Journal of Biomolecular Structure and Dynamics

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tbsd20

Immuno-informatics guided designing of a multi-epitope vaccine against Dengue and Zika

Aditi Bhardwaj, Ritika Sharma & Abhinav Grover

To cite this article: Aditi Bhardwaj, Ritika Sharma & Abhinav Grover (2021): Immuno-informatics guided designing of a multi-epitope vaccine against Dengue and Zika, Journal of Biomolecular Structure and Dynamics, DOI: 10.1080/07391102.2021.2002720

To link to this article: https://doi.org/10.1080/07391102.2021.2002720

JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS
https://doi.org/10.1080/07391102.2021.2002720

Immuno-informatics guided designing of a multi-epitope vaccine against Dengue and Zika

Aditi Bhardwaj[a*], Ritika Sharma[b*] and Abhinav Grover[b]

[a]School of Biosciences & Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India; [b]School of Biotechnology, Jawaharlal Nehru University (JNU), Delhi, India

Communicated by Ramaswamy H. Sarma

ABSTRACT

Dengue and zika are amongst the most prevalent mosquito-borne diseases caused by closely related members Dengue virus (DENV) and Zika virus (ZIKV), respectively, of the *Flaviviridae* family. DENV and ZIKV have been reported to co-infect several people, resulting in fatalities across the world. A vaccine that can safeguard against both these pathogens concurrently, can offer several advantages. This study has employed immuno-informatics for devising a multi-epitope, multi-pathogenic vaccine against both these viruses. Since, the two viruses share a common vector source, whose salivary components are reported to aid viral pathogenesis; antigenic salivary proteins from *Aedes aegypti* were also incorporated into the design of the vaccine along with conserved structural and non-structural viral proteins. Conserved B- and T-cell epitopes were identified for all the selected antigenic proteins. These epitopes were merged and further supplemented with β-defensin as an adjuvant, to yield an immunogenic vaccine construct. *In-silico* 3D modeling and structural validation of the vaccine construct was conducted, followed by its molecular docking and molecular dynamics simulation studies with human TLR2. Immune simulation study was also performed, and it further provided support that the designed vaccine can mount an effective immune response and hence provide protection against both DENV and ZIKV.

Abbreviations: CASTp: computed atlas of surface topography of proteins; DNA: deoxyribonucleic acid; 3D: threedimensional; FASTA: fast all; HABA: 4′-hydroxyazobenzene-2-carboxylic acid; IC_{50}: half maximum inhibitory concentration; HLA: human leukocyte antigen; Ig: immunoglobulin; IEDB: immune epitope database; MHC: major histocompatibility; NCBI: National Centre for Biotechnology Information; BLASTp: protein BLAST; ProSA: protein structural analysis; RNA: ribonucleic acid; RCSB: Research Collaboratory for Structural Bioinformatics; TLR2: Toll-like receptor 2

ARTICLE HISTORY
Received 18 June 2021
Accepted 28 October 2021

KEYWORDS
Multi-epitope; multi-pathogenic; immuno-informatics; Dengue virus; Zika virus; vaccine

1. Introduction

Dengue virus (DENV) and Zika virus (ZIKV) are closely related members of the *Flaviviridae* family that cause the diseases dengue and zika, respectively. Both dengue and zika represent a major health burden and are prevalent across the globe. Members of *Flaviviridae* are enveloped viruses having positive single-stranded RNA as their genetic material (Supriyono et al., 2020). The DENV has four genotypically distinct variants referred to as DENV serotypes viz. DENV1–DENV4, with a genomic identity of ≈65% (Estofolete et al., 2019), whereas ZIKV has two lineages: African and Asian (Dowd et al., 2016) but no serotypes. Both DENV and ZIKV are transmitted to humans mainly via *Aedes aegypti* mosquitoes. DENV infection causes an array of symptoms from mild dengue fever (DF) to grievous dengue hemorrhagic fever (DHF) that result in fatalities (Joob & Wiwanitkit, 2020); whereas fever, rashes, muscle and joint pain are common symptoms of ZIKV infection (Guanche Garcell et al., 2020).

Dengue affects around 50–100 million people every year, worldwide (Rodriguez-Morales et al., 2016). Approximately 40% of the world population inhabits dengue-prone areas and is thus at a high risk of transmission; around 100 countries are found to be endemic for this disease (Miah & Husna, 2021). Unlike dengue, zika is known to be transmitted sexually and vertically amongst humans (Comeau et al., 2020).

As both viruses are transmitted by a common vector viz. *A. aegypti*, co-circulation of DENV and ZIKV is quite prevalent in many geographical regions, especially the sub-tropical and tropical regions (Pessoa et al., 2016). Although the mortality rate of zika is not as high as dengue or any other arboviral disease, the infection rate is quite high. Also, zika is quite often linked to various abnormalities such as Guillain-Barre syndrome, and therefore the concerns regarding this disease have increased considerably (Grijalva et al., 2020). Due to the

CONTACT Abhinav Grover agrover@jnu.ac.in School of Biotechnology, Jawaharlal Nehru University (JNU), Delhi, 110067, India
*These authors contributed equally to the study.

Supplemental data for this article can be accessed online at https://doi.org/10.1080/07391102.2021.2002720.

presence of a common vector involved in the transmission, prospects of dengue–zika co-infections within human and mosquito populations are very prominent (Chaves et al., 2018). Hence, researchers across the globe are trying hard to conceive a secure and effective vaccine that may perhaps target both DENV and ZIKV simultaneously.

Though 'DENGVAXIA', the first-ever marketed vaccine for dengue got licensed in 2015, it still poses certain limitations. One such being, its efficacy requires prior exposure to at least one of the four DENV serotypes (Aguiar et al., 2016). Also, the use of a live attenuated form for immunization poses safety risks (Fatima & Syed, 2018). On the contrary, there is no licensed vaccine commercially available for zika currently.

Vaccine designing using immuno-informatics is a practical approach relying on accession and analysis of the data related to the immune system and the pathogens (Tahir Ul Qamar et al., 2020). The entire process is capable of cutting down the labor and cost of experimentation considerably, and simultaneously providing systematic screening of the pathogen proteomes (Jakhar & Gakhar, 2020). A multi-epitope vaccine offers several advantages over the conventional vaccines. These peptide or protein vaccines are regarded as safe, since they do not involve any part of the pathogen. Moreover, these vaccines can be designed in a time-saving manner as many wet-lab procedures such as microbial culturing can be surpassed in the initial stages (Yazdani et al., 2020). A vaccine construct needs to fulfill certain crucial requisites: high immunogenicity and antigenicity, optimal physicochemical properties, and it should also be non-allergenic and non-toxic, to become a potential candidate in vaccine discovery (Ayyagari et al., 2020). Here, immuno-informatics has been utilized to design a multi-pathogen vaccine against both dengue and zika. Although immune-informatics has been utilized to design separate vaccines against dengue and zika, there are very few reports where a single vaccine has been proposed to combat these diseases simultaneously.

The present literature available for both Dengue and Zika was searched to identify proteins that are involved in disease pathogenesis. This search was further narrowed down by considering the amino acid identity shared between the proteins across the DENV1–4 serotypes and ZIKV. Saliva from *A. aegypti* contains several crucial proteins that facilitate the replication of both DENV and ZIKV, via their ability to modulate the host immune response (Sri-In et al., 2019). Therefore, we opted to also incorporate crucial antigenic salivary proteins from *Aedes* in assembling the vaccine. Moreover, the presence of immunogenic salivary proteins from *A. aegypti* further enables the inclusion of additional epitopes that can assist in offering collective protection against not just DENV and ZIKV but also other infectious viruses such as chikungunya and yellow fever virus that are similarly spread by *A. aegypti* to humans (Narula et al., 2018).

The DENV structural envelope glycoprotein (E) and the non-structural (NS) proteins – NS3 and NS5 are reported amongst the most conserved proteins, across all the four DENV serotypes; and they also share significant sequence identity with their respective ZIKV counterparts (Abdul Ahmad et al., 2017; Swarbrick et al., 2017; Zhao et al., 2015). Moreover, these three proteins are critical in the viral infection and replication process inside the host cells, thus these viral proteins were selected for vaccine design (Elshahawi et al., 2019). Likewise, the *A. aegypti* salivary proteins – neutrophil stimulating factor 1 (NeSt1; Hastings et al., 2019), Venom allergen 3 and the 34 kDa putative secreted protein all play vital roles in the infection process of DENV and ZIKV (Sun, Nie, et al., 2020; Surasombatpattana et al., 2014). Thus, these three salivary proteins from *A. aegypti* were also selected for vaccine design.

Following the identification of target proteins; conserved, immunogenic, and non-allergenic epitopes were mined out for all the target proteins. These epitopes were then combined and also merged with the β-defensin adjuvant sequence by utilizing linkers, to finally build the vaccine construct.

The designed vaccine was found to exhibit desired physicochemical properties and a stable 3D structure on analysis by *in-silico* studies. The proposed vaccine also displayed antigenic potential via its interaction with the immune receptor molecule viz. human TLR2 (Toll-like receptor 2), as observed by molecular docking study. Immune simulation studies further supported the immunogenic capacity of the proposed vaccine; wherein, it was found that the designed vaccine can simultaneously generate an antibody-mediated and cellular immune response. Preliminary codon optimization and *in-silico* cloning analyses affirm the potential of producing the vaccine construct recombinantly for future *in-vitro* testing studies. Overall, the *in-silico* analysis conducted in this study affirms the viability of the proposed vaccine and provides a lead that can be pursued further for laboratory efficacy testing against both DENV and ZIKV.

2. Materials and methods

2.1. Literature survey and selection of proteins

The prevalence of multiple DENV serotypes makes the devising of an effective vaccine more difficult (Khan et al., 2008; Wen & Shresta, 2019). For a vaccine to be effective against dengue, protection must be provided against all the four DENV serotypes. However, in the case of zika only a single ZIKV serotype is known (Dowd et al., 2016). Therefore, we searched the literature for identifying the most conserved and antigenic proteins across all the four DENV serotypes and the ZIKV proteome. Also, because both ZIKV and DENV are transmitted by *A. aegypti* mosquitos, whose saliva contributes to their infection process (Oktarianti et al., 2015), a search for these vital salivary proteins was conducted in the literature already available.

The complete sequence of the selected proteins was retrieved from the NCBI (National Centre for Biotechnology Information) database (https://www.ncbi.nlm.nih.gov/; Pruitt et al., 2007) in FASTA format. The antigenicity of all these proteins was assessed by the ANTIGENpro tool (http://scratch.proteomics.ics.uci.edu/) of the SCRATCH prediction server (Cheng et al., 2005). To ensure that the selected proteins bear no homology with human proteins, BLASTp was

performed. For this, individually each selected protein was provided as a query and searched against the human proteome.

2.2. Identification and selection of epitopes

The prediction of epitopes specific for all the immune cell types was performed using different, freely available online servers (Desai & Kulkarni-Kale, 2014; Soria-Guerra et al., 2015). Cytotoxic T cells, helper T cells, and B cells, collectively play the most significant role in eliciting immune responses on encountering any antigen. Herein, B-cell, as well as T-cell epitopes, were mined so that both branches of acquired immunity get activated by the devised vaccine. These epitopes were individually identified for both the selected salivary proteins of *A. aegypti*; and the structural (Envelope) and NS proteins (NSP3 and NSP5) from across DENV1–4 and ZIKV. Since, the intention was to propose a multi-pathogenic, multi-epitope vaccine, capable of safeguarding against both the viruses; hence, identifying epitope sequences that are conserved among these two viruses and their strains was the focus in this study.

2.2.1. B-cell epitope prediction

The ABCPred server (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html) was utilized for the identification of B-cell epitope sequences. Predictions were made using the default settings provided for the different parameters, except for, a window length of 10 amino acid residues. ABCPred computes the amino acid stretches present within a protein, that possibly directs a humoral immune reaction on interacting with lymphocytic B cells under physiological settings (Sun et al., 2019).

