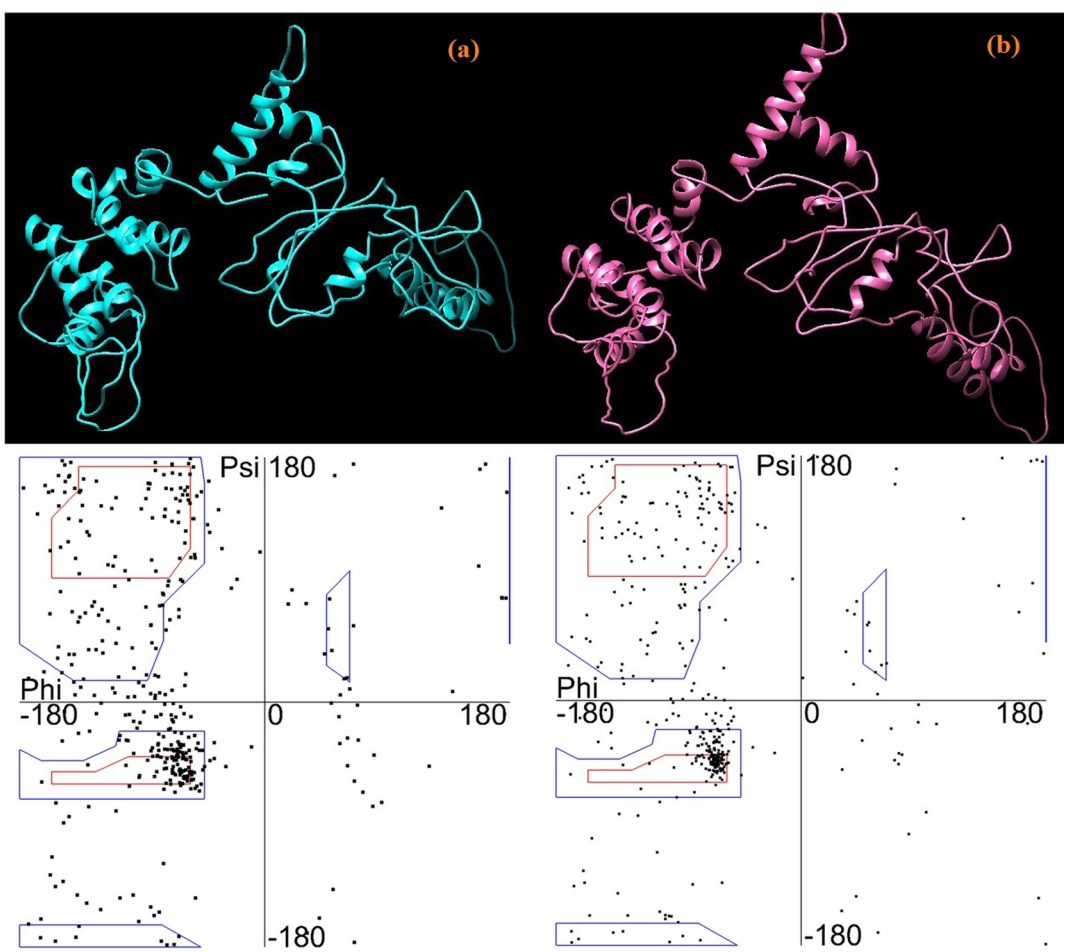

Fig. 6. 3D structure of primary (a) and refined model (b) of the final vaccine construct. Ramachandran plots showed that in the raw model 68.7%, 22.1% and 9.2% of residues were located in favored, allowed, and outlier regions, respectively (Fig. 5-a) while, in the refined model, 73.4%, 18.9%, and 7.6% of residues were positioned in favored, allowed, and outlier regions, respectively.

Table 8
Conformational B-cell epitopes from 3D model of proposed vaccine. A total number of seven epitopes were predicted. Epitopes No. 3 and 4 were considered as the broadest and smallest conformational B- cell epitopes with 78 and 5 amino acid residues.

