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Designing Multi-epitope Subunit Vaccine Candidate for Zika Virus Utilizing *In Silico* Tools

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ABSTRACT

Background: The arboviruses Zika virus (ZIKV) is a pathogen that threatens human health. Scientists have warned that a single mutation in the mosquito-borne ZIKV could spark another major outbreak of the disease in humans. Therefore, designing a suitable vaccine for this virus seems necessary. This study aimed to predict the protective epitopes of envelope protein from the Zika virus with bioinformatics methods for multi-epitope vaccine development.

Materials and Methods: Computational studies including the identification of potential *B-cell* and *T-cell* epitopes were used. For generating a multi-epitopic vaccine construct (MEVC), selected epitopes are connected by suitable linkers. To enhance protein immunogenicity, Maltose-bound protein was added to the MEVC after the prediction and refinement of the 3D structure of the designed vaccine. The binding mode of the MEVC with toll-like receptor was investigated by molecular docking technique. Finally, molecular dynamics and *in silico* cloning were performed for the designed vaccine.

Results: This study showed that this recombinant vaccine is nontoxic, nonallergenic, and thermostable and elicits immune responses against the Zika virus.

Conclusion: The computational data suggest that the MEVC has appropriate characteristics and a high-quality structure.

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Introduction

The arboviruses Zika virus (ZIKV) was 1st identified in the Zika forest in Uganda and then it was reported in humans. ZIKV disease was observed across Africa and parts of South-East Asia. The 1st epidemic of the Zika virus was reported in 2007 on the island of Yap in the Federated States of Micronesia. It was later recognized in Easter Islands and Brazil. Zika transmission persists in several countries but has generally been at low levels throughout 2018 to the present. However, scientists have warned that a mutation in the virus could cause another major outbreak [1-3].

The incubation period for this virus is around seven days. Symptoms include yellow fever, malaise, headache, dizziness, stomachache, anorexia, and maculopapular rash [2]. ZIKV infection can also lead to optic neuritis, blindness, chorioretinal atrophy, and lens subluxation. Multiple studies have shown that caspase-3 activation and apoptosis can be due to Zika virus infection [1, 4].

Zika virus belongs to the Flaviviridae family of viruses. The Flaviviridae family comprises four genera: Pestivirus, Flavivirus, Pegivirus, and Hepacivirus [5].

Zika virus has a single-stranded positive RNA genome. During infection, the Zika virus binds to special proteins and then the process of endocytosis occurs. The endoplasmic reticulum carries out the process of RNA genome translation [6]. RNA genome encodes structural and non-structural proteins. The genome is translated as a single polyprotein and then is proteolytically cleaved into individual proteins. The nonstructural proteins are found at the C-terminus and the structural proteins are located at the N-terminus of the polyprotein [7].

By nonstructural protein 1, flaviviruses elude from complement-mediated lysis [8]. Nonstructural protein 3 is protease and helicase and nonstructural protein 2B is a cofactor for protease activity. The most important protein, with nine hundred residues, is nonstructural protein 5 which is also involved in virus replication [9].

The capsid protein is known to associate with the viral RNA genome to form the virus particle. The precursor membrane is a critical part of the formation of mature pathogen [10]. The mature Zika virus has many copies of the E and M proteins in its membrane. The structures of the E protein have been determined. ZIKV E protein is composed of four sections including the stem-transmembrane domain pair, and three domains found outside of the membrane. The basic organizational unit of E protein in the mature virus is dimer [11, 12]. The E protein is a glycosylated protein that mediates viral entry. Studying the structural details of this protein is very important in recombinant vaccine design [11]. Envelope (E) protein facilitates the endocytosis process after binding to the specific parts of human cells and forming the ligand-receptor [10-13].

In recent years, bioinformatics has garnered much scientific attention [14]. Vaccines created through bioinformatics are more secure, more helpful, more effective, and less costly than traditional vaccines [15].

So far, many vaccines have been proposed for the Zika virus, but due to their ineffectiveness, investigations are still ongoing to design a suitable vaccine. On the other hand, the warning of the medical community about the possibility of another major outbreak of this virus makes it necessary to design new vaccines. The purpose of the present study is to design an effective recombinant vaccine against the Zika virus by utilizing *in silico* Tools.