For all the viral proteins, the B-cell epitopes determined by ABCPred were examined for sequence conservancy across all the four DENV serotypes and ZIKV. Only those epitopes that were above the threshold score and at the same time conserved, were taken further into the vaccine design. While in the case of *A. aegypti* salivary proteins, the top-scoring B-cell epitopes were selected for vaccine design.

2.2.2. Cytotoxic T-cell epitopes prediction

The epitopes corresponding to the cytotoxic T-lymphocytes (CTL)–MHC(I) interaction were obtained using the NetCTL 1.2 Server (http://www.cbs.dtu.dk/services/NetCTL/). This server utilizes artificial neural networks, by contemplating functions such as MHC(I) binding potency, proteasomal cleavage site accessibility, and other associated antigen processing and presentation activities to identify functional epitopes (Dar et al., 2016). Individually each selected protein sequence was submitted to the server and predictions were run using all default settings, except selecting only three of the 12 MHC(I) binding supertypes viz. A2, A3, and B7. This was done because these three MHC(I) supertypes are known to collectively account for more than 88% of the total global population (Lim & Khan, 2018).

Only those epitopes amongst the ones obtained by the server that displayed some level of conservancy across the different serotypes of DENV and that of ZIKV were selected for further utilization in vaccine construction. However, in the case of NeSt1, Venom Allergen 3, and 34 kDa putative secreted protein from *Aedes* saliva the top-scoring epitopes predicted for each of the three MHC supertypes were selected.

2.2.3. Helper T-cell epitopes prediction

The IEDB (immune epitope database) server (https://www.iedb.org/) was used for the identification of the Helper T-lymphocytes (HTL)–MHC(II) binding epitopes for all the target proteins (Dhanda et al., 2019). Each of the selected antigenic proteins was individually entered to the T-cell epitope MHC II binding tool of the IEDB server. Predictions were executed by keeping all the parameters as default, and by opting for the entire reference set of human HLA (human leucocyte antigen) alleles. The entire human HLA reference set was selected to acquire epitopes that are capable of covering all the MHC II alleles present globally (Chatterjee et al., 2020).

For all the viral proteins a minimum of two high-ranked epitopes, that were found to be conserved and had low IC_{50} (half maximum inhibitory concentration) values were selected (Khatoon et al., 2017). Likewise, for the three *Aedes* salivary proteins, only the top two scoring HTL epitopes were selected for vaccine design.

2.3. Vaccine design and construction

The predicted epitopes were combined using suitable linker amino acid sequences and an adjuvant to yield the vaccine construct. Initially, three different vaccine constructs were designed, differing only in their adjuvant sequence. The three different peptide adjuvants utilized were: bacterial L7/L12 ribosomal protein (NCBI accession: BAA98290.1), *Mycobacterium tuberculosis* HABA (4′-hydroxyazobenzene-2-carboxylic acid) protein (NCBI accession: AGV15514.1), and human β-defensin-3 (NCBI accession: NP_061131.1). Adjuvants enhance the immunogenicity of vaccines and are especially crucial for use with peptide vaccines.

The vaccine constructs were created by combining the above-identified epitopes in the order that follows – beginning with the adjuvant at the N terminal, followed by the EAAKK linker which connects the B-cell epitopes that are individually inter-connected using the linker sequence KK, then followed by the GPGPG linker that joins the HTL epitopes together and finally the AAY linker that combines the CTL epitopes. The linkers used in assembling the vaccine construct have defined functions that can help in providing additional stability to the vaccine construct. The KK linker is known to enhance proteasomal processing (Yano et al., 2005), while the GPGPG linker aids in improving structural flexibility of the protein (Livingston et al., 2002); likewise the EAAAK linker promotes the formation of α helix secondary structure in the designed vaccine (Srivastava et al., 2020).

2.4. Antigenicity, allergenicity, and toxicity evaluation

The VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) and ANTIGENpro (http://scratch.proteomics.ics.uci.edu/) servers were used for computing and comparing the individual antigenicity of the three assembled vaccine constructs. VaxiJen evaluates antigens on the measure of auto cross-covariance of the peptide sequences which is further centered on their physicochemical properties rather than sequence alignment (Doytchinova & Flower, 2007). ANTIGENPro on the other hand is an alignment-independent, sequence-based tool for protein antigenicity prediction, that does not consider the nature of the pathogen into account while computing the antigenic score (Lee et al., 2020). Further structure modeling and validation analyses were performed only with the vaccine construct that had the maximum antigenic potency as computed via the VaxiJen and ANTIGENPro servers.

For assessing the toxicity and allergenicity of the selected epitopes, the ToxinPred server (https://webs.iiitd.edu.in/raghava/toxinpred/algo.php) was used. It predicts the peptide toxicity based on the quantitative matrix-based, position-specific scores (Gupta et al., 2013). Each epitope was individually assessed for toxicity analysis.

AllerTop server (https://www.ddg-pharmfac.net/AllerTOP/) was used for evaluating the allergenic potency of the vaccine. AllerTop predictions utilize an alignment independent approach, wherein the chemical nature of the constitutive amino acid sequences of the protein is taken into account for identifying probable allergens (Dimitrov et al., 2014).

2.5. Physiochemical properties evaluation and solubility check

The ExPASy server's ProtParam tool (https://web.expasy.org/protparam/) was utilized for examining the physicochemical properties of the designed vaccine construct (Wilkins et al., 1999). These properties included determination of amino acid composition, number of negative and positive amino acid residues, molecular weight, aliphatic index, and estimated half-life to name a few.

Solubility assessment of the vaccine was performed using the SOLPro tool (http://scratch.proteomics.ics.uci.edu/) available at the SCRATCH server. It has been found that there is a significant opportunity of increasing the production of recombinant proteins via *in-silico* experimentation using different bioinformatics tools. Protein solubility prediction tools are one such means, as they provide an initial estimate of production viability and thus avoiding the non-promising trial and error process of the *in-vitro* experimentation (Magnan et al., 2009).

2.6. Structure prediction

The 3D modeling of the construct was performed using the I-TASSER (Iterative Threading ASSEmbly Refinement) server (https://zhanggroup.org/I-TASSER/; Yang et al., 2015). The server utilizes several threading alignments and iterative structural assembling to yield models of a protein's structure (Zheng et al., 2019). The major advantage that I-TASSER offers over other 3D structure prediction servers is that it constantly brings the initial template structures nearer to their original states via the fragment assembly procedure (Zhang, 2007). Default server settings were used while submitting the sequence of the designed vaccine construct for 3D modeling.

2.7. Model refinement and validation

In-silico 3D structure prediction is prone to errors such as conformational abnormalities in the amino acid side chains, abnormal bonds or bond lengths, angles, and contacts in the protein backbone. Therefore, before the predicted 3D structure is used for conducting any study, it has to be refined and corrected for such errors. The Galaxy refine server was used for structural refinement of the designed vaccine's structure as obtained by I-TASSER. The server upon job completion returns different models of the input structure (Blankenberg et al., 2010).

Further, the refined model having the lowest Molprobity score was examined for its structural stability using three different 3D structure validation tools – ProSA (protein structural analysis, https://prosa.services.came.sbg.ac.at/prosa.php), ERRAT (https://servicesn.mbi.ucla.edu/ERRAT/), and PROCHECK (https://servicesn.mbi.ucla.edu/PROCHECK/). ProSA evaluates the overall as well as the local quality of the model (Wiederstein & Sippl, 2007), likewise, PROCHECK assesses the stereochemical quality of the 3D structure (Laskowski et al., 1996) and the ERRAT server analyzes the non-bonded atomic interaction wherein the high scores indicate higher quality (Zobayer, 2019).

2.8. Molecular interaction with human TLR2

To predict the feasibility of an immunogenic collaboration between the designed vaccine and human TLR2, a molecular docking study was performed using the HADDOCK 2.2 server (https://milou.science.uu.nl/services/HADDOCK2.2/; Karaca et al., 2010). The crystal structure of human TLR2 was downloaded from the RCSB Protein Databank (https://www.rcsb.org/; PDB ID: 3A7B). HADDOCK requires information about the active binding sites or interacting amino acid residues at the time of job submission, the same was predicted for both the vaccine construct and the TLR2 using the CASTp server (http://sts.bioe.uic.edu/castp/index.html?1ycs; Tian et al., 2018). The docked complex was evaluated via PDBsum server (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html) for identifying the different binding interactions between the vaccine and the TLR-2 molecules.

2.9. Molecular dynamics simulation of the docked complex

The strength of the interaction between vaccine and TLR2 was assessed by simulating the biological environment, using GROMACS (GROningen MAchine for Chemical Simulations). GROMACS is a command-line program operated using Linux.

Table 1. List of the selected proteins used in the design of the vaccine and their NCBI accession numbers.

S. No.	Protein	Organism	Accession no.
1.	Envelope	DENV1	NP_722460.2
		DENV2	NP_739583.2
		DENV3	YP_001531168.2
		DENV4	NP_740317.1
		ZIKV	YP_009227198.1
2.	Non-structural-3	DENV1	NP_722463.1
		DENV2	YP_001531172.2
		DENV3	NP_739587.2
		DENV4	NP_740321.1
		ZIKV	YP_009227202.1
3.	Non-structural-5	DENV1	NP_722465.1
		DENV2	YP_001531176.2
		DENV3	NP_739590.2
		DENV4	NP_740325.1
		ZIKV	YP_009227205.1
4.	Venom allergen 3	*Aedes aegypti*	XP_001651000
5.	Putative 34 kDa family protein	*Aedes aegypti*	ABF18170
6.	Neutrophil stimulating factor 1 (NeSt1)	*Aedes aegypti*	XP_001657055

The GROMOS96 54a7 force field and the SPC216 water model were used in setting up the simulation. Simulation comprised of four key steps – energy minimization, temperature stabilization – number volume temperature (NVT) and number pressure temperature (NPT) equilibrations at normal temperature and pressure of 300 K and 1 atm, respectively, and the final MD run. After completion, analysis was carried out and parameters that define the stability of the docked complex on simulation such as root mean square deviation (RMSD), root mean square fluctuation (RMSF), the radius of gyration (R_g), and solvent accessibility surface area (SASA) were computed. All the simulation plots and graphs were analyzed using the Xmgrace tool of GROMACS. Until a stable RMSD profile was obtained, the simulation was extended in time steps of 10 ns.

2.10. Codon optimization and in-silico cloning of the construct in E. coli

Codon optimization was done using the Java codon adaptation tool (JCat, http://www.jcat.de/; Grote et al., 2005). Optimization is considered to be a crucial step for studying the expression of a synthesized gene construct in a particular host and involves the process of reverse translation (Lewis et al., 2020). While performing codon optimization, *E. coli* (K12 strain) was selected as the host and rho-independent transcription, restriction cleavage sites, and prokaryotic ribosome binding sites were avoided. The output was generated in terms of codon adaptation index (CAI) and %GC content. For *in-silico* cloning of the vaccine construct, the *E. coli* pET-28a (+) expression vector was chosen. The codon-optimized sequence obtained using JCat was cloned within the NdeI and BamHI restriction sites of this plasmid by utilizing the SnapGene software (https://www.snapgene.com/; Khan et al., 2021).

2.11. Immune simulation

The immunogenic effect of the proposed vaccine in the event of its administration in humans was evaluated by performing an immune simulation study. The C-ImmSim server (http://www.cbs.dtu.dk/services/) was used for performing the same. This server works on the position-specific scoring matrix (PSSM) and machine learning techniques for the identification of epitopes and prediction of associated cell and humoral-mediated immune responses (Castiglione et al., 2007).

All the default settings were used for setting the simulation except for the time steps that were set as 1, 84, and 170. A time step of value 1 implies the time of injection as zero and each time step is equivalent to 8 h. These parameters were used as they would ensure that three injections can be administered at an interval of 4 weeks for simulating exposure to the antigen in a repeated manner, which is typically observed in the areas of the endemic to explore for clonal selection.

