

A systematic and reverse vaccinology approach to design novel subunit vaccines against Dengue virus type-1 (DENV-1) and human Papillomavirus-16 (HPV-16)



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ABSTRACT

Currently, dengue fever and human cervical cancer are two of the most dangerous diseases around the world. Dengue fever is caused by four serotypes of dengue virus (DENV)- 1, 2, 3 and 4 and most of the human cervical cancer cases are found to be caused by human papillomavirus type-16 (HPV-16). In this study, potential epitope-based subunit vaccines were designed using numerous tools of reverse vaccinology, targeting the DENV-1 envelope protein E and HPV-16 major capsid protein L1. Reverse vaccinology is a genome-based approach of vaccine designing, identifying potential antigenic epitopes of a pathogen. After obtaining the target protein sequences, their antigenicity and physicochemical properties were determined. Thereafter, the possible T cell and B cell epitopes were predicted and after analyzing their antigenicity, allergenicity, toxicity, and conservancy, as well as docking results (docked with MHC class-I and class-II alleles), the best epitopes were selected for vaccine construction. Thereafter, two best vaccine constructs (one for each of the virus) were selected for further analysis based on analyzing the docking results (docked with various MHC alleles and TLR molecules) of the initially constructed six vaccines. The molecular dynamics simulation of the two selected docked complexes with the two best vaccines showed quite satisfactory results. Finally, after codon adaptation, the vaccine constructs were inserted into the pET-19b plasmid vector for *E. coli* strain K12 using an *in silico* cloning approach. However, more *in vitro* and *in vivo* studies might be required on the suggested vaccines for their proper validation.

1. Introduction

Dengue is the most common type of arthropod transmitted viral infection in humans. Every year, about 50–100 million new cases are estimated around the world. Besides dengue fever, about 250,000 to 500,000 new cases of dengue haemorrhagic fever (DHF) are also recorded every year [1]. Dengue fever and DHF are caused by four very closely related dengue virus (DENV) serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. However, most of their antigenic determinant portions are quite different from each other; for this reason, vaccination against one serotype of the virus do not provide protective immunity for other serotypes [2]. Dengue is transmitted by a mosquito called *Aedes aegypti*. This day-biting mosquito acts as the vector for this virus. Dengue is endemic on all the continents of the world except Europe, and almost two thirds of the world's population is currently living under the direct threat of dengue. At present, around 40% of the world's population is at

risk from dengue virus and 50–100 million dengue cases occur worldwide every year [3,4]. Most of the Asian and south-east Asian countries are some of the most dengue-prevalent regions in the world. According to the European Centre for Disease Prevention and Control, in Bangladesh, 101,354 cases were recorded in 2019, which was about a ten-fold increase compared to 2018. Significant increases in dengue cases in 2019 have also been recorded in other Asian countries including Cambodia, Sri Lanka, and Vietnam. (<https://www.ecdc.europa.eu/en/dengue-monthly>). Accessed on: 13 February 2020). DENV is a member of the *flaviviridae* family and infects the human body when the mosquito bites and takes blood from the body. It contains an antigen called NS1, which is the most important antigen for dengue manifestation. As a result, vaccine constructs targeting NS1 antigen are currently under development to fight dengue [5–7]. However, reports can also be found about the failure of such dengue vaccines that has caused much controversy over the years [8,9]. The dengue envelope protein E or the

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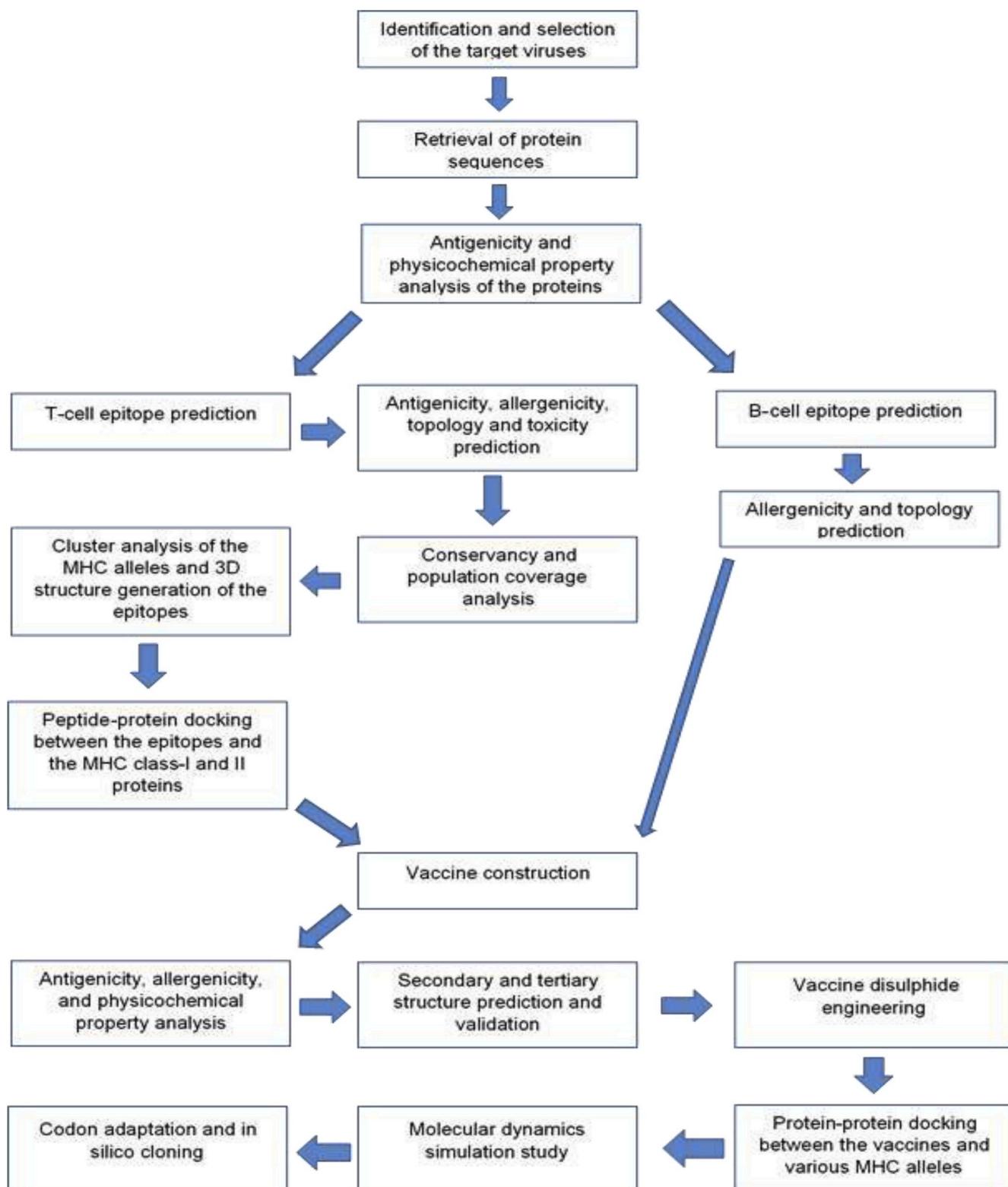


Fig. 1. Stepwise procedures adapted in the study to design the epitope-based subunit vaccines.

envelope glycoprotein E is an essential protein of the DENV envelope. This protein, along with another protein called premembrane protein (prM), plays very important roles in viral assembly, maturation, and induction of potential immunity [10,11]. For this reason, targeting the DENV envelope protein E could be a potential way for developing vaccines against DENV.