Materials and Methods

Protein sequences retrieval and identification of conserved regions

In the current study, firstly, the most immunologically significant protein of the Zika virus, the E protein, which directly involves infusion of virus membrane with host endosome membrane and attachment to host cell was selected. The UniProtKB database was exploited for retrieving the FASTA format of the ZikV envelope protein. We also performed multiple sequence alignments through CLUSTALW [16].

B-cell and *T-cell* epitope prediction

In the design of the recombinant vaccine, it is very important to use epitopes that generate strong immune responses. We used the Emini Surface Accessibility (ESA) and BepiPred-2.0 prediction methods in Immune Epitope Database (IEDB) server to predict *B-cell* epitopes [17]. The residues with scores above 0.5 are predicted to be part of an epitope [18]. RANKEPEP was used for the prediction of human leukocyte antigen [19].

Table 1. Allergenicity, antigenicity, and toxicity analysis

Predicted Epitopes	Number Epitope	Epitopes	Antigenicity	Allergenicity	Toxicity
	1	GTDGPCK	Non-antigen		
	2	GTPHWNN	Antigen		
	3	AGTDGPC	Non-antigen	No	
	4	HGSQHSG	Non-antigen		
	5	GGFGSLG	Antigen		
	6	YAGTDGP	Non-antigen		
	7	DGPCKVP	Non-antigen	Yes	
B-cell epitopes	8	GADTGTP	Non-antigen		
	9	TPNSPRA	Non-antigen	No	
	10	KGRLSSG	Non-antigen		
	11	TPHWNNK	Antigen	Yes	
	12	GSQHSGM	Non-antigen		
	13	TGTPHWN	Antigen	No	NT
	14	GRLSSGH	Non-antigen	Yes	
	15	VTPNSPR	Non-antigen		
	16	IVNDENRAK	Antigen	No	
	17	ITESTENSK	Antigen	Yes	
MHC-I epitopes	18	CTAAFTFTK	Non-antigen		
	19	GRLSSGHLK	Antigen	No	
	20	GLDFSDLYY	Antigen		
	21	LYYLTMNNK	Antigen		
	22	LSVHGSQHS	Non-antigen	No	
MHC-II epitopes	23	IVNDENRAK	Antigen		
	24	MLSVHGSQH	Non-antigen	Yes	
	25	VVVLGSQEG	Antigen	No	

Notes: NT: nontoxic.

Toxicity, allergenicity, and antigenicity analysis of the selected epitopes

Non-toxicity and non-allergenicity of the sequences are the main parameters for choosing different parts of the recombinant vaccine. Server AllerTOP was used to check the allergenicity of selected epitopes [20]. ToxinPred Server is an *in silico* approach to investigate the toxicity of peptide sequences. ToxinPred Server predicted toxic/non-toxic selected peptides based on Physicochemical properties [21]. The antigenicity of target peptides was studied by the VaxiJen tool [22].

Molecular docking study

Three-dimensional structures of epitopes were subsequently constructed, compared, and optimized with Hyperchem release 8 and PEP-FOLD 2.0. The HLA

Table 2. Molecular docking between selected epitopes and HLA

Number Epitope	Epitopes	Docking Score*	Ligand rmsd (Å)	Amino Acids of the Receptor in H-bonds	Amino Acids of the Epitope in H-bonds
16	IVNDENRAK	-172.06	67.04	TYR9, HR73, TYR99, ASN3	ASP4, GLU5
19	GRLSSGHLK	-180.74	138.56	TYR99, TRP147, TYR9 GLN70, ASP77	GLY1, SER4, ARG2, LEU3
20	GLDFSDLYY	-210.89	133.19	ARG6, GLU212, THR233, ASP30, SER57	ASP3, LEU7 TYR9, TYR8
21	LYYLTMNNK	-209.09	91.98	GLN9, ASN62, LYS7 GLU11, IS13, TYR30 GLN70	MET6, ASN7 TYR2, TYR3 LYS9
23	IVNDENRAK	-202.88	91.61	GLN9, HIS13, THR77 TYR78, ASN82, GLU55 GLN70	ARG7, LYS9 GLU5, ILE1 ASN6
25	VVVLGSQEG	-151.17	95.38	GLN70, SER53	GLU8, VAL1

Note: * Docking score calculates binding affinity between selected epitopes and receptors. A more negative docking score means a more possible binding model.