3. Results

3.1. Literature survey and selection of antigenic proteins

The different DENV serotypes (~65% genetically similar), exhibit significant structural and genetic similarity with the ZIKV. The organizational structure for both DENV and ZIKV bears a resemblance, this includes the arrangement of the envelope protein (E); which is also the principal target for antibody responses in Dengue and Zika infections. The response of cell-mediated immunity to DENV and ZIKV is majorly directed toward the NS proteins, which are more conserved than structural proteins in members of the Flaviviridae family (Wen & Shresta, 2019). While the CTL responses to DENV are directed mainly to NS3, NS4B, and NS5 (Weiskopf et al., 2013), the HTL responses target NS3, NS5, and the structural Capsid (C) protein (Grifoni et al., 2017; Rivino et al., 2013). In the case of ZIKV, cell-mediated responses are directed toward both structural proteins (E, C, and pre-Membrane) and NS proteins (NS1, NS2A, NS3, NS4B, and NS5; Elong Ngono et al., 2017; Koblischke et al., 2018; Wen et al., 2017).

The E protein shares substantial amino acid sequence identity across DENV1–4 and ZIKV; 60–75% identical among DENV1–4 and 54–59% identical between ZIKV and DENV1–4 (Diamond et al., 2019). Amongst the NS proteins, NS3 and

NS5 are the most conserved. NS3 is 77% identical across DENV1–4 and 66–67% amongst DENV and ZIKV, while NS5 is 74–84% identical amongst the four DENV serotypes and 67–69% identical when DENV and ZIKV are compared (Wen & Shresta, 2019). The NS3 and NS5 proteins contain important enzymatic activities necessary for flaviviral RNA replication (Keller et al., 2006; Li et al., 2005). Keeping the above facts in mind, the E, NS3, and NS5 proteins from ZIKV and DENV1–4 were selected for vaccine design.

Since salivary proteins of *A. aegypti* perform a crucial part in promoting the replication and transmission of both DENV and ZIKV in the host (McCracken et al., 2014; Sri-In et al., 2019) therefore we also chose to incorporate them in the design of the proposed vaccine. Several reports highlight the potential of targeting these proteins for protection against flaviviral infection (Manning et al., 2018). Passive immunization against *A. aegypti* salivary protein NeSt1 in mice was successful in ameliorating ZIKV induced pathogenesis (Hastings et al., 2019). The NeSt1 protein is implicated in the activation of neutrophils at the site of a mosquito bite, neutrophils are further involved in the activation of macrophages that become sites for viral infection. Likewise, the *A. aegypti* 34 kDa salivary protein is known to enhance the replication of DENV in keratinocytes (Surasombatpattana et al., 2014) and the venom allergen protein is involved in the transmission of both DENV and ZIKV in host cells by promotion of autophagy (Sun, Nie, et al., 2020). Based on these studies we chose to proceed with the salivary proteins viz. NeSt1, Venom Allergen, and 34 kDa protein in the design of the vaccine.

The complete amino acid sequence for all the selected proteins shown in Table 1 was downloaded from the NCBI database. The antigenic potential for each of the selected proteins was confirmed via ANTIGENpro (Supplementary Table S1). BLASTp analysis confirmed that none of the selected proteins had a significant resemblance with any human protein.

3.2. Identification of B-cell epitopes

The B-cell epitopes for the viral and *Aedes* salivary proteins were predicted using the ABCPred server. For the different viral proteins, amongst the identified epitopes only the top-scoring epitopes that presented significant conservancy were selected (Supplementary Table S2). Thus, we were able to derive one epitope each from the Envelope, NS3, and NS5 proteins of DENV1–4 and ZIKV. However, in the case of each salivary protein from *Aedes*; the top-scoring B-cell epitope identified by the ABCPred server was selected.

3.3. Identification of T_C cell epitope

The T_C cell epitope prediction for viral and *Aedes* salivary protein was executed using the NetCTL server where the input was the FASTA format of the sequence for all the selected proteins. The default server parameters were used, and only the A2, A3, and B7 supertypes were selected for predictions. This was done because these supertypes together comprise almost 88% of the world population (Lim & Khan, 2018).

The server sorts the epitopes identified based on their percentile score. A higher score signifies a better binding affinity of the peptide/epitope with the MHC (I) molecules. Only the top-ranking and significantly conserved CTL epitopes identified across the DENV1–4 serotypes and ZIKV for the selected viral proteins were chosen for vaccine design (Supplementary Table S3). In the case of *A. aegypti* salivary proteins, the top-ranked epitopes were selected, one from each of the selected HLA supertypes (Supplementary Table S3).

3.4. Identification of T_H epitopes

T-helper cell epitopes were obtained by using the IEDB server (Zhang et al., 2008). For the different viral proteins used in the study, the best-ranked epitopes that were predicted by the IEDB server and displaying lower IC_{50} values were checked for conservancy across the different Dengue serotypes and ZIKV. Only conserved epitopes were selected for further study (Supplementary Table S4). In the case of salivary proteins from *A. aegypti* the top two ranked epitopes were selected (Supplementary Table S4).

3.5. Design of the vaccine construct, antigenicity evaluation, and safety assessment

Initially, three vaccine constructs differing only in their adjuvant sequence (human β-defensin, HABA protein, and bacterial L7/L12 ribosomal protein) were designed. Each of these constructs was evaluated for its antigenicity potential using the VaxiJen and ANTIGENpro servers. The vaccine construct adjuvanted with human β-defensin-3 exhibited superior antigenicity based on higher scores obtained via ANTIGENpro and VaxiJen, as compared to the other two constructs (Supplementary Table S5).

To evaluate the safety of the designed vaccine, toxicity analysis was carried out. Each epitope sequence utilized in building the vaccine construct was individually analyzed using the ToxinPred server and acceptable results were obtained (Supplementary Tables S6–S8). Further, AllerTop server identified the designed vaccine construct to be non-allergic and hence safe for administration into humans.

3.6. Physiochemical properties assessment and safety prediction

The vaccine exhibited desirable physicochemical properties (Table 2). The Aliphatic index which indicates the thermostability of the protein was predicted to be 71.95; the grand average of hydropathicity (GRAVY) index which measures the hydrophilicity and hydrophobicity of the polypeptide was found to be −0.218. The instability index was predicted as 33.49 (which lies below the threshold value of 40), thus predicting the protein to be stable on expression in host systems. Furthermore, the SOLPro tool of the SCRATCH suite

Table 2. The predicted physicochemical properties of the proposed vaccine construct.

Feature	Computed value
Number of amino acids	575
Molecular weight	63100.56
Theoretical pI	10.08
Extinction coefficient ($M^{-1}\,cm^{-1}$)	91525
Estimated half-life	30 h (mammalian reticulocytes, *in vitro*)
Instability index	33.49 (stable)
Aliphatic index	71.95
Grand average of hydropathicity (GRAVY)	−0.218

also identified the protein as soluble, with a probability value of −0.5172.

3.7. 3D Modeling, structural refinement, and validation

I-TASSER server was used for generating a 3D model for the designed vaccine (Yang & Zhang, 2015). The server-generated five different structure predictions, of these predicted models the one having the best C-score (confidence score) of −1.26 was selected for further studies. An ideal C-score ranges between the values −5 to 2, the higher the score better is the quality of the generated structure.

Further, the best I-TASSER generated model was refined using the Galaxy Refine server. Out of the refined models produced by the Galaxy server, the model corresponding to the lowest Molprobity score (2.166) and RMSD (0.465) value was downloaded as the final 3D structure for the designed vaccine construct. ProSA, PROCHECK, and ERRAT analysis interpreted the predicted 3D structure to have acceptable structural quality as shown in Figure 1. Ramachandran analysis performed by PROCHECK indicated that 85.5% (Figure 1(B)) of the residues were present in the energetically favorable region (Pandey et al., 2019; Samad et al., 2020). Similarly, the ERRAT server predicted the overall quality factor for the 3D model as 88.70 (Figure 1(E)), and Z-score identified by ProSA for the vaccine model was found to be −3.72 (Figure 1(C)), well within the array of Z-scores of other similar proteins.

3.8. Molecular docking of the vaccine construct with human TLR2

TLR's are primary immunogenic receptors that contribute to improving vaccine efficacy. The designed vaccine utilizes human β-defensin-3 as an adjuvant which is a known agonist of TLR2 (Funderburg et al., 2007). Therefore, we chose to study the interaction between the vaccine and TLR2 via molecular docking using the HADDOCK webserver, Figure 2. HADDOCK clustered a total of 129 structures in 13 clusters. Each HADDOCK cluster provides an average of RMSD values; van der Waals, electrostatic and desolvation energy values, etc., and the one having the lowest *Z*-score is considered as the best cluster as it signifies considerably good interaction within the two molecules. Cluster 1 having the *Z*-score of −1.9 was identified as the best cluster with the details given in Table 3. PDBsum enabled the identification of key amino acid residues involved in binding interactions between the vaccine and TLR-2 molecules and pinpointed the formation of 12 Salt-bridges and 34 Hydrogen bonds within the two (Supplementary Figure S2).

Table 3. Summary of the best cluster obtained after successful molecular docking between the vaccine construct and TLR2.

HADDOCK score	−82.4 ± 2.0
Cluster size	32
RMSD from the overall lowest-energy structure	2.0 ± 1.3
van der Waals energy	−140.7 ± 20.5
Electrostatic energy	−772.1 ± 52.1
Desolvation energy	42.4 ± 19.8
Restraints violation energy	1703.3 ± 72.86
Buried surface area 5027.3	5155.7 ± 195.8
Z-score	−1.9

3.9. MD Simulation

Results of MD simulation performed on the TLR2, and vaccine docked complex were analyzed via trajectory analysis. The MD simulation was performed for a total time of 100 ns, within which the firmly steady trajectory was obtained, and the representative configurations were retrieved, this was followed by further analysis.

The fluctuation of the main chain atoms for the obtained simulated structures in comparison to the initial structure was used as a source and was measured through RMSD (Figure 3(A)). The RMSD plot indicates that the RMSD fluctuation gets stabilized somewhere between 15 and 50 ns during the MD simulation, with values ranging from 1.4 nm to 1.6 nm. This stable trajectory was used for further carrying out RMSF analysis (Figure 3(B)). RMSF is calculated by analyzing the fluctuations of the individual amino acid residues from their average position. The RMSF plot revealed only slight shifts in the side chains of amino acids, implying persistent interaction between the designed vaccine and the TLR-2 receptor. Furthermore, solvent accessible surface area (SASA) that examines the exposure of protein surface to the surrounding solvent molecules and the radius of gyration (R_g) analysis were also conducted. SASA values for the simulation reached a steady value of ≈590 au around 70 ns (Figure 3(C)). Also, they remained close to this range till the end of the simulation, indicating the presence of a stable structure with robust binding interactions between the TLR2 and vaccine. The radius of gyration (R_g) analysis was done to analyze the changes in the compression of TLR2 vaccine docked complex during the simulation. The R_g plot attained displays the decrease in the values of radius of gyration which is indicative of increased compaction of the protein complex, and this perseveres throughout the runtime (Figure 3(D)). The increased compactness of the complex as the simulation progresses further indicates stable interaction.