Cervical cancer is one of the main cancers that are responsible for the death of millions of women every year in the world. It is caused by the persistent infection of human papillomavirus (HPV) [12]. HPV is the most commonly transmitted virus that transmits through skin-to-skin contact during sexual intercourse. More than 100 types of HPV have been discovered so far in humans. Among them, thirty HPV types are genital and mucosal HPV viruses. The HPV-6, 11, 72, 81 etc. are classified as low-risk HPV types and the HPV-16, 18, 31, 33, 35 etc. are classified as high-risk HPV types. The HPV types 16, 18, 31, 33, 35 are mainly responsible for cervical cancer cases [13–16]. Among these HPV types, the HPV-16 is responsible for the majority of cervical cancers along with HPV-18. HPV-16 is also responsible for some other types of cancers like oropharyngeal cancer [17,18]. In fact, 50% of all the cervical cancers and 90% of the anogenital and oropharyngeal cancer cases are caused by HPV-16. The virus contains two types of capsid proteins i.e., major capsid protein, L1 and minor capsid protein, L2. Together, these two proteins form a 55-nm icosahedral capsid. The major capsid protein contains many epitopes that could be used as potential targets for vaccines [19]. The cervical cancer caused by HPV is most prevalent in South American and African countries. In some African countries with the most reported cervical cancer cases, the government has reported an age-standardized cervical cancer incidence rate per 100,000 women per year of around 56.3. However, in some Asian countries like India, Bangladesh, Myanmar, and Vietnam, the rate is around 30, which is far greater than many other countries in the world [20]. There are some commercial vaccines that are already available for HPV-16, targeting the whole L1 protein of the virus. However, these commercial vaccines are mainly based on virus-like particles (VLPs) [21–24]. The possible epitope sequences of the HPV-16 L1 protein were used in the study to construct epitope-based vaccines against the virus which might be more specific and antigenic than the VLP based vaccines.

In this experiment, potential vaccines were developed against DENV-1 and HPV-16, using different tools of reverse vaccinology and bioinformatics, targeting numerous epitopes of the DENV envelope protein E and HPV-16 major capsid protein L1. In recent years, the development of various computer software and tools has led the way to exploit computational methods in conducting research in cancer biology, genomics, proteomics, epigenomics, drug design, vaccine design, personalized medicine, synthetic biology and so on. The use of computational methods in vaccine design has been shown to be effective and promising in many studies. Immunoinformatics and reverse vaccinology are successfully utilized in designing different types of vaccines in recent years [23–26]. The reverse vaccinology approach can be defined as a potential process of vaccine development where the novel antigens of a virus or a pathogenic organism are identified by analyzing the genomic information of that particular pathogen or virus. In this approach, the tools of bioinformatics are used thoroughly to identify the novel antigens by dissecting the genome. Later, the selected antigens are used for vaccine construction. The reverse vaccinology approach of vaccine development is assistive to understand and identify antigenic segments of a pathogen that should be given a higher priority during the vaccine development. For developing novel vaccine candidates, reverse vaccinology attempts to combine immunogenetics and immunogenomics with bioinformatics. This method is a quick, easy, efficient, and cost-effective way to design possible vaccines [27,28]. Vaccines against many viruses like the Zika virus, Chikungunya virus etc. are designed successfully using a reverse

vaccinology approach [29]. In our study, numerous tools of reverse vaccinology and immunoinformatics were exploited to design epitope-based subunit vaccines against the DENV-1 and HPV-16. Although these two viruses are not related to each other, however, the two viruses were used in the study to design separate vaccines against each of the viruses. A flowchart of the vaccine development process used in this study is illustrated in Fig. 1.

2. Materials and methods

2.1. Strain identification and selection

The strains of the viruses were identified and selected by analyzing different entries of the National Center for Biotechnology Information of NCBI (<https://www.ncbi.nlm.nih.gov/>) database.

2.2. Retrieval of the protein sequences

The DENV-1 envelope protein E sequence (accession number: Q8BE39) and the HPV-16 major capsid protein L1 (accession number: Q9WLQ6) were retrieved from the UniProt (<https://www.uniprot.org/>) database.

2.3. Antigenicity prediction and physicochemical property analysis of the protein sequences

The antigenicity prediction of the protein sequences was carried out by the online server for antigenicity analysis, VaxiJen v2.0 (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>), keeping the threshold at 0.4 [30–32]. Various physicochemical properties of the protein sequences were determined by ExPASy's online tool ProtParam (<https://web.expasy.org/protparam/>) [33].

2.4. T cell and B cell epitope prediction

The T cell and B cell epitopes were predicted using the online epitope prediction server Immune Epitope Database or IEDB (<https://www.iedb.org/>). The IEDB is a database that contains a huge collection of experimental data on T cell epitopes and antibodies, and these data are collected from experiments carried out on human, non-human primates and other animals. It is a server that allows robust analysis on numerous epitopes in the context of some tools i.e., population coverage, conservation across antigens and clusters with similar sequences [34]. The MHC class-I restricted CD8⁺ cytotoxic T-lymphocyte (CTL) epitopes of the selected sequences were obtained using the NetMHCpan EL 4.0 prediction method for HLA-A*11-01 allele, keeping the sequence length 9. The MHC class-II restricted CD4⁺ helper T-lymphocyte (HTL) epitopes were obtained for HLA DRB1*04-01 allele using Sturniolo prediction method, keeping the sequence length 12. Twenty of the top forty MHC class-I epitopes were selected based on their percentile scores and antigenicity scores (AS). The B cell lymphocytic (BCL) epitopes were selected based on their lengths (the sequences with over ten amino acids in length were selected) and obtained using BepiPred linear epitope prediction method.

2.5. Transmembrane topology and antigenicity prediction of the selected epitopes

The transmembrane topology of the selected epitopes was determined using the transmembrane topology of protein helices determinant, TMHMM v2.0 server (<http://www.cbs.dtu.dk/services/TMHMM/>). The server predicts whether the epitope would be a

transmembrane peptide or it would remain inside or outside of the membrane [35]. The antigenicity of the selected epitopes were predicted using the VaxiJen v2.0 (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) server [30,31].

2.6. Allergenicity and toxicity prediction of the epitopes

The allergenicity of the selected epitopes was predicted using two online tools, AllerTOP v2.0 (<https://www.ddg-pharmfac.net/AllerTOP/>) and AllergenFP v1.0 (<http://ddg-pharmfac.net/AllergenFP/>) [36,37]. However, the results predicted by AllerTOP were given priority since the server has a better accuracy of 88.7% as compared to the AllergenFP server (87.9%) [36]. The toxicity prediction of the selected epitopes was performed by the ToxinPred server (<http://crdd.osdd.net/raghava/toxinpred/>), using the support vector (SVM-Swiss-Prot) based method, keeping all parameters at default.