Number	Residues	Number of residues	score
1	I2, L4, F6, G7, V8, F9, F10, T11, V12, L13, L14, S15, S16, A17, Y18, A19, H20, G21, T22, P23, Q24, N25, I26, T27, D28, L29, C30, A31, E32, H34, N35	31	0.787
2	V308, N309, I310, S311, S312, D313, V314, N315, K316, D317, S318, K319, G320, I321, Y322, I323, S324, S325, S326, A327, G328, K329, T330, F332, I333	25	0.746
3	D80, Q82, K83, K84, A85, I86, E87, R88, M89, K90, D91, T92, L93, R94, I95, A96, Y97, L98, T99, E100, A101, K102, V103, E104, K105, L106, C107, V108, W109, N110, N111, K112, T113, P114, H115, A116, A117, A118, A119, I120, S121, M122, A123, N124, E125, A126, A127, A128, K129, D130, E131, L132, K137, S138, D139, D140, P141, D142, V143, V144, A145, A146, G181, P182, G183, F184, L185, A186, P187, F188, I189, L190, L191, I192, G193, P194, G195, P196	78	0.714
4	Y339, P340, D341, N342, Y343	5	0.690
5	T282, Y283, A284, G285, V286, S287, K288, T289, F290, K291, D292, K293, C294, A295	14	0.688
6	L334, S345, N346, E347, M348, R349, K350, I351, A352, M353, A354, A355, V356, L357, S358, N359, V360, R361, V362, N363, L364, C365, A366, S367, E368, A369, Y370, T371, P372, N373, H374, V375, W376, A377, I378, E379, L380, A381, P382	39	0.686
7	I240, G241, P242, G243, P244, G245, P246, G247, Y248, L249, L250, I251, I252, V253, G254	15	0.592

epitopes was checked for their ability to binding to MHC-II alleles. After that, as primary screening, the T-cell epitopes with ability to bind to both MHC-I and II were selected for further analysis. Finally, five T-cell epitopes were screened based on antigenicity, population coverage, hydrophobicity and allergenicity. Chowell et al demonstrated that hydrophobicity is a hallmark of immunogenic T-cell epitopes and marks a step toward removing the requirement for experimental epitope testing for vaccine development [58]. Therefore, in the study, for first time we considered hydrophobicity of the predicted T-cell epitopes as a determining factor in epitope screening process.

B-cell epitopes have a pivotal role in boosting neutralizing-antibody responses in different infections. Consequently, currently identification of B-cell epitopes is highly considered for epitope-driven vaccine development and diagnostic reagents design [59,60]. With the aim of achieving a potent protective immunity against CCHF two top-ranked linear B-cell epitopes were selected based on their antigenicity, allergenicity, toxicity and hydrophilicity. The screened B- and T-cell epitopes were combined into our vaccine construct as the final immunodominant epitopes using GPGPGPG linker to retain both structural features and conformational dependent immunogenicity of the epitope vaccine.

Besides the various advantages of epitope-based vaccines, some weaknesses such as low immunogenicity and instability have faced the vaccine platform to serious challenge. Incorporating natural adjuvants in epitope-based vaccine construct can notably diminish these obstacles [19]. Hence, we merged the immunodominant epitopes with two natural adjuvants including CTxB and LT-IIc at N and C terminal using EAAAK linker. The EAAAK can increase stability as well as decrease the interaction between the vaccine domains and cause more effective separation [61,62].

After the organization of different domains of the vaccine, physicochemical, immunological and structural properties of our proposed vaccine were evaluated using different bioinformatics tools. The results revealed that the vaccine is stable, water-soluble, non-allergenic and highly antigenic.

From an empirical attitude, to achieve a high-level protein expression in E. coli, some key parameters such as CAI, GC, and CFD content of the gene should be optimized. Generally, a gene with CAI of > 0.8 is rated as good for expression in the desired host. The GC content of 30% to 70% is considered as ideal percentage range. The CFD value of 100 supports maximum protein expression in the desired host [19]. All the mentioned parameters of the optimized gene showed that our proposed vaccine could be expressed efficiently in E. coli host.