3.10. Codon optimization and in-silico cloning of the construct in E. coli

For evaluating the expression of the designed protein vaccine in *E. coli* codon optimization and *in-silico* cloning were performed. The CAI of the vaccine construct was found to be

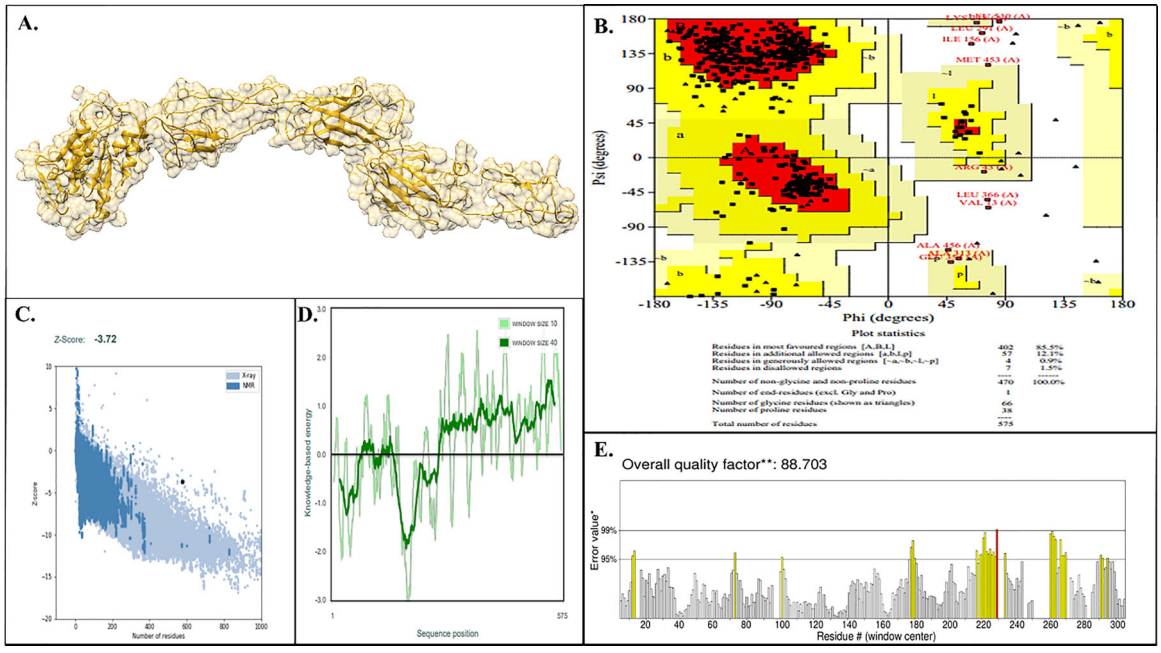

Figure 1. Structure validation analysis of the modeled vaccine construct. (A) The Refined tertiary structure of the protein vaccine. (B) Ramachandran plot produced using PROCHECK. The zones displayed using different colors viz. red, yellow, and light yellow depict the most favored regions (85.5%), additional allowed regions (12.1%), and disallowed regions (0.9%), respectively. (C) ProSA Z-score (Overall model quality) was predicted as −3.72, within the ideal range. (D) ProSA graphical plot (Local model quality) (E) The overall quality of the model is predicted by the ERRAT server.

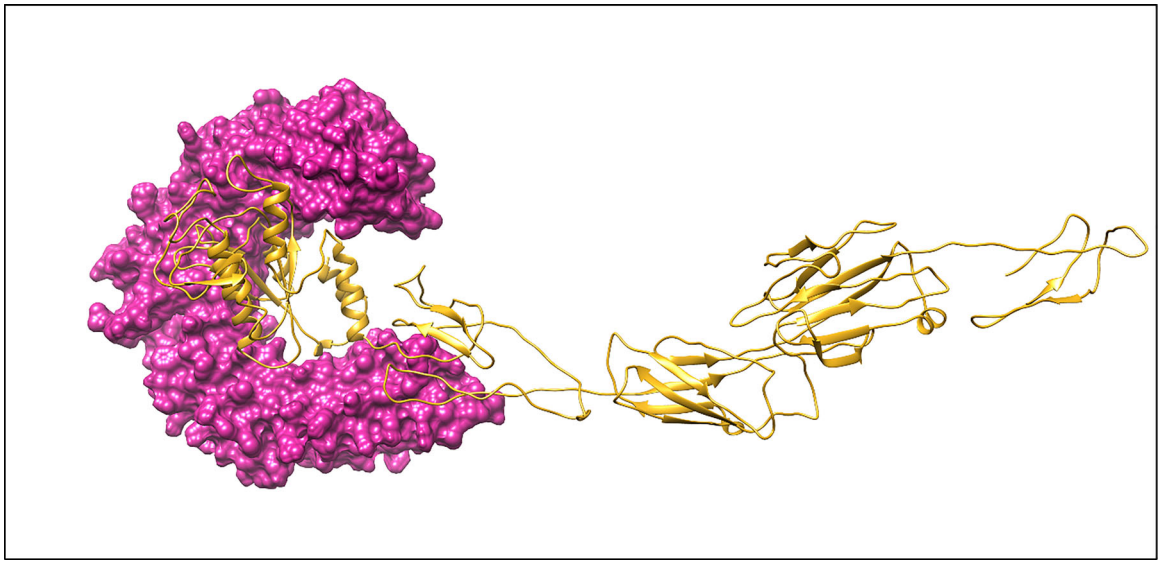

Figure 2. Molecular interaction of the designed vaccine candidate with the immune receptor molecule – TLR2 using High Ambiguity Driven protein–protein docking (HADDOCK) server.

satisfactory at a value of 0.93, likewise, the GC content of the construct was found to be 52.11%. These values indicate that the vaccine construct is amenable to a high level of recombinant protein expression in *E. coli* (Fu et al., 2020). The optimized coding sequence of the vaccine was successfully cloned in-silico into the pET-28a (+) expression vector using the SnapGene software (Figure 4).

3.11. Immune simulation

The immune simulation study was conducted using the C-ImmSim server (Rapin et al., 2010) that depicts the probable immune response generated on the administration of the vaccine into the mammalian host. The antigen peak shows the primary immune response that peaks around 1–2 days on injecting the vaccine in its multiple doses. Both the IgG (1 and 2) and IgM peaks show the secondary and tertiary immune reaction along with IgG+IgM, which acquires the peak somewhere around 10–15 days after each dose of vaccination (Figure 5(A)). The level of different cytokines primarily IFN-γ, TGF-β, IL-10, and IFN-β was also elevated in response to the vaccination (Figure 5(B)). These cytokines play key roles in generating an immune response against viruses. The levels of active B cells (Figure 6(A,B)), helper T cells (Figure 6(C,D)), and cytotoxic T cells (Figure 6(E,F)) were also elevated, which suggest a desirable secondary immune response.

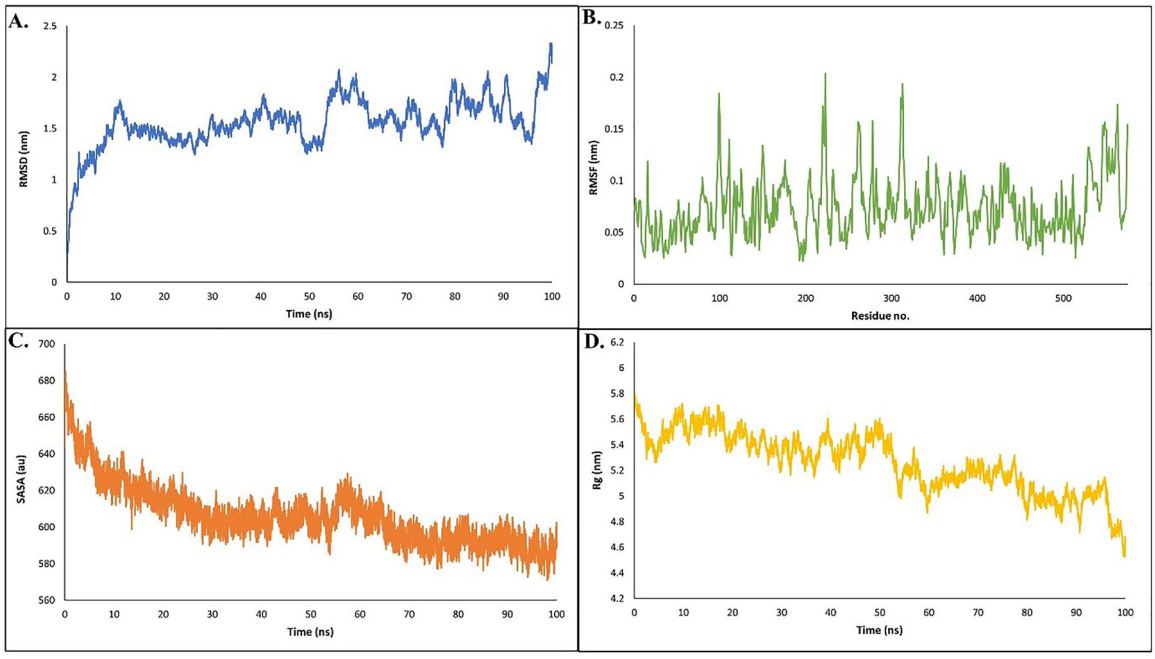

Figure 3. The stability of the interaction between the TLR-2 and the designed vaccine was analyzed by a molecular dynamics simulation study. (A) Root mean square deviation (RMSD) profile. (B) Root mean square fluctuation (RMSF) profile. (C) Solvent accessible surface area (SASA) profile, and the (D) The Radius of gyration (R_g) profile.

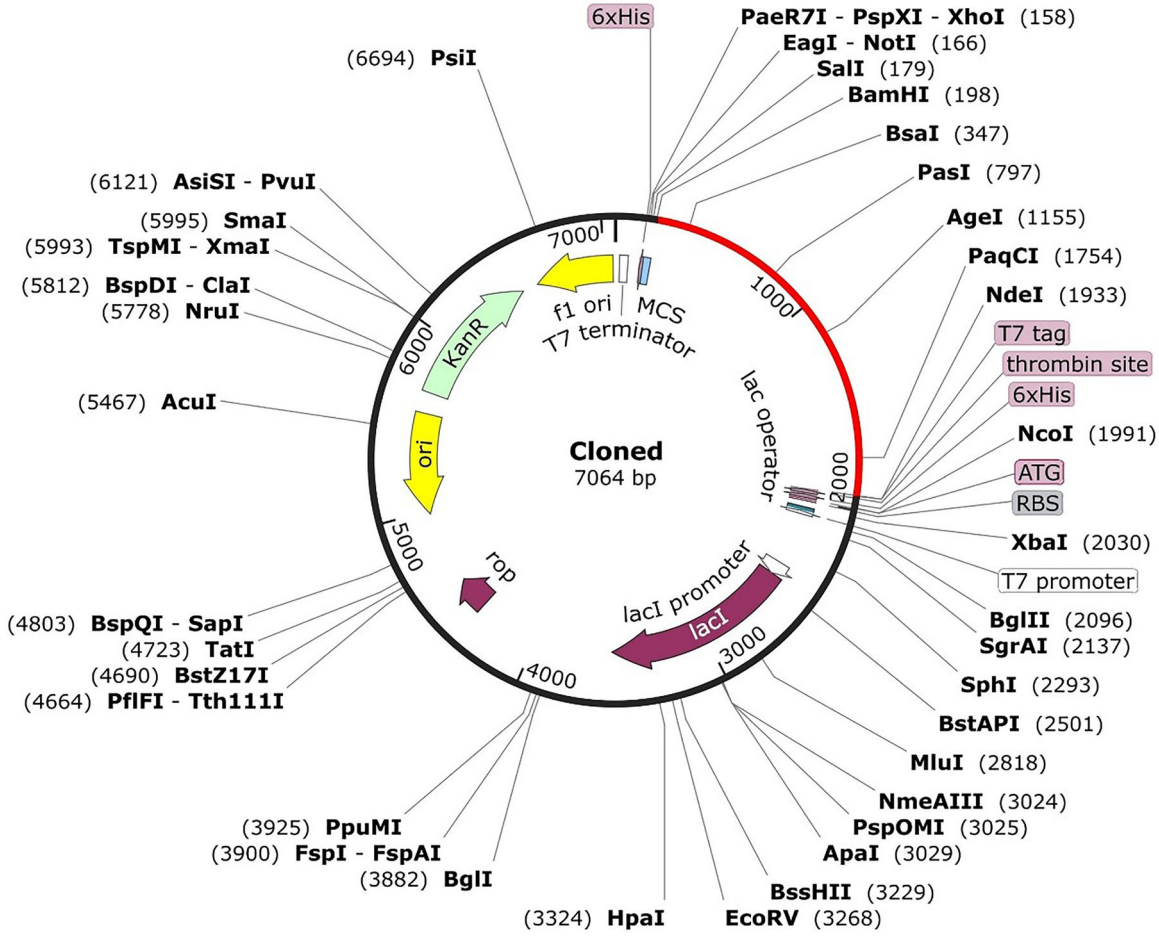

Figure 4. *In-silico* cloning of the vaccine construct (depicted in red) in *E. coli* (Strain K12) using the pET-28 a (+) vector, having restriction sites NdeI and BamHI at the N- and C-terminal, respectively, using the SnapGene software.