DRB1*04-01 (PDB ID: 5JLZ) and HLA-A*11-01 allele (PDB ID: 5WJL) were chosen as receptors and the crystal structure was downloaded from the protein data bank. Discovery Studio Visualizer (DSV) was used to remove co-crystallized ligands and water molecules. Finally, the HDOCK server was used to perform the docking operation. Through this server, the affinity of the selected peptides to the receptor and the interactions between them and the binding energy were obtained. The pdb output file from the HDOCK server was analyzed by DSV.

Epitope screening and MEVC design

Based on docking results, suitable epitopes were selected and then connected by KK linkers for the vaccine construction. The KK linker is made of two lysine residues increasing the immunogenic activity of MEVC. In this recombinant vaccine construct, Maltose-bound protein (MBP) was used as an adjuvant in the N terminal of the structure [23]. The connection of MBP to the initial part of the vaccine was done by a rigid Pro-rich linker to improve antigen-specific immune responses.

A multi-epitope vaccine antigenicity and allergenicity assessment

To evaluate the antigenicity and allergenicity of the multi-epitope vaccine, online servers were used. The recombinant construction antigenicity was estimated by the VaxiJen tool. The AllergenFP version 1.0 server was another platform utilized to estimate the allergenicity of the recombinant vaccine.

Calculating physicochemical properties of the Multi-epitope vaccine

The Protparam database was used to calculate the physicochemical properties of the novel vaccine including the number of residues in structure, the number of

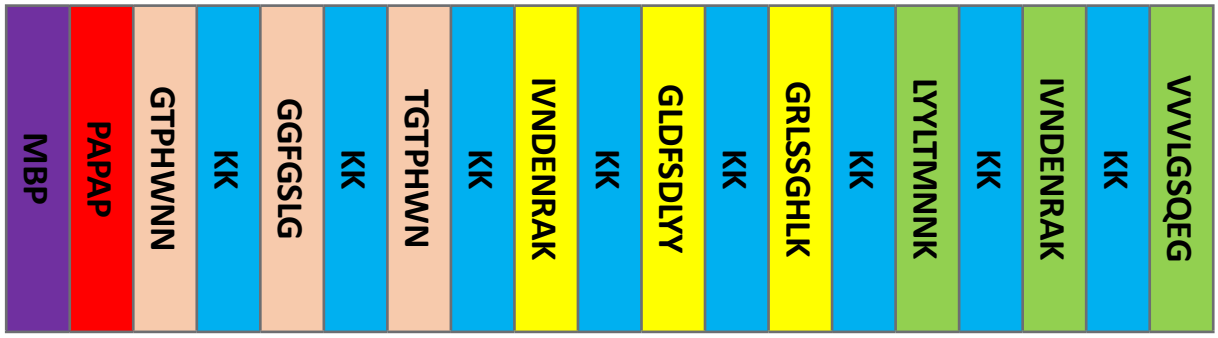

Figure 1. The arrangement of different parts of MEVC. At the amino-terminus end of the vaccine, the adjuvant (purple) is linked by the PAPAP linker (red). *B-cell* epitopes (orange), class I MHC epitopes (yellow), and class II MHC epitopes (green) are linked using KK linkers (blue).