After organization of the vaccine elements, the 3D structure of it

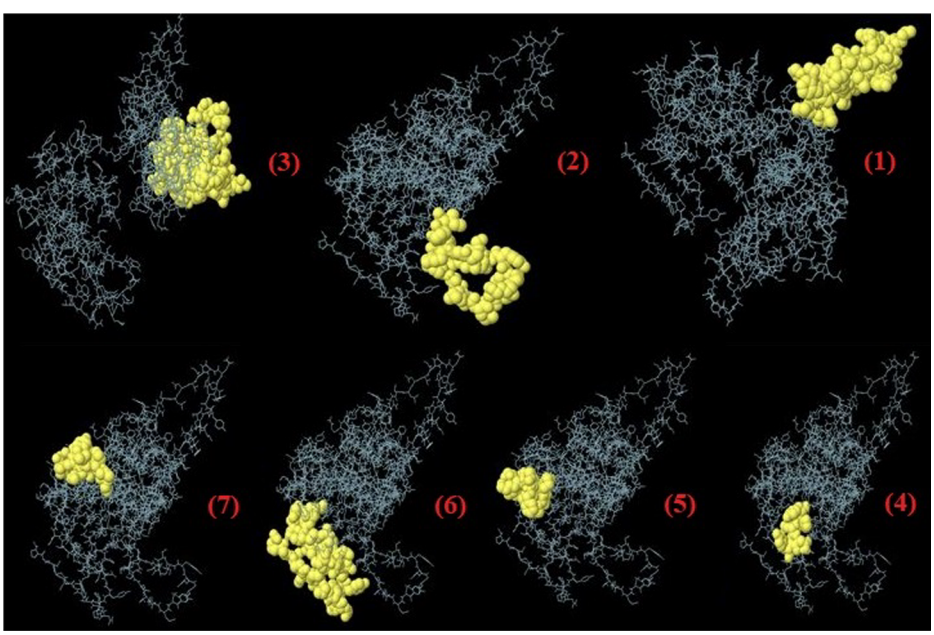

Fig. 7. Predicted conformational B-cell epitopes of the final vaccine construct. A total number of seven conformational B-cell epitopes were predicted from the vaccine, which included 207 out of 382 residues.

Table 9
The predicted IFN-γ inducing epitopes from the proposed vaccine. A total number of 40 positive IFN-γ inducing epitopes with a score of *greater than* or *equal* to one were predicted from the vaccine. The amino acid residues of the vaccine, which are located in 126–141 region showed highest score (1.21).

Number	Sequence	Start-End	score
1	GTPQNITDLCAEYHN	20–35	1
2	TPQNITDLCAEYHNT	21–36	1
3	PQNITDLCAEYHNTQ	22–37	1
4	QNITDLCAEYHNTQI	23–38	1
5	NITDLCAEYHNTQIH	24–39	1
6	ITDLCAEYHNTQIHT	25–40	1.02
7	TDLCAEYHNTQIHTL	26–41	1.09
8	NDKIFSYTESLAGKR	41–56	1
9	DKIFSYTESLAGKRE	42–57	1
10	KIFSYTESLAGKREM	43–58	1
11	IFSYTESLAGKREMA	44–59	1
12	FSYTESLAGKREMAI	45–60	1
13	SYTESLAGKREMAII	46–61	1
14	YTESLAGKREMAIIT	47–62	1
15	AAAKDELTVHVKSDD	125–140	1
16	AAKDELTVHVKSDDP	126–141	1.21
17	AKDELTVHVKSDDPD	127–142	1
18	KDELTVHVKSDDPDV	128–143	1
19	LTVHVKSDDPDVVAA	131–146	1
20	TVHVKSDDPDVVAAS	132–147	1
21	VHVKSDDPDVVAASS	133–148	1
22	HVKSDDPDVVAASSS	134–149	1
23	AGVSKTFKDKCASTT	283–298	1
24	SVQLVNISSDVNKDS	303–318	1
25	VQLVNISSDVNKDSK	304–319	1
26	QLVNISSDVNKDSKG	305–320	1
27	LVNISSDVNKDSKGI	306–321	1
28	VNISSDVNKDSKGIY	307–322	1
29	NISSDVNKDSKGIYI	308–323	1
30	IPGGQYYPDNYLSNE	332–347	1
31	PGGQYYPDNYLSNEM	333–348	1
32	GGQYYPDNYLSNEMR	334–349	1
33	GQYYPDNYLSNEMRK	335–350	1
34	QYYPDNYLSNEMRK	336–351	1
35	YYPDNYLSNEMRKIA	337–352	1.05
36	YPDNYLSNEMRKIAM	338–353	1
37	PDNYLSNEMRKIAMA	339–354	1
38	DNYLSNEMRKIAMAA	340–355	1
39	NYLSNEMRKIAMAAV	341–356	1
40	FTVLLSSAYAHGTPQ	9–24	1

was predicted using I-TASSER webserver. The primary 3D structure was subjected to the refining process to achieve a high-quality 3D structure. Quality of the refined model was assigned by Ramachandran plot, ERRAT quality factor, and ProSA z-score, and the results demonstrated that the quality of the refined model was significantly improved.