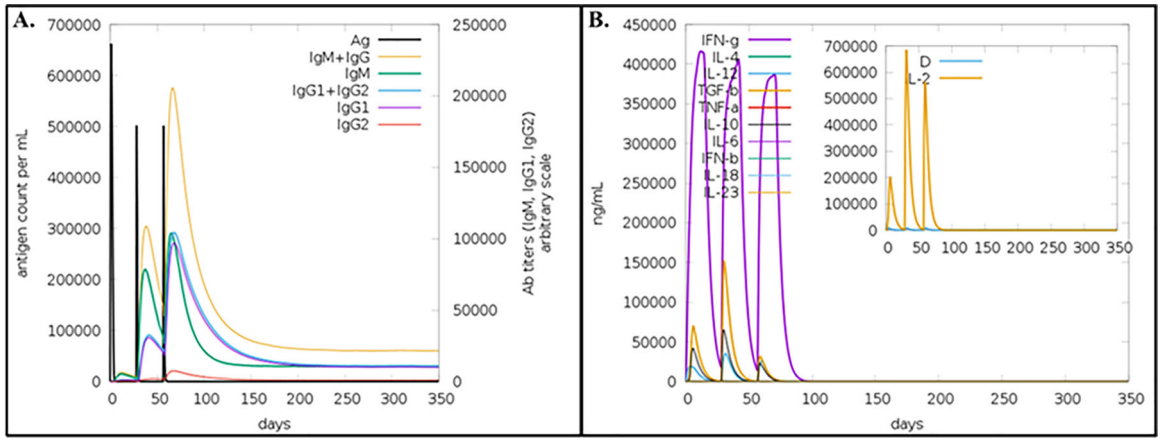

Figure 5. The immunoglobulin and cytokine response expected on vaccine administration as assessed by the c-ImmSim server (A) The increased antibody response to the vaccine inoculations (black colored lines), the different classes of immunoglobulins are indicated by colored lines. (B) Increase in the level of different cytokines after different doses of vaccine administration. The inset graph illustrates the Simpson Index, D of IL-2. Simpson Index, D was interpreted as the measurement of diversity.

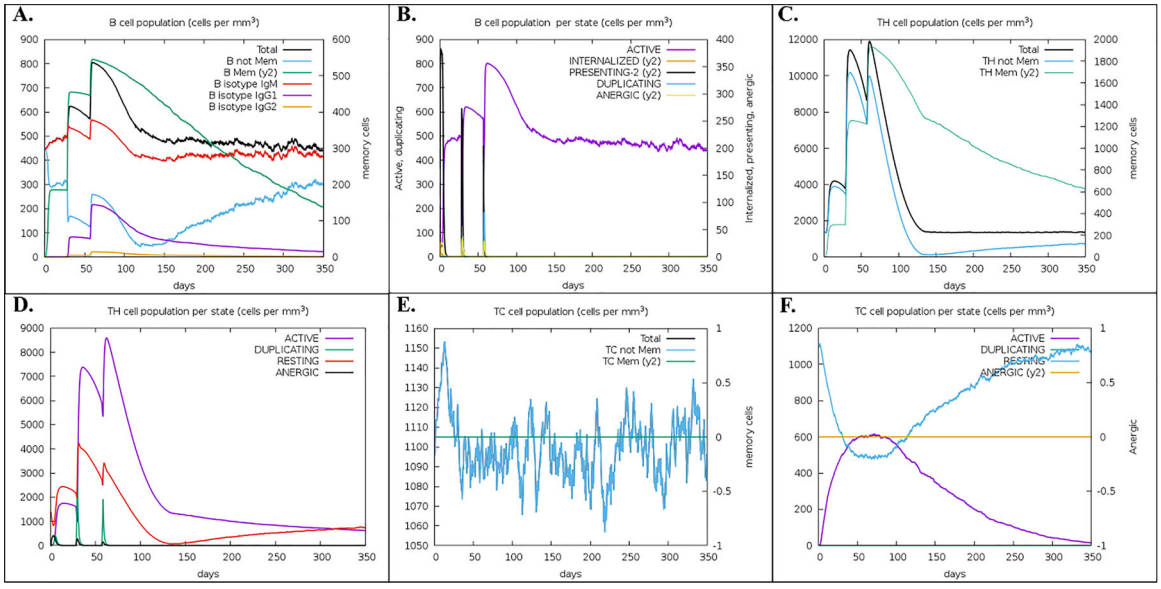

Figure 6. The likely influence of vaccine administration on the population of different immune cells as determined via immune simulation performed using the C-ImmSim server. (A) The B-cell population emerging after each dose of vaccine administration. (B) Increase of the B-cell population per state throughout vaccination. (C) Increase in the helper T-cell population after each dose of vaccine administration. (D) Elevation of the helper T-cell population per state. (E) Increase in the cytotoxic T-lymphocyte population throughout the vaccine administration. (F) The cytotoxic T-cell population per state was influenced as a result of different doses of vaccine administration injections Various subclasses of immunoglobulin are depicted as colored peaks. In the graphs, RESTING implies to the cells, which were not presented to the antigens while ANERGIC indicates the tolerance level of antigen.

4. Discussion

The increasing burden of Dengue and Zika infections and their co-infection mandates the development of effective treatment, especially in tropical regions of the world endemic to *Aedes* mosquitos. Researchers have not been successful in formulating either an effective drug for treatment against or a vaccine that could prevent these two diseases. The use of 'DENGVAXIA', the first-ever vaccine for dengue has several shortcomings (Fatima & Syed, 2018) and has also been found controversial due to health risks associated with it (Rapin et al., 2010; Thomas & Yoon, 2019). As per a recent study, people who have never been infected with Dengue previously, are prone to a considerably higher risk of a more severe form of Dengue on 'DENGVAXIA' administration (Mendoza, 2020).

In-silico vaccine designing or reverse vaccinology offers the advantage of expediting the otherwise tedious and exhaustive process of vaccine development. Immuno-informatics makes use of bioinformatics strategies for exploiting various immunological events and formulating algorithms for epitopes prediction. This has found use in designing multi-epitope subunit vaccines, as it is quite economic against several infectious pathogens, having higher specificity, safety, and stability (Rahmat Ullah et al., 2021). This approach has been used for designing vaccines against a wide range of pathogens including *Vibrio cholera* (Nezafat et al., 2016), *Leishmanias* (Ojha et al., 2020), Chikungunya (Jadoon et al., 2020) to name a few.

Here, we have utilized immuno-informatics for designing a novel multi-epitope, multi-pathogenic subunit vaccine against Dengue and Zika by utilizing structural and NS

proteins from DENV1–4 and ZIKV. Since both DENV and ZIKV are transferred to humans majorly via the *A. aegypti* mosquitos, whose salivary proteins are integral in viral pathogenesis; we have also targeted its selective salivary proteins in designing the vaccine. The viral proteins were selected based on their sequence similarity across the four Dengue serotypes and ZIKV, namely – envelope (E), non-structural protein 3 (NS3), and non-structural protein 5 (NS5); these three are reported to be the most conserved proteins across both the viruses and their different serotypes (Wen & Shresta, 2019). The *A. aegypti* salivary proteins were selected based on their reported relevance to viral pathogenesis, viz. Venom allergen 3 (Sun, Nie, et al., 2020), NeST1 (Hastings et al., 2019), and the putative 34 kDa family protein (Surasombatpattana et al., 2014). Thus, a total of six proteins were selected for vaccine design; all these proteins were checked for their antigenic potential and lack of homology to the human proteins via ANTIGENpro and BLASTp analysis, respectively.

An ideal vaccine should trigger both branches of acquired immunity to fight against a pathogen. Therefore, the selected proteins were used for the prediction of both B- and T-cell epitopes. For all the viral proteins only the epitopes that were significantly conserved across both the viruses were selected further for vaccine design, where no consensus could be reached the predicted epitopes were left from inclusion into the vaccine construct. In the case of *A. aegypti* salivary proteins, the top-ranked epitopes as predicted by the servers were included in designing the vaccine. The selected epitopes were combined and supplemented with three different peptide adjuvants (human β-defensin, HABA protein from *M. tuberculosis*, and bacterial L7/L12 ribosomal protein) using linker sequences to create three separate vaccine constructs. The three vaccine constructs differed in their sequence only by the adjuvant used. Since the β-defensin adjuvanted vaccine construct exhibited superior antigenic potential on analysis by ANTIGENpro and VaxiJen servers, we proceeded further in the study with just this construct.

The 3D structure for the lead vaccine construct as predicted by the I-TASSER server was refined using Galaxy refine server and on further inspection by structure validation tools viz. ProSA, ERRAT, and PROCHECK were found to have desired structural quality. The designed vaccine construct was found to exhibit suitable physicochemical properties and was predicted to be soluble by ProtParam and SOLPro tools, respectively. Moreover, the toxicity and allergenicity of the designed vaccine were evaluated and it was found to be both non-toxic and non-allergenic to humans.

To examine the capability of the vaccine construct in interacting with immunogenic receptors in humans, molecular docking with human TLR2 was conducted using the HADDOCK server. Human β-defensin 3 is known for its role in activating the professional antigen-presenting cells through via interactions with TLR2. The interaction of human β-defensin 3 with TLR1 and 2 activates the myeloid dendritic cells and monocytes (Funderburg et al., 2007). The designed vaccine construct was found to stably interact with TLR2 and exhibited a good docking score. Further molecular dynamics simulation was performed, to determine the stability of this interaction under physiological conditions. The docking complex of TLR2 and the vaccine construct was found to be stabilized as observed through RMSD analysis at around 15–50 ns in the total 100 ns time frame of the simulation. The trajectory of the simulation displayed minimal topological variations in the range of 1.4–1.6 nm. Similarly, RMSF, R_g and SASA analysis of the simulation suggested the docked complex to have a strong and stabilized interaction.

Codon optimization yielded CAI and %GC values of 0.93 (optimum value 1) and 52.11% (optimal range 30–70%; Khatoon et al., 2017), respectively, warranting that the synthesized vaccine sequence is composed of codons that are favorable for its successful expression in *E. coli* (strain K12; Shey et al., 2019). Further *in-silico* cloning of the vaccine construct within the pET-28 a (+) was also successful (Ghosh et al., 2021).

Ultimately, immune simulation analysis confirmed the designed vaccine to be efficacious. Both humoral and cellular immune responses were generated adequately with an appropriate dosage of vaccine, and they lasted for several months.

In addition to Dengvaxia the approved vaccine against dengue, numerous additional vaccine candidates against dengue are under evaluation in clinical trials. Prominent amongst these is one subunit protein vaccine (Phase I), two recombinant live attenuated vaccines (Phase III), and one DNA vaccine (Phase I; Deng et al., 2020; Sun, Du, et al., 2020). However, none of the vaccine candidates against Zika have till now advanced to the Phase III trials, though there are promising candidates currently in Phase I and II trials (Pattnaik et al., 2020). These include DNA vaccines (Phase I and II), RNA vaccines (Phase I), Live attenuated (Phase I) and Inactivated virus vaccines (Phase I). Designing peptide vaccines against DENV and ZIKV by utilizing immuno-informatics approaches has also been projected; however, such vaccines are only in the early pre-clinical stage of development.

Immuno-informatics has been utilized for designing multi-epitope vaccines separately against DENV (Ali et al., 2017; Krishnan et al., 2021) and also ZIKV (Mittal et al., 2020; Prasasty et al., 2019). In these studies where DENV has been targeted the designed vaccine has been developed by considering only a single viral serotype (Ali et al., 2017; Krishnan et al., 2021), thus limiting their application. There has also been a recent report where immune-informatics has been utilized to generate a multi-pathogen vaccine against both DENV and ZIKV (Sarkar et al., 2020). The vaccine proposed by Sarkar et al. utilizes only the structural envelope protein from DENV and ZIKV in its design. However, the present study puts forth a novel vaccine that targets DENV1–4 and ZIKV simultaneously by making use of a combination of conserved epitopes from structural as well as NS viral proteins. Additionally, we have included antigenic epitopes from *A. aegypti* salivary proteins to enhance the multi-pathogenic potential of the vaccine.