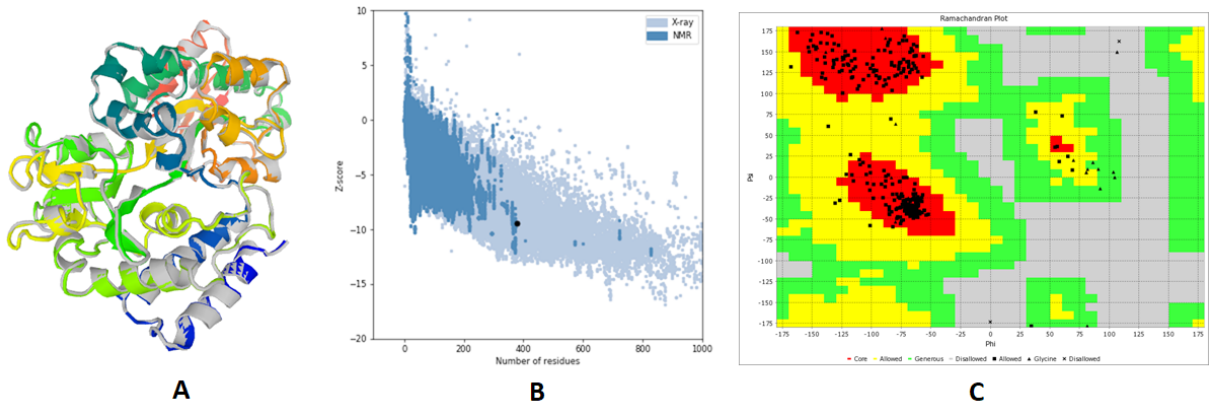

Figure 2 (A). Final vaccine construct after refinement through galaxyrefine server; (B) Z-score plot. (C) Ramachandran plot analysis of the final tertiary structure model.

positively and negatively charged amino acids, extinction coefficient, half-life, protein resistance, alpha helix percentage, and GRAVY.

Secondary and tertiary structure prediction

The secondary structure of the recombinant construct was predicted by Garnier–Osguthorpe–Robson server [24]. The 3D structure of the multi-epitope peptide vaccine was predicted by Phyre2. Phyre2 is a widely used web-based tool for protein structure prediction and analysis [25]. DSV was used for viewing the final structure.

Tertiary structure refinement and validation

To obtain tertiary structure refinement of the recombinant vaccine, GalaxyRefine was used. Then, the validation process was done using two Vadar and ProsA servers. The VADAR web server is freely accessible and calculates φ and ψ torsion angles for each residue in the MEVC. To evaluate the quality of the tertiary structure of the vaccine, the ProSA-web server was used.

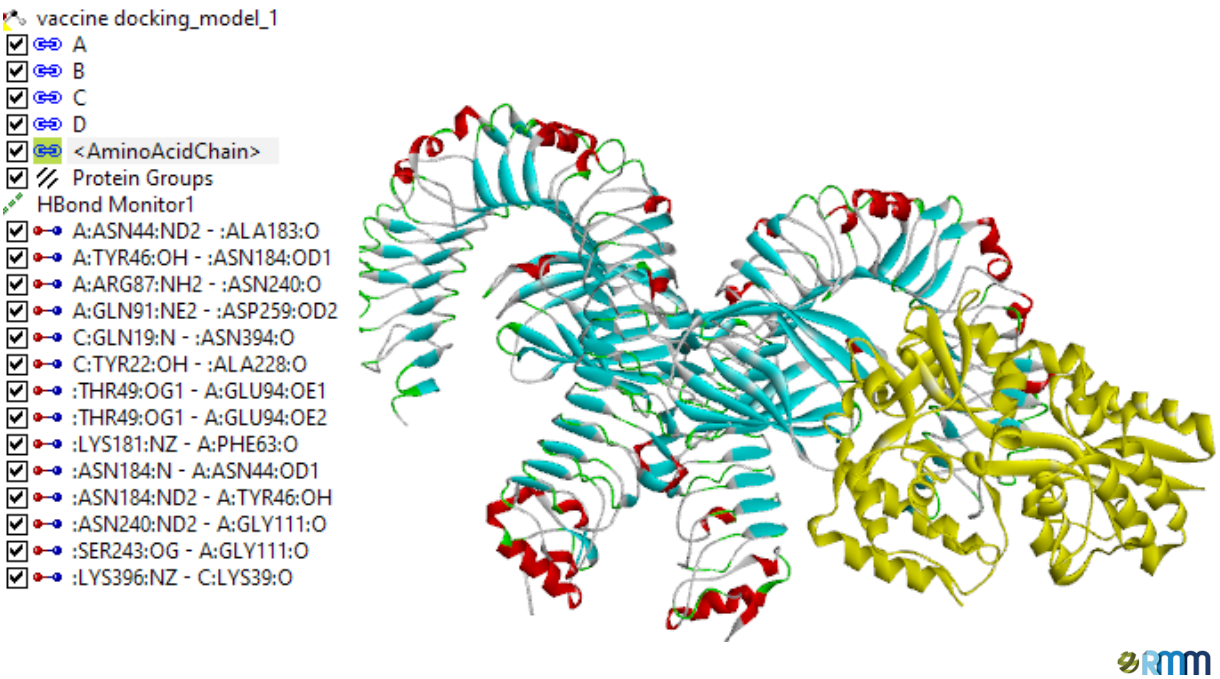

Figure 3. Best docked complex of TLR4/MD2 receptor and the designed vaccine with a list of residues forming H-bonding. MEVC is shown in yellow.