Since discontinuous B-cell epitopes as well as IFN-γ inducing epitopes have a crucial role in humoral and cell-mediated adaptive immune responses by producing antibodies and stimulating macrophages and natural killer cells, prediction of the epitopes currently are highly considered for vaccine design [13]. Subsequently, prediction of discontinuous B-cell epitopes and IFN-γ inducing epitopes were performed. For this, the refined 3D model was subjected to prediction as input, and results showed that 207 residues out of 382 residues of the vaccine were defined as a conformational B-cell epitope. Furthermore, the results indicated that there were 40 positive IFN-γ inducing epitopes in our vaccine, which shows the vaccine can provoke a strong antibody secretion, cell-mediated responses and long-running protection against CCHFV.

Antigen presentation is a required step in the recognition of antigens by immune system. APCs presenting the antigen using MHC I and II molecules [63]. Accordingly, the high affinity of a multi-epitope vaccine to MHC molecules can be considered as a determining factor. Therefore, we evaluated the binding affinity of the vaccine to MHC-I and II using molecular docking study. The results confirmed that our vaccine has high affinity to both MHC molecules. To gain more insight into the vaccine-MHCs interaction and evaluate their stability, top-ranked complexes of the vaccine-MHC-I as well as vaccine-MHC-II were subjected to MD stimulations. The results confirmed the suitable stability of the studied complexes demonstrating persistent interactions between MHC molecules and our proposed vaccine.

The results of our study showed that the proposed vaccine could be considered as a good candidate for more in vitro and in vivo evaluations. In general, after proposing an in silico designed vaccine candidate some in vitro and in vivo validation such as potential toxicity, probable allergenicity, cross-reactivity, stability, determination of effective dose, total antibody titers and proliferation of lymphocytes, challenge in animal model must be done to clarify the vaccine efficacy[64]. Vaccine development is a time-consuming, complex and costly process that can be simplified in to two main stages including pre-clinical and clinical developments. In two mentioned stages selection of the right antigen(s), vaccine efficacy in test tubes and animals, formulation, safety for human use, good manufacturing practice standards, immunity against

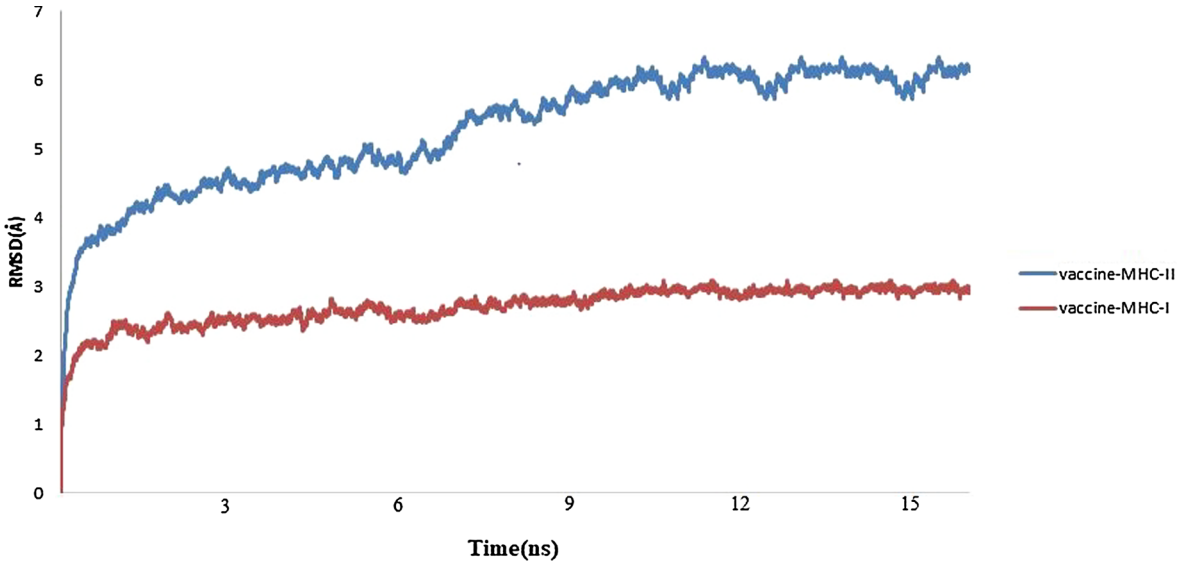

Fig. 8. RMSD plots of docked vaccine-MHCs complexes. The results indicated the stable microscopic interaction between the vaccine and MHCs molecules.