The immuno-informatics approach of designing multi-epitope vaccines against a number of pathogens has been widely accepted in recent years and there are also some reports where lead candidates identified via reverse

vaccinology have been validated through experimental evaluation for ensuring their efficacy and immunogenicity (Bazhan et al., 2019; Foroutan et al., 2020; Li et al., 2008). Likewise, the preliminary results obtained by this computational study also need further extensive wet-lab validations such as virus neutralization assays, and serology testing amongst others for establishing the actual efficacy of the proposed vaccine. Although the robustness, reliability, and accuracy of bioinformatics studies are recognized, yet it remains crucial that the computational results are confirmed using *in-vitro* and *in-vivo* experiments.

5. Conclusion

This study proposes a vaccine that can be effective in protecting against both dengue and zika. The vaccine has been designed keeping in mind the prevalence of multiple DENV serotypes, which has been one of the reasons behind the failure of producing a safe and efficacious vaccine against Dengue. Only significantly conserved epitopes from all the four DENV serotypes and ZIKV have been utilized in designing the vaccine construct. Moreover, we have also utilized *A. aegypti* salivary proteins to identify immunogenic epitopes and used them in the design of the vaccine, providing further cross-protection against infection from both these diseases. Preliminary *in-silico* results suggest that the designed vaccine construct exhibits all the desirable qualities required in a candidate vaccine. Based on our preliminary results the proposed vaccine can be pushed as a candidate in vaccine discovery against dengue and zika.

Acknowledgment

R.S. is thankful to Indian Council of Medical Research (ICMR), India for the Senior Research Fellowship. A.G. is thankful to University Grant Commission, India for the faculty Recharge position.

Disclosure statement

The authors declare no conflicting interests.

Funding

This research did not receive specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

All datasets generated for this report are included in the article/ Supplementary Material.

ORCID

Abhinav Grover http://orcid.org/0000-0002-3296-7860

Author contributions

A.B. and R.S. conceptualized and visualized the whole study. A.B. and R.S. designed and conducted the experimental studies and drafted the manuscript. A.G. managed resources and reviewed the manuscript. All authors read and approved the manuscript.

References

Abdul Ahmad, S. A., Palanisamy, U. D., Tejo, B. A., Chew, M. F., Tham, H. W., & Syed Hassan, S. (2017). Geraniin extracted from the rind of *Nephelium lappaceum* binds to dengue virus type-2 envelope protein and inhibits early stage of virus replication. *Virology Journal*, *14*(1), 229. https://doi.org/10.1186/s12985-017-0895-1

Aguiar, M., Stollenwerk, N., & Halstead, S. B. (2016). The risks behind Dengvaxia recommendation. *The Lancet. Infectious Diseases*, *16*(8), 882–883. https://doi.org/10.1016/S1473-3099(16)30168-2

Ali, M., Pandey, R. K., Khatoon, N., Narula, A., Mishra, A., & Prajapati, V. K. (2017). Exploring dengue genome to construct a multi-epitope based subunit vaccine by utilizing immunoinformatics approach to battle against dengue infection. *Scientific Reports*, *7*(1), 9232. https://doi.org/10.1038/s41598-017-09199-w

Ayyagari, V. S., T, C. V., K, A. P., & Srirama, K. (2020). Design of a multi-epitope-based vaccine targeting M-protein of SARS-CoV2: an immunoinformatics approach. *J Biomol Struct Dyn*, 1–15. https://doi.org/10.1080/07391102.2020.1850357

Bazhan, S. I., Antonets, D. V., Karpenko, L. I., Oreshkova, S. F., Kaplina, O. N., Starostina, E. V., Dudko, S. G., Fedotova, S. A., & Ilyichev, A. A. (2019). In silico designed ebola virus T-cell multi-epitope DNA vaccine constructions are immunogenic in mice. *Vaccines*, *7*(2), 34. https://doi.org/10.3390/vaccines7020034

Blankenberg, D., Von Kuster, G., Coraor, N., Ananda, G., Lazarus, R., Mangan, M., Nekrutenko, A., & Taylor, J. (2010). Galaxy: A web-based genome analysis tool for experimentalists. *Current Protocols in Molecular Biology*, Chapter*19:Unit 19*(21), 10–11. https://doi.org/10.1002/0471142727.mb1910s89

Castiglione, F., Duca, K., Jarrah, A., Laubenbacher, R., Hochberg, D., & Thorley-Lawson, D. (2007). Simulating Epstein-Barr virus infection with C-ImmSim. *Bioinformatics*, *23*(11), 1371–1377. https://doi.org/10.1093/bioinformatics/btm044

Chatterjee, D., Priyadarshini, P., Das, D. K., Mushtaq, K., Singh, B., & Agrewala, J. N. (2020). Deciphering the structural enigma of HLA class-II binding peptides for enhanced immunoinformatics-based prediction of vaccine epitopes. *Journal of Proteome Research*, *19*(11), 4655–4669. https://doi.org/10.1021/acs.jproteome.0c00405

Chaves, B. A., Orfano, A. S., Nogueira, P. M., Rodrigues, N. B., Campolina, T. B., Nacif-Pimenta, R., Pires, A., Junior, A. B. V., Paz, A. D. C., Vaz, E., Guerra, M., Silva, B. M., de Melo, F. F., Norris, D. E., de Lacerda, M. V. G., Pimenta, P. F. P., & Secundino, N. F. C. (2018). Coinfection with zika virus (ZIKV) and dengue virus results in preferential ZIKV transmission by vector bite to vertebrate host. *The Journal of Infectious Diseases*, *218*(4), 563–571. https://doi.org/10.1093/infdis/jiy196

Cheng, J., Randall, A. Z., Sweredoski, M. J., & Baldi, P. (2005). SCRATCH: A protein structure and structural feature prediction server. *Nucleic Acids Research*, *33*(Web Server issue), W72–W76. https://doi.org/10.1093/nar/gki396

Comeau, G., Zinna, R. A., Scott, T., Ernst, K., Walker, K., Carriere, Y., & Riehle, M. A. (2020). Vertical transmission of zika virus in *Aedes aegypti* produces potentially infectious progeny. *The American Journal of Tropical Medicine and Hygiene*, *103*(2), 876–883. https://doi.org/10.4269/ajtmh.19-0698

Dar, H., Zaheer, T., Rehman, M. T., Ali, A., Javed, A., Khan, G. A., Babar, M. M., & Waheed, Y. (2016). Prediction of promiscuous T-cell epitopes in the zika virus polyprotein: An in silico approach. *Asian Pacific Journal of Tropical Medicine*, *9*(9), 844–850. https://doi.org/10.1016/j.apjtm.2016.07.004

Deng, S. Q., Yang, X., Wei, Y., Chen, J. T., Wang, X. J., & Peng, H. J. (2020). A review on dengue vaccine development. *Vaccines*, *8*(1), 63. https://doi.org/10.3390/vaccines8010063

Desai, D. V., & Kulkarni-Kale, U. (2014). T-cell epitope prediction methods: An overview. *Immunoinformatics*, *1184*, 333–364. https://doi.org/10.1007/978-1-4939-1115-8_19

Dhanda, S. K., Mahajan, S., Paul, S., Yan, Z., Kim, H., Jespersen, M. C., Jurtz, V., Andreatta, M., Greenbaum, J. A., Marcatili, P., Sette, A., Nielsen, M., & Peters, B. (2019). IEDB-AR: Immune epitope database-analysis resource in 2019. *Nucleic Acids Research*, *47*(W1), W502–W506. https://doi.org/10.1093/nar/gkz452

Diamond, M. S., Ledgerwood, J. E., & Pierson, T. C. (2019). Zika virus vaccine development: Progress in the face of new challenges. *Annual Review of Medicine*, *70*, 121–135. https://doi.org/10.1146/annurev-med-040717-051127

Dimitrov, I., Bangov, I., Flower, D. R., & Doytchinova, I. (2014). AllerTOP v.2 – A server for in silico prediction of allergens. *Journal of Molecular Modeling*, *20*(6), 2278. https://doi.org/10.1007/s00894-014-2278-5

Dowd, K. A., DeMaso, C. R., Pelc, R. S., Speer, S. D., Smith, A. R. Y., Goo, L., Platt, D. J., Mascola, J. R., Graham, B. S., Mulligan, M. J., Diamond, M. S., Ledgerwood, J. E., & Pierson, T. C. (2016). Broadly neutralizing activity of zika virus-immune sera identifies a single viral serotype. *Cell Reports*, *16*(6), 1485–1491. https://doi.org/10.1016/j.celrep.2016.07.049

Doytchinova, I. A., & Flower, D. R. (2007). VaxiJen: A server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics*, *8*(1), 4. https://doi.org/10.1186/1471-2105-8-4

Elong Ngono, A., Vizcarra, E. A., Tang, W. W., Sheets, N., Joo, Y., Kim, K., Gorman, M. J., Diamond, M. S., & Shresta, S. (2017). Mapping and role of the $CD8^+$ T cell response during primary zika virus infection in mice. *Cell Host & Microbe*, *21*(1), 35–46. https://doi.org/10.1016/j.chom.2016.12.010

Elshahawi, H., Syed Hassan, S., & Balasubramaniam, V. (2019). Importance of zika virus NS5 protein for viral replication. *Pathogens*, *8*(4), 169. https://doi.org/10.3390/pathogens8040169

Estofolete, C. F., Terzian, A. C. B., Colombo, T. E., de Freitas Guimaraes, G., Ferraz, H. C. J., da Silva, R. A., Greque, G. V., & Nogueira, M. L. (2019). Co-infection between zika and different dengue serotypes during DENV outbreak in Brazil. *Journal of Infection and Public Health*, *12*(2), 178–181. https://doi.org/10.1016/j.jiph.2018.09.007

Fatima, K., & Syed, N. I. (2018). Dengvaxia controversy: Impact on vaccine hesitancy. *Journal of Global Health*, *8*(2), 010312. https://doi.org/10.7189/jogh.08-020312

Foroutan, M., Ghaffarifar, F., Sharifi, Z., & Dalimi, A. (2020). Vaccination with a novel multi-epitope ROP8 DNA vaccine against acute *Toxoplasma gondii* infection induces strong B and T cell responses in mice. *Comparative Immunology, Microbiology & Infectious Diseases*, *69*, 101413. https://doi.org/10.1016/j.cimid.2020.101413

Fu, H., Liang, Y., Zhong, X., Pan, Z., Huang, L., Zhang, H., Xu, Y., Zhou, W., & Liu, Z. (2020). Codon optimization with deep learning to enhance protein expression. *Scientific Reports*, *10*(1), 17617. https://doi.org/10.1038/s41598-020-74091-z

Funderburg, N., Lederman, M. M., Feng, Z., Drage, M. G., Jadlowsky, J., Harding, C. V., Weinberg, A., & Sieg, S. F. (2007). Human β-defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(47), 18631–18635. https://doi.org/10.1073/pnas.0702130104

Ghosh, P., Bhakta, S., Bhattacharya, M., Sharma, A. R., Sharma, G., Lee, S. S., & Chakraborty, C. (2021). A novel multi-epitopic peptide vaccine candidate against *Helicobacter pylori*: In-silico identification, design, cloning and validation through molecular dynamics. *International Journal of Peptide Research and Therapeutics*, *27*(2), 1149–1118. https://doi.org/10.1007/s10989-020-10157-w

Grifoni, A., Angelo, M. A., Lopez, B., O'Rourke, P. H., Sidney, J., Cerpas, C., Balmaseda, A., Silveira, C. G. T., Maestri, A., Costa, P. R., Durbin, A. P., Diehl, S. A., Phillips, E., Mallal, S., De Silva, A. D., Nchinda, G., Nkenfou, C., Collins, M. H., de Silva, A. M., … Weiskopf, D. (2017). Global assessment of dengue virus-specific $CD4^+$ T cell responses in dengue-endemic areas. *Frontiers in Immunology*, *8*, 1309. https://doi.org/10.3389/fimmu.2017.01309