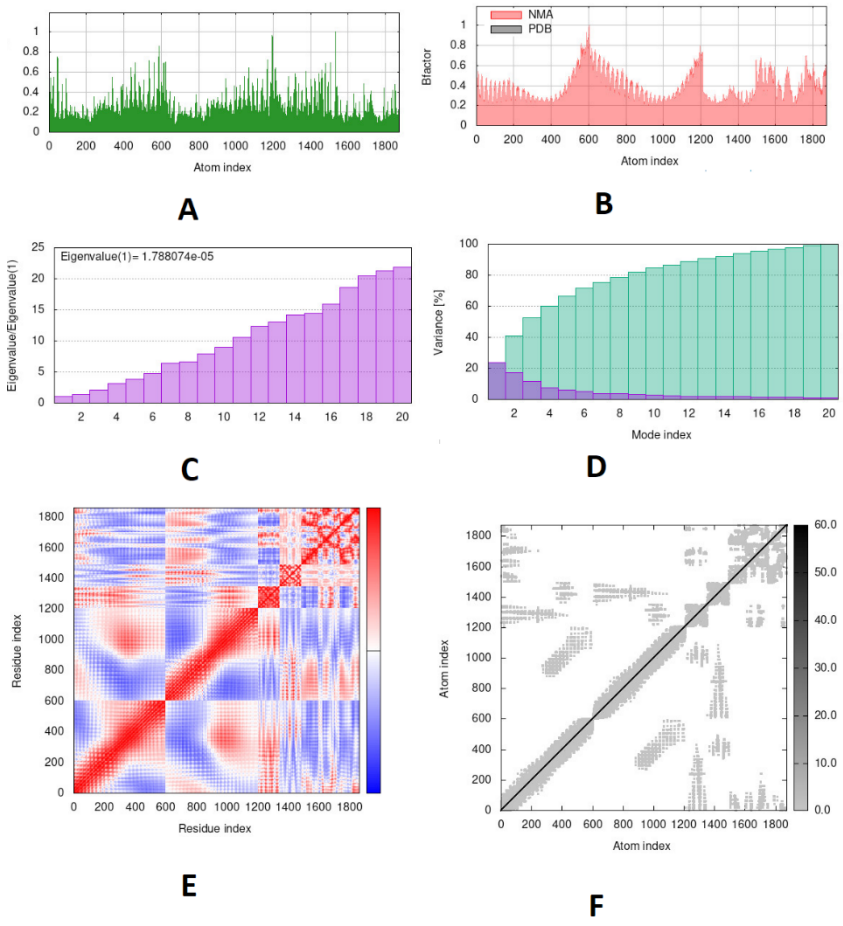

Figure 4. MDS of the complex of TLR4/MD2 receptor and the designed vaccine. deformability (A), B-factor values (B), eigenvalue (C), variance (D), and covariance of residue index (E), and elastic network analysis (F)

Investigation of the interaction of the designed structure and TLR4/MD2 receptor through the docking process

To examine the affinity of the MEVC to toll-like receptors on immune cells, the TLR4 was docked with the vaccine using the HDOCK server. In this process, the pdb file of the TLR4/MD2 receptor with reference number 4G8A was mined from the RCSB server. Then, the best-docked complex generated by the HDOCK server and DSV.

Molecular dynamic simulation

Molecular dynamic simulation (MDS) analysis was performed by the iMODS server. This server is accessible to all users and does not require registration. The iMODS server measured four main factors: covariance, eigenvalues, B-factors, and deformability.

Codon optimization and *in silico* cloning

In the final stage of vaccine design, *in silico* codon optimization and cloning were performed. The sequence manipulation suite server was used for reverse translation and Java Adaptation Tool (JCat) was utilized for codon optimization to clone MEVC in *E. coli* (strain UTI89/UPEC).