2.7. Conservancy prediction and population coverage analysis

The conservancy analysis of the selected epitopes was performed using the ‘epitope conservancy analysis’ tool of IEDB server (<http://tools.iedb.org/conservancy/download/>) [34]. The sequence identity threshold was kept ‘ ≥ 50 ’. For the conservancy analysis of the DENV-1 epitopes, the envelope protein E from other types of dengue viruses, DENV, 2, 3 and 4 (UniProt accession numbers: Q8BE39, Q66394, Q7TGD1, Q7TGC7, respectively), were used for comparison. Since 100% conserved sequences were used for vaccine construction, so the constructed vaccines might also confer immunity towards the DENV serotypes- 2, 3 and 4 along with DENV-1. Moreover, to determine the conservancy of the HPV epitopes, the major capsid protein L1 from five different types of HPV viruses- 11, 92, 16, 32, 57b (UniProt accession numbers: P04012, Q8B5B0, Q9WLQ6, P36737, P89427, respectively), were used for comparison. Like the DENV vaccines, the 100% conserved epitopes of the HPV-16 were used for the HPV vaccine construction; as a result, these vaccines might also provide potential immunity towards HPV-11, 92, 16, 32 and 57b along with HPV-16.

For population coverage analysis, the population coverage calculation tool of IEDB server (<http://tools.iedb.org/population/>) was used [34]. The coverage analysis was conducted for several ethnic groups and demographic regions of the world, using the epitopes selected for the molecular docking study.

Based on the antigenicity, allergenicity, toxicity and conservancy analysis, the best epitopes were selected for further analysis and vaccine construction. The epitopes that showed high antigenicity, non-allergenicity, non-toxicity, and 100% conservancy as well as more than a 50% minimum identity, were considered as the best selected epitopes.

2.8. Cluster analysis of the MHC alleles

Cluster analysis of the MHC alleles helps to identify the alleles of the MHC class-I and class-II molecules that have similar binding specificities. The cluster analysis of the MHC alleles was carried out by the online tool, MHCcluster 2.0 (<http://www.cbs.dtu.dk/services/MHCcluster/>) [38]. During the analysis, the number of peptides to be included was maintained at 50,000, the number of bootstrap calculations was set at 100, and all the HLA supertype representatives (MHC class-I) and HLA-DR representatives (MHC class-II) were selected. For analyzing the MHC class-I alleles, the NetMHCpan-2.8 prediction method was used. The server generated the results in the form of the MHC specificity tree and MHC specificity heat-map.

2.9. Generation of the 3D structures of the selected epitopes

The 3D structures of the selected best epitopes were generated using online with the 3D structure generating tool PEP-FOLD3 (<http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>). The server is a tool for generating de novo 3D structures of peptides [39–41].

2.10. Molecular docking of the selected epitopes

The molecular docking of the selected epitopes was carried out by an online docking tool PatchDock (<https://bioinfo3d.cs.tau.ac.il/PatchDock/>). PatchDock contains algorithms that divide the Connolly dot surface representation of the molecules into concave, convex, and flat patches. Thereafter, the complementary patches were matched for generating potential candidate transformations. Then each candidate transformation was evaluated by a scoring function and finally, an RMSD (root mean square deviation) clustering score was applied to the candidate solutions for discarding the redundant solutions, and the top scored solutions would become the top ranked solutions. After docking by PatchDock, the docking results were refined flexibly and re-scored by FireDock server (<http://bioinfo3d.cs.tau.ac.il/FireDock/>). The FireDock server generates global energies upon the refinement of the best solutions from the PatchDock server and ranks them based on global energies, and the lowest global energy is always appreciable [42–45]. The molecular docking experiment was performed using the HLA-A*11-01 allele (PDB ID: 5WJL) and HLA DRB1*04-01 (PDB ID: 5JLZ) as receptors and the ligands were the best selected MHC-I and MHC-II epitopes (the epitopes that followed the selection criteria of high antigenicity, non-allergenicity, non-toxicity and 100% conservancy) of both viruses. The receptors were downloaded from the RCSB-Protein Data Bank (<https://www.rcsb.org/>) server. The best results were visualized using Discovery Studio Visualizer [46]. Release 4.1. Accelrys Inc., San Diego, CA) [47].

2.11. Vaccine construction

Three vaccines against each of the viruses (DENV-1 and HPV-16) were constructed. For vaccine construction, the best selected CTL, HTL and BCL epitopes were combined together. All the vaccines were constructed following the sequence: adjuvant, PADRE sequence, CTL epitopes, HTL epitopes and BCL epitopes. Three different adjuvant sequences were used for vaccine construction i.e., beta-defensin, L7/L12 ribosomal protein and HABA protein (*M. tuberculosis*, accession number: AGV15514.1). Beta-defensin adjuvant can activate the toll-like receptors (TLRs) 1, 2, and 4 by acting as agonist and both L7/L12 ribosomal protein and HABA protein have the capability to activate TLR-4. The three vaccines for each of the viruses differ from each other only in their adjuvant sequences. Three vaccines for each of the viruses were constructed to determine the two best vaccines (one from each virus group) with the best adjuvant sequences (by molecular docking), that might elicit potential immunogenic responses. During vaccine construction, different linkers were used i.e., EAAAK linkers were used to conjugate the adjuvant and PADRE sequence, GGGG linkers were used to attach the PADRE sequence with the CTL epitopes and the CTL epitopes with the other CTL epitopes, GPGPG linkers were used to join the CTL with HTL epitopes and also the HTL epitopes among themselves and KK linkers for conjugating the HTL and the BCL epitopes as well as the BCL epitopes among themselves [48–55]. The PADRE sequence improves the CTL response of the vaccines that contain it [56]. A total six vaccines were constructed in the experiment.

2.12. Antigenicity, allergenicity and physicochemical property analysis of the constructed vaccines

The antigenicity of the constructed vaccines were again determined by the online server VaxiJen v2.0 (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>). The threshold of the prediction was kept at 0.4 [30–32]. AlgPred (<http://crdd.osdd.net/raghava/algpred/>) and AllerTop v2.0 (<https://www.ddg-pharmfac.net/AllerTOP/>) were used for the prediction of the allergenicity of the vaccine constructs. The AlgPred server predicts the possible allergens based on the similarity of known epitopes of any of the known regions of the protein [57]. The MEME/MAST motif prediction approach was used in the allergenicity prediction of the vaccines by AlgPred. Moreover, numerous physicochemical properties of the vaccines were examined by the online server ProtParam (<https://web.expasy.org/protparam/>) [33].