Grijalva, I., Grajales-Muniz, C., Gonzalez-Bonilla, C., Borja-Aburto, V. H., Paredes-Cruz, M., Guerrero-Cantera, J., Gonzalez-Ibarra, J., Vallejos-Paras, A., Rojas-Mendoza, T., Santacruz-Tinoco, C. E., Hernandez-Bautista, P., Arriaga-Nieto, L., Garza-Sagastegui, M. G., Vargas-Ramos, I., Sepulveda-Nunez, A., Campos-Villarreal, O. I., Corrales-Perez, R., Azuara-Castillo, M., Sierra-Gonzalez, E., … Cervantes-Ocampo, A. A. (2020). Zika and dengue but not chikungunya are associated with Guillain-Barré syndrome in Mexico: A case-control study. *PLoS Neglected Tropical Diseases*, *14*(12), e0008032. https://doi.org/10.1371/journal.pntd.0008032

Grote, A., Hiller, K., Scheer, M., Münch, R., Nörtemann, B., Hempel, D. C., & Jahn, D. (2005). JCat: A novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Research*, *33*(Web Server issue), W526–W531. https://doi.org/10.1093/nar/gki376

Guanche Garcell, H., Gutierrez Garcia, F., Ramirez Nodal, M., Ruiz Lozano, A., Perez Diaz, C. R., Gonzalez Valdes, A., & Gonzalez Alvarez, L. (2020). Clinical relevance of zika symptoms in the context of a zika dengue epidemic. *Journal of Infection and Public Health*, *13*(2), 173–176. https://doi.org/10.1016/j.jiph.2019.07.006

Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., & Raghava, G. P., Open Source Drug Discovery Consortium. (2013). In silico approach for predicting toxicity of peptides and proteins. *PLoS One*, *8*(9), e73957. https://doi.org/10.1371/journal.pone.0073957

Hastings, A. K., Uraki, R., Gaitsch, H., Dhaliwal, K., Stanley, S., Sproch, H., Williamson, E., MacNeil, T., Marin-Lopez, A., Hwang, J., Wang, Y., Grover, J. R., & Fikrig, E. (2019). *Aedes aegypti* NeSt1 protein enhances zika virus pathogenesis by activating neutrophils. *Journal of Virology*, *93*(13), e00395-19. https://doi.org/10.1128/JVI.00395-19

Jadoon, M. H., Rehman, Z., Khan, A., Rizwan, M., Khan, S., Mehmood, A., & Munir, A. (2020). In silico T-cell and B-cell epitope based vaccine design against alphavirus strain of chikungunya. *Infectious Disorders – Drug Targets*, *20*(4), 523–530. https://doi.org/10.2174/1871526519666190521100521

Jakhar, R., & Gakhar, S. K. (2020). An Immunoinformatics Study to Predict Epitopes inthe Envelope Protein of SARS-CoV-2. *Can J Infect Dis Med Microbiol, 2020*, 7079356. https://doi.org/10.1155/2020/7079356

Joob, B., & Wiwanitkit, V. (2020). Clinical relevance of zika symptoms in the context of a zika dengue epidemic. *Journal of Infection and Public Health*, *13*(1), 158. https://doi.org/10.1016/j.jiph.2019.10.001

Karaca, E., Melquiond, A. S., de Vries, S. J., Kastritis, P. L., & Bonvin, A. M. (2010). Building macromolecular assemblies by information-driven docking: Introducing the HADDOCK multibody docking server. *Molecular & Cellular Proteomics*, *9*(8), 1784–1794. https://doi.org/10.1074/mcp.M000051-MCP201

Keller, T. H., Chen, Y. L., Knox, J. E., Lim, S. P., Ma, N. L., Patel, S. J., Sampath, A., Wang, Q. Y., Yin, Z., & Vasudevan, S. G. (2006). Finding new medicines for flaviviral targets. *Novartis Foundation Symposia*, *277*, 102–114; discussion 114-9, 251-3. https://www.ncbi.nlm.nih.gov/pubmed/17319157

Khan, A., Khan, S., Saleem, S., Nizam-Uddin, N., Mohammad, A., Khan, T., Ahmad, S., Arshad, M., Ali, S. S., Suleman, M., & Wei, D. Q. (2021). Immunogenomics guided design of immunomodulatory multi-epitope subunit vaccine against the SARS-CoV-2 new variants, and its validation through in silico cloning and immune simulation. *Computers in Biology and Medicine*, *133*, 104420. https://doi.org/10.1016/j.compbiomed.2021.104420

Khan, A. M., Miotto, O., Nascimento, E. J., Srinivasan, K. N., Heiny, A. T., Zhang, G. L., Marques, E. T., Tan, T. W., Brusic, V., Salmon, J., & August, J. T. (2008). Conservation and variability of dengue virus proteins: Implications for vaccine design. *PLoS Neglected Tropical Diseases*, *2*(8), e272. https://doi.org/10.1371/journal.pntd.0000272

Khatoon, N., Pandey, R. K., & Prajapati, V. K. (2017). Exploring Leishmania secretory proteins to design B and T cell multi-epitope subunit vaccine using immunoinformatics approach. *Scientific Reports*, *7*(1), 8285. https://doi.org/10.1038/s41598-017-08842-w

Koblischke, M., Stiasny, K., Aberle, S. W., Malafa, S., Tsouchnikas, G., Schwaiger, J., Kundi, M., Heinz, F. X., & Aberle, J. H. (2018). Structural influence on the dominance of virus-specific CD4 T cell epitopes in zika virus infection. *Frontiers in Immunology*, *9*, 1196. https://doi.org/10.3389/fimmu.2018.01196

Krishnan, S., Joshi, A., Akhtar, N., & Kaushik, V. (2021). Immunoinformatics designed T cell multi epitope dengue peptide vaccine derived from non structural proteome. *Microbial Pathogenesis*, *150*, 104728.

Laskowski, R. A., Rullmannn, J. A., MacArthur, M. W., Kaptein, R., & Thornton, J. M. (1996). AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR. *Journal of Biomolecular NMR*, *8*(4), 477–486. https://doi.org/10.1007/BF00228148

Lee, E. K., Tian, H., & Nakaya, H. I. (2020). Antigenicity prediction and vaccine recommendation of human influenza virus A (H3N2) using convolutional neural networks. *Human Vaccines & Immunotherapeutics*, *16*(11), 2690–2708. https://doi.org/10.1080/21645515.2020.1734397

Lewis, C. J., Dixit, B., Batiuk, E., Hall, C. J., O'Connor, M. S., & Boominathan, A. (2020). Codon optimization is an essential parameter for the efficient allotopic expression of mtDNA genes. *Redox Biology*, *30*, 101429. https://doi.org/10.1016/j.redox.2020.101429

Li, J., Lim, S. P., Beer, D., Patel, V., Wen, D., Tumanut, C., Tully, D. C., Williams, J. A., Jiricek, J., Priestle, J. P., Harris, J. L., & Vasudevan, S. G. (2005). Functional profiling of recombinant NS3 proteases from all four serotypes of dengue virus using tetrapeptide and octapeptide substrate libraries. *Journal of Biological Chemistry*, *280*(31), 28766–28774. https://doi.org/10.1074/jbc.M500588200

Li, H. X., Mao, X. H., Shi, Y., Ma, Y., Wu, Y. N., Zhang, W. J., Luo, P., Yu, S., Zhou, W. Y., Guo, Y., Wu, C., Guo, G., & Zou, Q. M. (2008). Screening and identification of a novel B-cell neutralizing epitope from *Helicobacter pylori* UreB. *Vaccine*, *26*(52), 6945–6949. https://doi.org/10.1016/j.vaccine.2008.09.089

Lim, W. C., & Khan, A. M. (2018). Mapping HLA-A2, -A3 and -B7 supertype-restricted T-cell epitopes in the ebolavirus proteome. *BMC Genomics*, *19*(S1), 42. https://doi.org/10.1186/s12864-017-4328-8

Livingston, B., Crimi, C., Newman, M., Higashimoto, Y., Appella, E., Sidney, J., & Sette, A. (2002). A rational strategy to design multiepitope immunogens based on multiple Th lymphocyte epitopes. *Journal of Immunology*, *168*(11), 5499–5506. https://doi.org/10.4049/jimmunol.168.11.5499

Magnan, C. N., Randall, A., & Baldi, P. (2009). SOLpro: Accurate sequence-based prediction of protein solubility. *Bioinformatics*, *25*(17), 2200–2207.

Manning, J. E., Morens, D. M., Kamhawi, S., Valenzuela, J. G., & Memoli, M. (2018). Mosquito saliva: The hope for a universal arbovirus vaccine? *The Journal of Infectious Diseases*, *218*(1), 7–15. https://doi.org/10.1093/infdis/jiy179

McCracken, M. K., Christofferson, R. C., Grasperge, B. J., Calvo, E., Chisenhall, D. M., & Mores, C. N. (2014). *Aedes aegypti* salivary protein "aegyptin" co-inoculation modulates dengue virus infection in the vertebrate host. *Virology*, *468–470*, 133–139. https://doi.org/10.1016/j.virol.2014.07.019

Mendoza, K. P. (2020). Vaccine hesitancy and the cultural politics of trust in the dengvaxia controversy. *Austrian Journal of South-East Asian Studies*, *13*(2), 267–274.

Miah, M. A., & Husna, A. (2020). Coinfection, coepidemics of COVID-19, and dengue in dengue-endemic countries: A serious health concern. *Journal of Medical Virology*, *93*(1), 161–162. https://doi.org/10.1002/jmv.26269

Mittal, A., Sasidharan, S., Raj, S., Balaji, S., & Saudagar, P. (2020). Exploring the zika genome to design a potential multiepitope vaccine using an immunoinformatics approach. *International Journal of Peptide Research and Therapeutics*, *26*, 2231–2240. https://doi.org/10.1007/s10989-020-10020-y

Narula, A., Pandey, R. K., Khatoon, N., Mishra, A., & Prajapati, V. K. (2018). Excavating chikungunya genome to design B and T cell multi-epitope subunit vaccine using comprehensive immunoinformatics approach to control chikungunya infection. *Infection, Genetics and Evolution*, *61*, 4–15. https://doi.org/10.1016/j.meegid.2018.03.007

Nezafat, N., Karimi, Z., Eslami, M., Mohkam, M., Zandian, S., & Ghasemi, Y. (2016). Designing an efficient multi-epitope peptide vaccine against *Vibrio cholerae* via combined immunoinformatics and protein interaction based approaches. *Computational Biology and Chemistry*, *62*, 82–95. https://doi.org/10.1016/j.compbiolchem.2016.04.006

Ojha, R., Pandey, R. K., & Prajapati, V. K. (2020). Vaccinomics strategy to concoct a promising subunit vaccine for visceral leishmaniasis targeting sandfly and leishmania antigens. *International Journal of Biological Macromolecules*, *156*, 548–557. https://doi.org/10.1016/j.ijbiomac.2020.04.097

Oktarianti, R., Senjarini, K., Hayano, T., Fatchiyah, F. & Aulanni'am, (2015). Proteomic analysis of immunogenic proteins from salivary glands of *Aedes aegypti*. *Journal of Infection and Public Health*, *8*(6), 575–582. https://doi.org/10.1016/j.jiph.2015.04.022

Pandey, R. K., Dahiya, S., Mahita, J., Sowdhamini, R., & Prajapati, V. K. (2019). Vaccination and immunization strategies to design *Aedes aegypti* salivary protein based subunit vaccine tackling Flavivirus infection. *International Journal of Biological Macromolecules*, *122*, 1203–1211. https://doi.org/10.1016/j.ijbiomac.2018.09.071

Pattnaik, A., Sahoo, B. R., & Pattnaik, A. K. (2020). Current status of zika virus vaccines: Successes and challenges. *Vaccines*, *8*(2), 266. https://doi.org/10.3390/vaccines8020266

Pessoa, R., Patriota, J. V., Lourdes de Souza, M., Felix, A. C., Mamede, N., & Sanabani, S. S. (2016). Investigation into an outbreak of dengue-like illness in Pernambuco, Brazil, revealed a cocirculation of zika, chikungunya, and dengue virus type 1. *Medicine*, *95*(12), e3201. https://doi.org/10.1097/MD.0000000000003201

Prasasty, V. D., Grazzolie, K., Rosmalena, R., Yazid, F., Ivan, F. X., & Sinaga, E. (2019). Peptide-based subunit vaccine design of T- and B-cells multi-epitopes against zika virus using immunoinformatics approaches. *Microorganisms*, *7*(8), 226. https://doi.org/10.3390/microorganisms7080226

Pruitt, K. D., Tatusova, T., & Maglott, D. R. (2007). NCBI reference sequences (RefSeq): A curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Research*, *35*(Database issue), D61–D65.