Results

Protein sequences retrieval and epitope prediction analysis

The E protein sequences were retrieved from the UniProtKB database in FASTA format (UniProtID: W8QIP7) and then used for *B-cell* and *T-cell* binding epitopes prediction by Immune Epitope Database (IEDB) and RANKEPEP server, respectively.

Allergenicity, toxicity, and antigenicity of potential epitopes

Allergenicity, toxicity, and antigenicity of selected *B-cell* and *T-cell* epitopes were determined. Results are shown in Table 1.

Molecular docking study

The epitopes selected by the above methods were validated by molecular docking. Docking experiments were performed by the HDOCK server between selected epitopes and HLA. Docking score, RMSD in Angstrom (Å), and interaction with amino acid residues are shown in Table 2.

Selection of epitopes and production of multi-epitope peptide vaccine

Finally, based on the ranking of epitopes, allergenicity, toxicity, antigenicity, and docking results of potential epitopes, GTPHWNN, GGFGSLG, and TGTPHWN sequences were selected from *B-cell*, the IVNDENRAK, GRLSSGHLK, and GLDFSDLYY sequences were selected from MHCI, and LYYLTMNNK, IVNDENRAK, and VVVLGSQEG sequences were selected from MHCII. The definitive epitopes from each antigen were fused by linkers. At the amino-terminus end of the vaccine, adjuvant MBP was added by linker PAPAP. The arrangement of different parts of MEVC is shown in Figure 1.

A multi-epitope vaccine antigenicity and allergenicity assessment

Using the servers explained in the methods section, it was determined that the designed vaccine was not an allergen and its antigenicity was 0.5470.

Physicochemical properties of MEVC

The Protparam database predicted that the vaccine with a molecular weight of 55 kDa has a suitable half-life. The instability index was 27.66, the aliphatic index of the protein was estimated to be 71.73, and GRAVY was calculated to be -0.483.

Secondary and tertiary structure prediction

Garnier Osguthorpe Robson server showed that MEVC was composed of 17.35% extended strand, 42.11% alpha-helix (H), and 40.55% random coil (C) secondary structural elements.

The tertiary structure of MEVC was analyzed by Phyre2. The Phyre2 server showed that 72% of the entire vaccine sequence (368 amino acids (was modeled with 100.0% confidence by the single highest-scoring template.

Refinement and validation of the 3rd structure

After checking the 3D structure with the GalaxyRefine tool, we reached the final 5 models. Model 1 is shown as the best structure in Figure 2A. The global distance test's high accuracy was 0.9741 and the root-mean-square deviation was 0.342 for this model. Therefore, model 1 was used for further investigations according to its appropriate parameters. The value obtained for the Z-score with ProSA-web was -9.44 (Figure 2B).

VADAR server was used to analyze the Ramachandran diagram. The server indicated that 95% of amino acid residues were located in the phipsi core region and 3% of them were located in the phipsi allowed region (Figure 2C).

Investigation of the interaction of the designed structure and TLR4/MD2 receptor through the docking process

The designed structure was docked into the binding site of the TLR4/MD2 Receptor. The estimated docking score and Ligand rmsd were -273.73 and 90.36 (Å), respectively. Figure 3 shows the interaction of the multi-epitope vaccine with the TLR4-MD-2 receptor.

Molecular dynamic simulation

The MDS of the complex of TLR4/MD2 receptor and the designed vaccine are shown in Figure 4. The deformability of the ligand-receptor complex and B-factor are shown in Figures 4A and 4B, respectively. Figure 4C shows the eigenvalue of the TLR4/MD2- MEVC complex. The estimated eigenvalue is 1.788, and the colored bars seen in Figure 4D determine the type of variance. The green bars show the cumulative variance and the purple bars show the individual variances. The covariance matrix is in Figure 4E. Figure 4F shows the elastic network analysis.

Codon optimization and *in silico* cloning

After reverse translation, GC content and the Codon Adaptation Index (CAI) of the designed vaccine were evaluated CAI value was 1, and the GC content of the optimized sequence was obtained as 52.59 % in *E. coli* (strain UTI89/UPEC).

Discussion

Prevention of Zika virus infection is a fundamental problem in human societies [26]. Scientists have warned that a small change in the genome of the Zika virus could cause a large and widespread outbreak. Therefore, designing a suitable vaccine is an effective strategy to prevent ZikV disease.