2.13. Secondary and tertiary structure prediction of the vaccine constructs

The secondary structures of the vaccine constructs were generated using online tool PRISPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>). PRISPRED is a simple secondary structure generator that can be used to predict the transmembrane topology, transmembrane helix, fold and domain recognition etc. along with the secondary structure prediction [58,59]. The PRISPRED 4.0 prediction method was used to predict the secondary structure of the vaccine proteins. The β -sheet structure of the vaccines were determined by another online tool, NetTurnP v1.0 (<http://www.cbs.dtu.dk/services/NetTurnP/>) [60]. The tertiary or 3D structures of the vaccines were generated using online tool RaptorX (<http://raptordx.uchicago.edu/>) server. The server is a fully annotated tool for the prediction of protein structure as well as the property and contact prediction, sequence alignment etc. [61–63]. The 3D structures of the vaccine constructs were visualized by Discovery Studio Visualizer [46]. Release 4.1. Accelrys Inc., San Diego, CA) [47].

2.14. 3D structure refinement and validation

The 3D structures of the constructed vaccines were refined using the online refinement tool, 3Drefine (<http://sysbio.rnet.missouri.edu/3Drefine/>). The server is a quick, easy and efficient tool for protein structure refinement [64]. The vaccine proteins were then validated by analyzing the Ramachandran plot, generated using the online server PROCHECK (<https://services.mbi.ucla.edu/PROCHECK/>) [65–67].

2.15. Vaccine protein disulfide engineering

The vaccine protein disulfide engineering was performed by the online tool Disulfide by Design 2 v12.2 (<http://cptweb.cpt.wayne.edu/DbD2/>). The disulfide engineering was conducted to improve the stability of the proteins. The server predicts the possible sites within a protein structure that may undergo disulfide bond formation [68]. When engineering the disulfide bonds, the intra-chain, inter-chain and C_β for glycine residue, were selected. The χ_3 Angle was kept -87° or $+97^\circ \pm 10$ and $C_\alpha-C_\beta-S_\gamma$ Angle was kept $114.6^\circ \pm 10$.

2.16. Protein-protein docking

In protein-protein docking, the binding affinities of the constructed DENV-1 and HPV-16 vaccines were analyzed by docking with various MHC alleles and TLRs. One best vaccine would be selected from each of the two vaccine groups based on their superior performances in the docking experiment. When viral infections occur, the viral particles or antigens are recognized by various portions of the MHC complex. These portions of MHC molecules are encoded by different alleles. Therefore, the vaccines should have good binding affinity towards these MHC portions encoded by different alleles [69]. All the vaccine constructs

were docked with six selected MHC alleles to test their binding affinity i.e., DRB1*0101 (PDB ID: 2FSE), DRB3*0202 (PDB ID: 1A6A), DRB5*0101 (PDB ID: 1H15), DRB3*0101 (PDB ID: 2Q6W), DRB1*0401 (PDB ID: 2SEB), and DRB1*0301 (PDB ID: 3C5J). Moreover, studies have shown that TLR-8 present on the immune cells is responsible for mediating the immune responses against the RNA viruses and TLR-3 of the immune cells mediates immune responses against the DNA viruses [70–72]. The DENV-1 is a RNA virus and HPV-16 is a DNA virus [73,74]. Therefore, the vaccine constructs of DENV-1 were also docked with TLR-8 (PDB ID: 3W3M) and the vaccine constructs of HPV-16 were docked with TLR-3 (PDB ID: 2AOZ). The docking was carried out three times by three different online servers for improving the accuracy of the docking. First, the docking was carried out by ClusPro 2.0 (<https://cluspro.bu.edu/login.php>). The server ranks the clusters of docked complexes based on their energy scores where the lower energy score corresponds to the better binding affinity [75–77].

The ClusPro server calculates the energy score based on the following equation:

$$E = 0.40E_{\text{Rep}} + (-0.40E_{\text{att}}) + 600E_{\text{elec}} + 1.00ED_{\text{ARS}} \quad (\text{Kozakov et al., 2017; Kozakov et al., 2013})$$

Thereafter, the docking was again performed by PatchDock (<http://bioinfo3d.cs.tau.ac.il/PatchDock/>) and later refined and re-scored by FireDock server (<http://bioinfo3d.cs.tau.ac.il/FireDock/>). The FireDock server ranks the docked complexes based on their global energies and the lower score represents the better result [42–45]. Finally, the docking was performed using the HawkDock server (<http://cadd.zju.edu.cn/hawkdock/>). The Molecular Mechanics/Generalized Born Surface Area (MM-GBSA) study was also carried out using the HawkDock server. According to the server, the lower scores and lower energy corresponds to better scores [78–81]. The HawkDock server generates several models of docked complexes and ranks them by assigning HawkDock scores in ascending order. For each of the vaccines and their respective targets, the score of model 1 was taken for analysis. Furthermore, the model 1 of every complex was also analyzed for molecular mechanics/generalized born surface area (MM-GBSA) study which was performed by the HawkDock server.

From the docking experiment, two best vaccines (one from each of the virus categories) were selected for further analysis. The best vaccines docked with TLRs were visualized by the Discovery Studio Visualizer [46]. Release 4.1. Accelrys Inc., San Diego, CA) [47].

2.17. Molecular dynamics simulation study

The molecular dynamics simulation study was conducted for the best vaccines (one vaccine from DENV-1 and one vaccine from HPV-16), by the online server iMODS (<http://imods.chaconlab.org/>). The server is a fast, user-friendly and effective molecular dynamics simulation tool that can be used to investigate the structural dynamics and stability of the protein complexes. The server provides the values of deformability, B-factor (mobility profiles), eigenvalues, variance, co-variance map and elastic network. For a complex or protein, the deformability depends on the ability to deform at each of its amino acid residues. The eigenvalue has relation with the energy which is required to deform the given structure and the lower eigenvalue corresponds to easier deformability of the complex. Moreover, the eigenvalue also represents the motion stiffness of a protein complex. The server is a fast and easy server for determining and measuring the protein flexibility [82–86]. For analysing the molecular dynamics simulation of the vaccine-TLR8 (for DENV-1) and vaccine-TLR3 (for HPV-16) docked complexes were used. The docked PDB files were uploaded to the iMODS server and the results were displayed keeping all the parameters as default.

Table 1

The antigenicity prediction and physicochemical property analysis of the two selected viral proteins. AN; antigenicity, AI; aliphatic index, GRAVY; grand average of hydropathicity.

Name of the protein	AN (threshold 0.4)	Number of amino acids	Molecular weight	Theoretical pi	Ext. coefficient (in M ⁻¹ cm ⁻¹)	Instability index	AI	GRAVY
Dengue virus type 1 envelope protein E	Antigenic	495	53776.92	7.51	67670	Stable (17.60)	85.62	-0.043
Human papillomavirus type 16 major capsid protein L1	Antigenic	505	56278.19	8.55	72030	Stable (36.57)	74.51	-0.347

Table 2

List of the predicted MHC class-I epitopes of both the DENV-1 envelope protein E and HPV-16 major capsid protein L1. AS; antigenicity score.