Rahmat Ullah, S., Majid, M., Rashid, M. I., Mehmood, K., & Andleeb, S. (2021). Immunoinformatics driven prediction of multiepitopic vaccine against *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* coinfection and its validation via in silico expression. *International Journal of Peptide Research and Therapeutics*, *27*(2), 987–913. https://doi.org/10.1007/s10989-020-10144-1

Rapin, N., Lund, O., Bernaschi, M., & Castiglione, F. (2010). Computational immunology meets bioinformatics: The use of prediction tools for molecular binding in the simulation of the immune system. *PLoS One*, *5*(4), e9862. https://doi.org/10.1371/journal.pone.0009862

Rivino, L., Kumaran, E. A., Jovanovic, V., Nadua, K., Teo, E. W., Pang, S. W., Teo, G. H., Gan, V. C., Lye, D. C., Leo, Y. S., Hanson, B. J., Smith, K. G., Bertoletti, A., Kemeny, D. M., & MacAry, P. A. (2013). Differential targeting of viral components by $CD4^+$ versus $CD8^+$ T lymphocytes in dengue virus infection. *Journal of Virology*, *87*(5), 2693–2706. https://doi.org/10.1128/JVI.02675-12

Rodriguez-Morales, A. J., Villamil-Gomez, W. E., & Franco-Paredes, C. (2016). The arboviral burden of disease caused by co-circulation and co-infection of dengue, chikungunya and zika in the Americas. *Travel Medicine and Infectious Disease*, *14*(3), 177–179. https://doi.org/10.1016/j.tmaid.2016.05.004

Samad, A., Ahammad, F., Nain, Z., Alam, R., Imon, R. R., Hasan, M., & Rahman, M. S. (2020). Designing a multi-epitope vaccine against SARS-CoV-2: An immunoinformatics approach. *Journal of Biomolecular Structure and Dynamics*, 1–17. https://doi.org/10.1080/07391102.2020.1792347

Sarkar, B., Ullah, M. A., Araf, Y., Das, S., & Hosen, M. J. (2020). Blueprint of epitope-based multivalent and multipathogenic vaccines: Targeted against the dengue and zika viruses. *Journal of Biomolecular Structure and Dynamics*, *39*(18), 6882-6902.10.1080/07391102.2020.1804456

Shey, R. A., Ghogomu, S. M., Esoh, K. K., Nebangwa, N. D., Shintouo, C. M., Nongley, N. F., Asa, B. F., Ngale, F. N., Vanhamme, L., & Souopgui, J. (2019). In-silico design of a multi-epitope vaccine candidate against onchocerciasis and related filarial diseases. *Scientific Reports*, *9*(1), 1–18. https://doi.org/10.1038/s41598-019-40833-x

Soria-Guerra, R. E., Nieto-Gomez, R., Govea-Alonso, D. O., & Rosales-Mendoza, S. (2015). An overview of bioinformatics tools for epitope prediction: Implications on vaccine development. *Journal of Biomedical Informatics*, *53*, 405–414.

Sri-In, C., Weng, S. C., Chen, W. Y., Wu-Hsieh, B. A., Tu, W. C., & Shiao, S. H. (2019). A salivary protein of *Aedes aegypti* promotes dengue-2 virus replication and transmission. *Insect Biochemistry and Molecular Biology*, *111*, 103181. https://doi.org/10.1016/j.ibmb.2019.103181

Srivastava, S., Verma, S., Kamthania, M., Kaur, R., Badyal, R. K., Saxena, A. K., Shin, H. J., Kolbe, M., & Pandey, K. C. (2020). Structural basis for designing multiepitope vaccines against COVID-19 infection: In silico vaccine design and validation. *JMIR Bioinformatics and Biotechnology*, *1*(1), e19371. https://doi.org/10.2196/19371

Sun, J., Du, S., Zheng, Z., Cheng, G., & Jin, X. (2020). Defeat dengue and zika viruses with a one-two punch of vaccine and vector blockade. *Frontiers in Microbiology*, *11*, 362. https://doi.org/10.3389/fmicb.2020.00362

Sun, P., Guo, S., Sun, J., Tan, L., Lu, C., & Ma, Z. (2019). Advances in in-silico B-cell epitope prediction. *Current Topics in Medicinal Chemistry*, *19*(2), 105–115. https://doi.org/10.2174/1568026619666181130111827

Sun, P., Nie, K., Zhu, Y., Liu, Y., Wu, P., Liu, Z., Du, S., Fan, H., Chen, C. H., Zhang, R., Wang, P., & Cheng, G. (2020). A mosquito salivary protein promotes flavivirus transmission by activation of autophagy. *Nature Communications*, *11*(1), 260. https://doi.org/10.1038/s41467-019-14115-z

Supriyono, Kuwata, R., Torii, S., Shimoda, H., Ishijima, K., Yonemitsu, K., Minami, S., Kuroda, Y., Tatemoto, K., Tran, N. T. B., Takano, A., Omatsu, T., Mizutani, T., Itokawa, K., Isawa, H., Sawabe, K., Takasaki, T., Yuliani, D. M., Abiyoga, D., Hadi, U. K., … Maeda, K. (2020). Mosquito-borne viruses, insect-specific flaviviruses (family Flaviviridae, genus Flavivirus), Banna virus (family Reoviridae, genus Seadornavirus), Bogor virus (unassigned member of family Permutotetraviridae), and alphamesoniviruses 2 and 3 (family Mesoniviridae, genus Alphamesonivirus) isolated from Indonesian mosquitoes. *Journal of Veterinary Medical Science*, *82*(7), 1030–1041. https://doi.org/10.1292/jvms.20-0261

Surasombatpattana, P., Ekchariyawat, P., Hamel, R., Patramool, S., Thongrungkiat, S., Denizot, M., Delaunay, P., Thomas, F., Luplertlop, N., Yssel, H., & Misse, D. (2014). *Aedes aegypti* saliva contains a prominent 34-kDa protein that strongly enhances dengue virus replication in human keratinocytes. *The Journal of Investigative Dermatology*, *134*(1), 281–284. https://doi.org/10.1038/jid.2013.251

Swarbrick, C. M. D., Basavannacharya, C., Chan, K. W. K., Chan, S. A., Singh, D., Wei, N., Phoo, W. W., Luo, D., Lescar, J., & Vasudevan, S. G. (2017). NS3 helicase from dengue virus specifically recognizes viral RNA sequence to ensure optimal replication. *Nucleic Acids Research*, *45*(22), 12904–12920. https://doi.org/10.1093/nar/gkx1127

Tahir Ul Qamar, M., Rehman, A., Tusleem, K., Ashfaq, U. A., Qasim, M., Zhu, X., Fatima, I., Shahid, F., & Chen, L. L. (2020). Designing of a next generation multiepitope based vaccine (MEV) againstSARS-COV-2: Immunoinformatics and in silico approaches. *PLoS One*, *15*(12), e0244176.. https://doi.org/10.1371/journal.pone.0244176

Thomas, S. J., & Yoon, I. K. (2019). A review of Dengvaxia®: Development to deployment. *Human Vaccines & Immunotherapeutics*, *15*(10), 2295–2314. https://doi.org/10.1080/21645515.2019.1658503

Tian, W., Chen, C., Lei, X., Zhao, J., & Liang, J. (2018). CASTp 3.0: Computed atlas of surface topography of proteins. *Nucleic Acids Research*, *46*(W1), W363–W367. https://doi.org/10.1093/nar/gky473

Weiskopf, D., Angelo, M. A., de Azeredo, E. L., Sidney, J., Greenbaum, J. A., Fernando, A. N., Broadwater, A., Kolla, R. V., De Silva, A. D., de Silva, A. M., Mattia, K. A., Doranz, B. J., Grey, H. M., Shresta, S., Peters, B., & Sette, A. (2013). Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for $CD8^+$ T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(22), E2046–E2053. https://doi.org/10.1073/pnas.1305227110

Wen, J., Ngono, A. E., Regla-Nava, J. A., Kim, K., Gorman, M. J., Diamond, M. S., & Shresta, S. (2017). Dengue virus-reactive $CD8^+$ T cells mediate cross-protection against subsequent zika virus challenge. *Nature Communications*, *8*(1), 1–11. https://doi.org/10.1038/s41467-017-01669-z

Wen, J., & Shresta, S. (2019). Antigenic cross-reactivity between zika and dengue viruses: Is it time to develop a universal vaccine? *Current Opinion in Immunology*, *59*, 1–8.

Wiederstein, M., & Sippl, M. J. (2007). ProSA-web: Interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Research*, *35*(Web Server issue), W407–W410. https://doi.org/10.1093/nar/gkm290

Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D., & Hochstrasser, D. F. (1999). Protein identification and analysis tools in the ExPASy server. *Methods in Molecular Biology*, *112*, 531–552. https://doi.org/10.1385/1-59259-584-7:531

Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., & Zhang, Y. (2015). The I-TASSER Suite: Protein structure and function prediction. *Nature Methods*, *12*(1), 7–8. https://doi.org/10.1038/nmeth.3213

Yang, J., & Zhang, Y. (2015). I-TASSER server: New development for protein structure and function predictions. *Nucleic Acids Research*, *43*(W1), W174–W181. https://doi.org/10.1093/nar/gkv342

Yano, A., Onozuka, A., Asahi-Ozaki, Y., Imai, S., Hanada, N., Miwa, Y., & Nisizawa, T. (2005). An ingenious design for peptide vaccines. *Vaccine*, *23*(17–18), 2322–2326. https://doi.org/10.1016/j.vaccine.2005.01.031

Yazdani, Z., Rafiei, A., Irannejad, H., Yazdani, M., & Valadan, R. (2020). Designing a novel multiepitope peptide vaccine against melanoma using immunoinformatics approach. *J Biomol Struct Dyn*, 1–13. https://doi.org/10.1080/07391102.2020.1846625

Zhang, Y. (2007). Template-based modeling and free modeling by I-TASSER in CASP7. *Proteins: Structure, Function, and Bioinformatics*, *69*(S8), 108–117. https://doi.org/10.1002/prot.21702

Zhang, Q., Wang, P., Kim, Y., Haste-Andersen, P., Beaver, J., Bourne, P. E., Bui, H. H., Buus, S., Frankild, S., Greenbaum, J., Lund, O., Lundegaard, C., Nielsen, M., Ponomarenko, J., Sette, A., Zhu, Z., & Peters, B. (2008). Immune epitope database analysis resource (IEDB-AR). *Nucleic Acids Research*, *36*(Web Server issue), W513–W518. https://doi.org/10.1093/nar/gkn254

Zhao, Y., Soh, T. S., Zheng, J., Chan, K. W., Phoo, W. W., Lee, C. C., Tay, M. Y., Swaminathan, K., Cornvik, T. C., Lim, S. P., Shi, P. Y., Lescar, J., Vasudevan, S. G., & Luo, D. (2015). A crystal structure of the dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication. *PLoS Pathogens*, *11*(3), e1004682. https://doi.org/10.1371/journal.ppat.1004682

Zheng, W., Zhang, C., Bell, E. W., & Zhang, Y. (2019). I-TASSER gateway: A protein structure and function prediction server powered by XSEDE. *Future Generation Computer Systems*, *99*, 73–85. https://doi.org/10.1016/j.future.2019.04.011

Zobayer, M. (2019). *In silico characterization and homology modeling of histamine receptors*. Khulna University of Engineering & Technology (KUET).