The immunoinformatics approach could be a substantial way for planning multi-epitope-based vaccines against infectious diseases [27]. MEVC can be designed for the antigenic proteins of a pathogen by distinguishing the B- and *T-cell* epitopes.

The envelope (E) protein plays an important role in receptor binding and receptor-mediated endocytosis during virus entry. This surface protein is a major component of the ZIKV virion. [28]. Therefore, in the present study, we identified *B-cell* and *T-cell* epitopes of the Zika virus by targeting the envelope protein using online servers. After screening the predicted epitopes for allergenicity, antigen, and toxicity, to convert the peptide sequence into the 3D structure, we used the PEP-FOLD server. A molecular docking study was performed for further screening based on the binding energy level. Finally, nine epitopes were selected and connected for use in MEVC with the help of linkers. Adjuvants are central to the efficacy of subunit vaccines. To enhance the immunogenicity of the protein vaccine, Maltose-binding protein was selected as an adjuvant and its sequence was extracted from the UniProt database. Maltose-binding protein can bind to disaccharide maltose and transport it from the periplasmic space of microorganisms [29].

We predicted the allergenicity and antigenicity of the vaccine and found that the vaccine designed in this study is not an allergen to humans and possessed proper immunogenicity.

The ProtParam server showed that the physicochemical properties of MEVC are suitable. To carry out the purification process, the molecular weight of the protein must be less than110 kDa. The molecular weight of the vaccine was 55.45 kDa. The aliphatic index of a protein is one of the important factors in protein stability. The designed vaccine was thermostable with an aliphatic index of 71.73. The pH at which the designed vaccine carries no net electrical charge was 8.45. The grand average of hydropathicity is used to indicate the degree of hydrophobicity of a peptide. This factor calculates the hydrophobicity values of all amino acids divided by the sequence length. GRAVY of the vaccine was -0.483. The long half-life of the designed recombinant protein makes it a suitable vaccine against the immune system. The instability index must be less than 40 for the protein to be classified as a stable protein. The instability index of protein was estimated at 27.66 [30-32].

After confirming the stability of the designed vaccine by online software ProtParam, the final multi-epitope subunit vaccine model was generated and refined using the Phyre2 web server and GalaxyRefine, respectively.

Toll-like receptors play an important role during the attack of microorganisms by inducing cytokines and interferons [33]. Vanwalscappel et al. showed that TLR agonists may be useful for the prophylactic or therapeutic treatment of ZIKV [34]. Other studies have also demonstrated Zika virus activates TLR by using nonstructural protein1 to inhibit the innate immune response [10, 35, 36]. Due to the importance of the Toll-like receptor for the Zika virus, this receptor was chosen for final docking.

Molecular docking is a suitable tool to check the affinity of the ligand to the receptor [37-39]. To study the binding affinity of the designed vaccine with TLR4, a molecular docking technique was performed. Through molecular docking studies, the best 3D predicted model of the vaccine was found and then the interaction with amino acid residues and docking energy score were analyzed.

The docking energy score of the best-bound conformation of the vaccine was -273.73 and TYR46, ARG87, GLN91, GLN19, TYR22, GLU94, PHE63, ASN44, TYR46, GLY111, and LYS39 of TLR4 were the sites for hydrogen bonding interactions with multi-epitope subunit vaccine. The results of the studies showed that the designed vaccine has high efficiency and is strongly attached to the TLR4. MDS revealed that MEVC has low deformability. *In silico* studies showed that *E. coli* (strain UTI89/UPEC) is the suitable host for the designed vaccine.

Conclusion

In this study, the coating protein of the virus was used to design the vaccine, and docking screening was done in two steps: once for epitopes and again for the recombinant vaccine. The results of the present study demonstrated a high affinity of the designed vaccine to TLR4/MD2 in the innate immune system. With complementary both *in vitro* and *in vivo* studies, the present study can be useful in eliminating Zika virus disease and problems.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed about the purpose of the research and its implementation stages; they were also assured about the confidentiality of their information; moreover, they were free to leave the study whenever they wished, and if desired, the research results would be available to them.

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Authors contribution's

All authors were equally contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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