Dengue virus type 1						HPV type 16					
Epitope	Start	End	Topology	AS	Percentile scores	Epitope	Start	End	Topology	AS	Percentile scores
VTNPAPLVRK	50	58	Inside	0.966	0.01	TTYKNTNFK	353	361	Inside	0.939	0.01
TTIFAGHLK	276	284	Inside	0.897	0.02	YTFWEVNLK	444	452	Inside	0.862	0.04
KALKLSWFK	385	393	Inside	0.709	0.13	ISGHPLLNK	117	125	Outside	0.773	0.09
GSCVTTMAK	28	36	Inside	0.601	0.23	TSDAQIFNK	301	309	Inside	0.750	0.1
KIVQYENLK	128	136	Inside	0.587	0.24	RIHLPDPNK	74	82	Inside	0.746	0.1
PTLDIELLK	39	47	Outside	0.488	0.34	YLPPVPVSK	12	20	Outside	0.703	0.14
MVLLTMKEK	16	24	Inside	0.466	0.37	ISTSETTYK	348	356	Inside	0.664	0.17
SLITCAKFK	112	120	Inside	0.458	0.38	TSSTTSTAK	491	499	Inside	0.637	0.21
GTVLVQVKY	318	326	Inside	0.432	0.35	YVARTNIYY	27	35	Inside	0.618	0.22
ALKLSWFKK	386	394	Inside	0.416	0.47	STSTTAKRK	493	501	Inside	0.528	0.3
CTGSFKLEK	302	310	Inside	0.403	0.49	TSQAIACQK	422	430	Inside	0.470	0.37
GGVFTSVGK	426	434	Outside	0.325	0.66	RLLAVGHPY	41	49	Outside	0.375	0.54
LTWGLLNSR	463	471	Outside	0.289	0.78	AANAGVDNR	136	144	Inside	0.336	0.63
IVQYENLKY	129	137	Inside	0.288	0.78	VGHPYFPK	45	53	Outside	0.181	1.3
TTSQETWNR	225	233	Inside	0.282	0.80	NTNFKEYLR	357	365	Inside	0.148	1.5
KLEGKIVQY	124	132	Inside	0.282	0.81	SGLQYRVFR	66	74	Inside	0.123	1.7
YIVVGAGEK	277	385	Inside	0.259	0.87	FVTVVDTTR	330	338	Inside	0.083	2.2
FSGVSWTMK	448	456	Outside	0.238	0.94	TTAKRKRRK	496	504	Outside	0.064	2.6
ISNTTTDSR	65	73	Inside	0.142	1.5	PIKKPNNK	51	59	Inside	0.064	2.6
FTSVGKLVH	429	437	Outside	0.035	3.5	DSLFFYLLR	244	252	Outside	0.044	3.2

2.18. Codon adaptation and in silico cloning

In codon adaptation, the best vaccine constructs were reverse translated to the possible DNA sequences and then the DNA sequences were adapted according to the desired organism, so that the molecular mechanisms of that specific organism could use the codons of the adapted DNA sequences efficiently and provide better production of the desired product. Codon adaptation is a necessary step of *in silico* cloning because the same amino acid can be encoded by different codons in different organisms (codon biasness). Moreover, the cellular mechanisms of an organism may be completely different from another organism. As a result, codon(s) for a specific amino acid in one organism may not work in another organism. Therefore, codon adaptation predicts the suitable and best codon that can encode a specific amino acid in a specific organism [87,88]. The predicted protein sequences of the best selected vaccines were used for codon adaptation by the Java Codon Adaptation Tool or JCat server (<http://www.jcat.de/>) [87]. Eukaryotic *E. coli* strain K12 was selected and rho-independent transcription terminators, prokaryotic ribosome binding sites and SgrA1 and SphI cleavage sites of restriction enzymes, were avoided. In the JCat server, the protein sequences were reverse translated to the optimized possible DNA sequences. The optimized DNA sequences were then taken and SgrA1 and SphI restriction sites were conjugated at the N-terminal and C-terminal sites, respectively. Finally, the SnapGene restriction cloning module was used to insert the newly adapted DNA sequences between the SgrA1 and SphI restriction sites of the pET-19b vector [89].

3. Results

3.1. Selection of viruses and retrieval of viral proteins

Two viruses i.e., DENV-1 and HPV-16, were selected as the potential targets for vaccine designing in the experiment. The viruses were selected by analyzing numerous data from the National Center for Biotechnology Information or NCBI database. The DENV-1 envelope protein E and the HPV-16 major capsid protein L1 were selected as target proteins. The amino acid sequence of these proteins were:

3.1.1. Dengue virus type 1 envelope protein E (accession number: Q8BE390)

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MRCVGIGNRDFVEGLSGATWVDVVLEHGSCVTTMAKDPTL-DIEL-
LKTEVTNPAPLVRKLCIEA-
KISNTTDSRCPTQEATLVEEQDANFVCRRTFVDRGWNGCGLFGKG-
SLITCAKFKVTKLEGKIVQYENLKYSVIVTVHTGDHQVGNES-
TEHGTATITPQAPTSEIQLTDY-
GALTLDSPRTGLDFNEMVLLTMKEKSWLVHKQWFLDPLPWTS-
GATTSQETWNRQDLLVTFKTAHAKKQEVVVLGSQEGAMHTALTGA-
TEIQTSGTTTIFAGHLKCRKMDKTLKGMSYVMCTGSFKLEKE-
VAETQHGTVLVQVKYEGTDAPCK-
IPFSTQDEKGVTQNGRVITANPIVTDKEKPVNIEAEPPFGESYIVVGAGE-
KALKLSWFKK-
STIGKMFEA-
```

Table 3

List of the predicted MHC class-II epitopes of both the DENV-1 envelope protein E and HPV-16 major capsid protein L1. AS; antigenicity score.

Dengue virus type 1						HPV-16					
Epitope	Start	End	Topology	AS	Percentile scores	Epitope	Start	End	Topology	AS	Percentile scores
LLKTEVTNPVAL	45	56	Outside	4.10	0.78	FVRHLFNRAGAV	256	267	Outside	3.40	2.10
WFLLDPLPWTSG	212	223	Outside	2.90	3.60	PPLELINTVIQD	186	197	Outside	3.38	2.20
VQVKYEGTDAPC	322	333	Inside	2.70	4.00	YRVFRILHPDPN	70	81	Inside	3.10	2.80
VGNESTEHGTTA	151	162	Outside	2.60	4.70	VFRIHLPDPNKF	72	83	Outside	2.98	3.30
IQTSGTTTIFAG	270	281	Inside	2.50	5.30	YIHSMNSTILED	390	401	Inside	2.90	3.60
VVVLGSQEGAMH	250	261	Outside	2.40	5.60	YPDYIKMVSEPY	231	242	Outside	2.20	6.90
YGVLFSGVSWTM	444	455	Outside	2.20	7.00	MDFITLQANKSE	208	219	Outside	2.10	7.60
TLDIELLKTEVT	40	51	Outside	1.80	9.60	RKFLLQAGLKAK	466	477	Outside	2.00	8.20
YSVIVTVHTGDQ	137	148	Inside	1.78	10	TNIYVHAGTSRL	31	42	Inside	1.80	9.80
RLKMDKLTGKM	286	297	Inside	1.40	13	YWLQRQAQGHNNNG	311	322	Inside	1.78	9.90
VIVTVHTGDHQ	139	150	Inside	1.30	14	VRHLFNRAVAVG	257	268	Inside	1.30	14
WWDVVLEHGSCV	20	31	Outside	1.20	15	LQFIFQLCKITL	372	383	Outside	1.20	14
WFKKGSTIGKMF	391	402	Inside	1.18	15	WLPSEATVYLP	4	15	Inside	1.18	15
VLFGVSWMTKI	446	457	Outside	1.10	16	YFPPTSGSMVTS	291	302	Outside	1.10	16
YENLKYSIVTV	132	143	Inside	0.980	17	TLQANKSEVPLD	212	223	Inside	1.00	17
WTSGATTSQETW	220	231	Inside	0.900	18	SMVTSDAQIFNK	298	309	Inside	0.90	18
QETWNQRQDLLVT	228	239	Inside	0.700	21	LKKYTFWEVNLK	441	452	Inside	0.80	20
WLGLNSRSTSLS	465	476	Outside	0.600	22	YDLQFIFQLCKI	370	381	Outside	0.70	21
KISNTTDSRCP	64	75	Inside	0.500	23	YVARTTNIYYHAG	27	28	Inside	0.58	22
KIGIGVLLTWLG	456	463	Outside	0.300	25	WNFGLQPPPGBT	402	413	Outside	0.60	22

TARGARRMAILGDTAWDFGSIGGVFTSVGKLVHQIFG-TAYGVLFSGVSWTMKI-GIGVLLTWLGLNSRSTSLSMTCIAVGLVTLYLGVMVQA

3.1.2. Human papillomavirus type 16 major capsid protein L1 (accession number: Q9WLQ6)

MSLWLPSEATVYLPPVPVSKVVSTDEYVARTNIYY-HAGTSRLAVGHPYFPIKPKPNNNKILVPKVSGLQYRV-FRIHLPDPNKGFPDTSFYNPDTQRLVWACVGVEVRGQPLGV-GISGHPLLNLKDDTENASAAYANAGVDNRECISMDYKQTQLCLIGCKPPI-GEHWGKGSPCTNVAVNPGDCPPL-EINTVIQDGMVDTGFGAMDFTTLQANKSEVPLDICTSICK-YPDYIKMVSEPYGDSLFFYLRREQMFRHLFNRAVAVGENVPD-DLYIKGSG-STANLASSNYFPTPSGSMVTSDA-QIFNKPYWLQRAQGHNNNGICWGNQLFVTVVDTTRSTNMSLCAAISTSET-TYKNTNFKEYLRHGEEYDLQFIFQLCK-ITLTADVMTYIHSMN-STI-LEDWNFGLQPPPGBTLEDTYRFVTSQAIACQKHTPPAPKEDPLKKYTF-WEVNKEKFSADLDQFPLGRKFLLQAGLKAKPKFTLGKRKATPTTSST-TAKRKKRKL

3.2. Antigenicity and physicochemical property analysis of the viral protein sequences

Both selected proteins were found to be potentially antigenic. Both of them had quite similar number of amino acids (495 amino acids for DENV-1 and 505 amino acids for HPV-16). DENV-1 had a molecular weight of 53776.92 and HPV-16 had a molecular weight of 56278.19. The extinction coefficients of DENV-1 and HPV-16 were 53776.92 and 56278.19, respectively, and both of them had quite a high aliphatic index. Moreover, the grand average of hydropathicity (GRAVY) of DENV-1 was -0.043 and HPV-16 was estimated to be -0.347. The results of the antigenicity and physicochemical analysis are listed in Table 1.

3.3. T-cell and B-cell epitope prediction and topology determination of the epitopes

The T-cell epitopes of MHC class-I of both the DENV-1 and HPV-16 were determined by the IEDB server. The server generated over 100 such epitopes. However, based on analyzing the antigenicity scores (AS)

and percentile scores, twenty potential epitopes from the top forty epitopes were selected for antigenicity, allergenicity, toxicity and conservancy tests. The server ranks the epitopes based on the increasing order of percentile scores, and lower percentile score always corresponds to good binding affinity of the epitopes to their alleles. The T-cell epitopes of MHC class-II (HLA DRB1*04-01 allele) of both DENV-1 and HPV-16 were also determined by the IEDB server. Since several epitopes generated a similar AS score as well as the percentile score, only one epitope from the group of epitopes that showed similar results were selected. Moreover, the B-cell epitopes were selected using the BepiPred linear epitope prediction method of the IEDB server and the epitopes were selected based on their higher lengths. Moreover, the topology prediction of the selected epitopes showed that more than half of the epitopes might reside in the interior of the cell membrane. Table 2 and Table 3 list the potential T-cell epitopes with their respective topologies. The potential B cell epitopes are listed in Table 4 (Fig. 2).

3.4. Antigenicity, allergenicity, toxicity, conservancy and population coverage analysis

In the antigenicity, allergenicity, toxicity and conservancy analysis, the epitopes that were found to be highly antigenic, non-allergenic, non-toxic and had conservancy of over 100%, were selected for further analysis and vaccine construction. Among the twenty selected MHC class-I epitopes and twenty selected MHC class-II epitopes of DENV-1, a total of ten epitopes (five epitopes from each of the MHC class-II and class-II category) were selected based on the mentioned criteria. However, all of the B-cell epitopes of DENV-1 were non-allergenic, and for this reason, these epitopes were selected for vaccine construction against DENV-1 and molecular docking analysis.