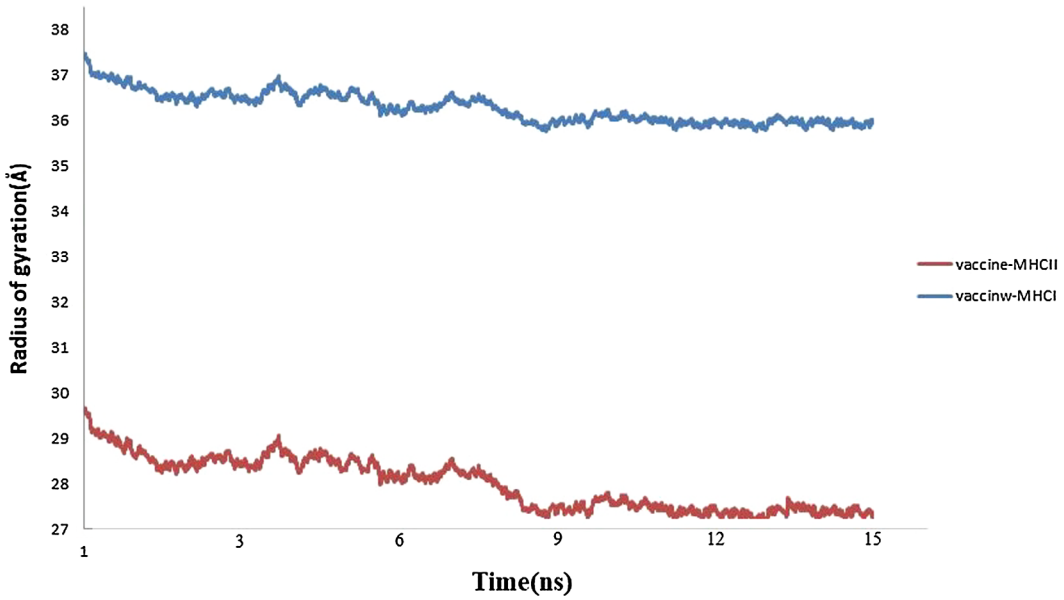

Fig. 9. Rg plots of docked the vaccine-MHCs complexes. The results reflected that the complex were compact and stable during stimulations.

artificial infection and clinical disease, vaccine efficacy under natural disease conditions and the long-term efficacy are assessed. However, the vaccine development time, may be affected by some other parameters such as nature of target pathogen, equipment and facilities available, technical and manufacturing hurdles [65,66]. Therefore, determining the exact time to introduce a vaccine is very difficult; but using computational methods and in silico validations the required time can be reduced.

Due to the encouraging results of the study, the proposed vaccine could synthesized by chemical methods such as coupling short peptides or expressed in different hosts such as bacteria, yeast, mammalian and plant cells for more in vitro and in vivo evaluations. Moreover, the efficacy of the proposed vaccine may be improved using novel antigen delivery systems such as Nano delivery platforms [67–69].

5. Conclusion

In conclusion, the present study indicated that computer-aided vaccine design could consider as a promising strategy to accelerate vaccine development against highly pathogenic pathogens. Based on this method, linear B-cell and T-cell epitopes from Gc and Gn of CCHFV were identified and selected as a core of a multi-epitope recombinant vaccine for CCHF. The results of this study suggest that our proposed protein vaccine can stimulate both humoral and cellular immune responses against CCHFV and could serve as a potential vaccine against CCHF. However, in vitro and in vivo immunological experiments are needed to validate the efficacy of the vaccine. Furthermore, based on the promising results of this study our proposed vaccine candidate can be subjected to an experimental cloning and purification as well as next in vitro and in vivo evaluations such as in vitro and in vivo stability and toxicity, the ability to enhance total antibody titer, human lymphocyte proliferation and creating immunity against the infection.

Conflict of interest

The authors declared that there is no conflict of interest.

Declaration of interests

The authors declare that they have no known competing financial interests.

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