On the other hand, only three of the selected twenty epitopes of MHC class-I and seven of the selected twenty epitopes of MHC class-II of HPV-16 obeyed the mentioned criteria and therefore, they were selected for vaccine construction after molecular docking. Five of the selected seven HPV-16 B-cell epitopes were found to be non-allergenic, for this reason these five B-cell epitope sequences were used for vaccine construction (Table 4, Table 5, Table 6).

The population coverage analysis predicted that the DENV-1 envelope protein E had MHC class-I coverage of around 80–90% in the world, whereas, MHC class-II coverage of around 70–80%. On the other hand, HPV-16 major capsid protein L1 had MHC class-I coverage of around 70–80% in most of the regions of the world and MHC class-II coverage of around 90% (Table 7).

Table 4
List of the predicted B cell epitopes of both the DENV-1 envelope protein E and HPV-16 major capsid protein L1 along with their allergenicity and topology. AG; allergenicity, TT; transmembrane topology.

Dengue virus type 1	Sequence	HPV-16					
		Start	End	Length	AG	TT	Remarks
TGDOHQYGNESTEHGTATITPQAPTSQL	145	175	31	Non-allergen	Inside	Selected	PPIGEHWGKGSPCTNVAVNPGDCPP
YEGTDAPKRPFSTQDEKGVTQN	326	348	23	Non-allergen	Inside	Selected	PDNIRKFGPPDTSFYNPDT
SNTTIDSRCPQTQGEATLVEE	66	85	20	Non-allergen	Inside	Selected	KRKATPTISSTSTFAKRK
PWTSGATTISQETWN	219	232	14	Non-allergen	Outside	Selected	DDTENASAYAAANAGVD
IVTDKEKPVNIEAEPFGE	357	375	19	Non-allergen	Inside	Selected	TVLPPPPVKVVS
—	—	—	—	—	—	—	SNVFTPTPSGSMTV
—	—	—	—	—	—	—	YIKGSGSTANIA

3.5. Cluster analysis of the MHC alleles

The cluster analysis of the possible MHC class-I and MHC class-II alleles that may interact with the predicted epitopes were carried out by the online tool MHCcluster 2.0 . The tool generates the clusters of the alleles in a phylogenetic manner. Fig. 3 illustrates the outcome of the experiment, where the red zones indicate strong interactions and the yellow zones correspond to weaker interactions.

3.6. Generation of the 3D structures of the epitopes and peptide-protein docking

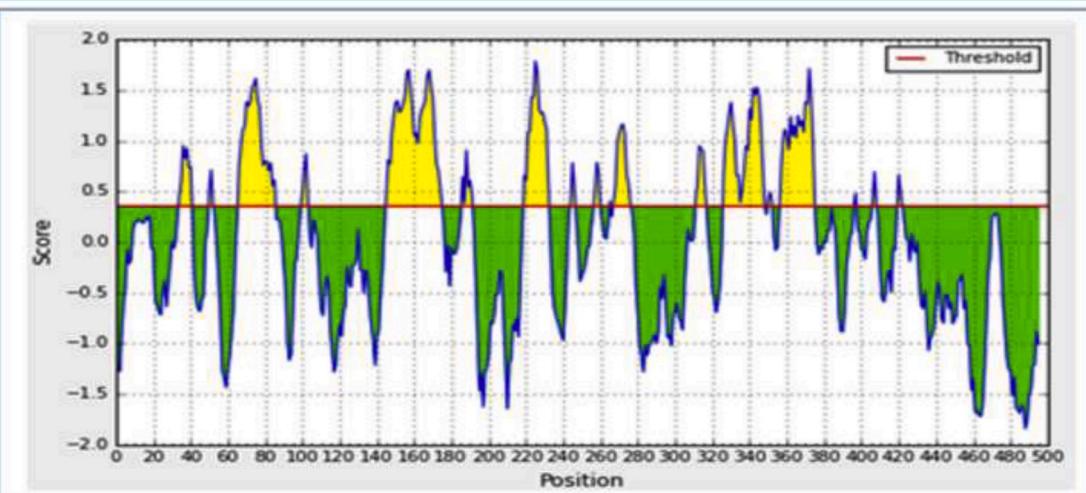
All of the T-cell epitopes (ten epitopes from each of the DENV-1 and HPV-16 viral proteins) were subjected to 3D structure generation for peptide-protein docking. In the docking study, all the best selected epitopes successfully generated global energies; therefore, all of the epitopes were docked successfully with their respective targets. FTSVGKLHV generated the lowest global energy of -38.88 and hence generated the best score among the epitopes from DENV-1 virus protein that were docked with the MHC class-I allele. VQVKYEGTDAPC generated the lowest and best global energy score of -49.55 among the epitopes of DENV-1 envelope protein E that were docked with the MHC class-II allele. On the other hand, SGLQYRVFR generated the best global energy of -49.76 among the epitopes from HPV-16 major capsid protein L1 that were docked with the MHC class-I allele and YIHSMN-STILED exhibited a global score of -18.54 , which was the best among the epitopes of HPV-16 viral protein that were docked with the MHC class-II allele (Fig. 4). The results of the docking experiment are listed in Table 8.

3.7. Vaccine construction

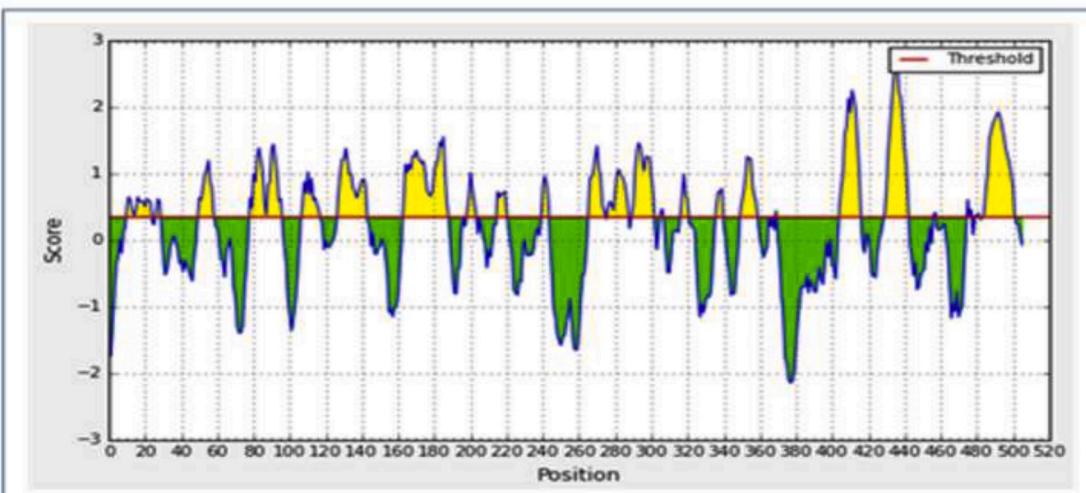
After successful docking, the vaccines were constructed against the two viruses. Against each of the viruses, three different vaccines were constructed. For the vaccine construction, first adjuvant sequences were linked with the PADRE sequence by EAAAK. Next, the PADRE sequence was connected to the CTL epitopes by GGGS linkers. The GGGS linkers were also used to connect the CTL epitopes among themselves. Later, HTL epitopes were conjugated with GPGPG linkers and the BCL epitopes were conjugated by KK linkers. Three different adjuvants were used to construct three different vaccines for each of the viruses. Therefore, the three vaccines of DENV-1 differ from each other only in their adjuvant sequences and three of them had the same epitopes. Similarly, the three vaccines of HPV-16 also differ from each other only in their adjuvant sequences. For the construction of DENV-1 vaccine, five CTL epitopes, five HTL epitopes and five BCL epitopes were used. Furthermore, three CTL epitopes, seven HTL epitopes and five BCL epitopes were used to construct the vaccines for HPV-16. The three dengue vaccines were denoted as DV-1, DV-2, DV-3, respectively, and the three HPV-16 vaccines were denoted as HV-1, HV-2 and HV-3, respectively (Table 9).

3.8. Antigenicity, allergenicity and physicochemical property analysis

All the vaccine constructs of the two viruses were found to be non-allergenic and probable antigenic. HV-3 construct had the highest number of amino acids (449). DV-3 had the highest molecular weight of 49502.81 as well as the highest extinction coefficient of $51.575\text{ M}^{-1}\text{ cm}^{-1}$. All the vaccine constructs were also found to be stable and had a predicted half-life of 1 h in the mammals. Moreover, HV-2 had the highest aliphatic index (76.29) and the lowest GRAVY value of -0.149 . The results of the allergenicity, antigenicity, and physicochemical property analysis of the vaccine constructs are listed in Table 10.



(a)



(b)

Fig. 2. The graphs predicting the results of the B-cell epitope prediction of dengue vaccine type-1 envelope protein E1 (a) and HPV-16 major capsid protein L1 (b), using the Bipiperid linear epitope prediction method. In the graphs, the most potent regions (above the threshold) are shown by yellow color.

Table 5 The antigenicity, allergenicity, toxicity and conservancy analysis of MHC class-I epitopes of DENV-1 envelope protein E and HPV-16 major capsid protein L1. AN; antigenicity, AG; allergenicity, MI; minimum identity, CV; conservancy.

Dengue virus-1		HPV-16												
Epitope	AN	AG	Toxicity	CV	MI	Remarks	Epitope	AN	AG	Toxicity	CV	MI	Remarks	
VINPAVLRK	Non-antigen	Non-allergen	Non-toxic	75%	33.33%	Not-selected	TYKNTNFK	Antigen	Non-allergen	Non-toxic	60%	44.44%	Not-selected	
TTIFAGHLK	Non-antigen	Non-allergen	Non-toxic	100%	55.56%	Not-selected	YTFWVFVNLLK	Antigen	Allergen	Non-toxic	100%	55.56%	Not-selected	
KALKSWFK	Antigen	Non-allergen	Allergen	100%	55.56%	Selected	ISGHPLLNK	Non-allergen	Allergen	Non-toxic	100%	66.67%	Non-selected	
GSCVTTMVK	Antigen	Non-allergen	Allergen	100%	77.78%	Not-selected	TSDAQIFNK	Non-allergen	Allergen	Non-toxic	100%	55.56%	Not-selected	
KIVQYENLK	Antigen	Non-allergen	Allergen	100%	55.56%	Not-selected	RHLPLDFNK	Antigen	Allergen	Non-toxic	100%	66.67%	Not-selected	
PTLDIELLK	Non-antigen	Non-allergen	Allergen	100%	77.78%	Not-selected	YLPPVPYSK	Non-allergen	Allergen	Non-toxic	80%	44.44%	Not-selected	
MVILTMKEK	Antigen	Non-allergen	Non-toxic	100%	55.56%	Selected	ISTSETTYK	Antigen	Allergen	Non-toxic	40%	33.33%	Not-selected	
SLITCAFKF	Antigen	Non-allergen	Non-toxic	75%	44.44%	Not-selected	TSSTTAK	Antigen	Allergen	Non-toxic	20%	44.44%	Not-selected	
GTIVLVQVRY	Non-antigen	Non-allergen	Non-toxic	75%	44.44%	Not-selected	YVARVTRYY	Non-allergen	Allergen	Non-toxic	100%	66.67%	Not-selected	
ALKLSWFKK	Antigen	Non-allergen	Non-toxic	100%	55.56%	Selected	STSTARRK	Antigen	Non-allergen	Non-toxic	60%	44.44%	Not-selected	
CTGSFKLEK	Non-antigen	Non-allergen	Allergen	50%	44.44%	Not-selected	TSOAIACQK	Non-allergen	Non-allergen	Non-toxic	80%	33.33%	Not-selected	
GGVFTSVGK	Non-antigen	Non-allergen	Allergen	100%	66.67%	Not-selected	RLLAVGHGPY	Antigen	Allergen	Non-toxic	100%	88.89%	Not-selected	
LTVLGLNSR	Non-antigen	Non-allergen	Allergen	100%	55.56%	Not-selected	AANAVGDNR	Antigen	Non-allergen	Non-toxic	60%	33.33%	Not-selected	
IVQYENLKY	Antigen	Allergen	Non-toxin	100%	66.67%	Not-selected	VGHYPFPIK	Antigen	Allergen	Non-toxic	100%	66.67%	Not-selected	
TTSQETWNRR	Antigen	Non-allergen	Non-toxin	50%	33.33%	Not-selected	NTNKEYLRL	Antigen	Non-allergen	Non-toxic	80%	44.44%	Not-selected	
KLEGKIVQY	Antigen	Allergen	Non-toxin	75%	44.44%	Not-selected	SGLQYRVFR	Antigen	Non-allergen	Non-toxic	100%	66.67%	Selected	
YIVVGAGEK	Antigen	Non-allergen	Allergen	75%	33.33%	Not-selected	FVTVDITR	Antigen	Non-allergen	Non-toxic	100%	66.67%	Selected	
FSGVSWTMK	Antigen	Non-allergen	Non-toxin	100%	55.56%	Not-selected	TTARKKKRK	Antigen	Non-allergen	Non-toxic	60%	33.33%	Not-selected	
ISNTTIDSR	Antigen	Non-allergen	Non-toxin	100%	66.67%	Selected	PIKPKNNNK	Antigen	Non-Toxic	40%	33.33%	Not-selected		
FTSVGKUHV	Antigen	Non-allergen	Non-toxin	100%	55.56%	Selected	DSLFVYLR	Antigen	Non-allergen	Non-toxic	100%	66.67%	Selected	

3.9. Secondary and tertiary structure prediction of the constructed vaccines

From the secondary structure analysis, it was analyzed that the DV-1 had the lowest amount of amino acids (5.3%) in the alpha helix formation, and the highest amount of the amino acids (42.6%) in the beta sheet formation, among the dengue vaccines. However, DV-2 had the highest amount of the amino acids, 54.6%, in the coil formation. Among the HPV-16 vaccines, HV-3 had the highest percentage of the amino acids in the alpha helix formation (42.7%). Furthermore, HV-1 had 20.2% of its amino acids, HV-2 had 12.1% of its amino acids and 9.5% of the amino acids, in the beta sheet formation. And among the HPV-16 vaccine, HV-1 had the highest percentage of amino acids in the coil structure (62.3%) (Fig. 5a, b, 5c and Table 11).

In the 3D structure prediction experiment, all three dengue vaccines were found to have 3 domains and DV-1 had the lowest p-value of 1.35e-09. The homology modeling of the three dengue vaccine constructs were carried out using 4GT0A as template from the protein data bank (<https://www.rcsb.org/>). Among the HPV-16 vaccine constructs, only HV-1 vaccine had 3 domains. The homology modeling of all the vaccines were conducted using 5J6RA as the template sequence from the protein data bank. However, HV-2 had the lowest p-value of 9.06e-12 among the HPV-16 vaccines (Fig. 6a, b, 6c). The results of the 3D structure analysis are listed in Table 12 and illustrated in Fig. 7.

3.10. 3D structure refinement and validation

The 3D protein structures generated by the RaptorX server were refined using the 3Drefine server and then the refined structures were analyzed by Ramachandran plots generated by the PROCHECK server. The analysis showed that DV-1 vaccine had 72.9% of the amino acids in the most favored regions, 23.8% of the amino acids in the additional allowed regions, 2.2% of the amino acids in the generously allowed regions, and 1.1% of the amino acids in the disallowed regions. The DV-2 vaccine had 78.4% of the amino acids in the most favored regions, 18.3% of the amino acids in the additional allowed regions, 1.7% of the amino acids in the generously allowed regions, and 1.7% of the amino acids in the disallowed regions.

The HV-1 vaccine had 84.9% amino acids in the most favored regions, 14.3% of the amino acids in the additional allowed regions, and 0.8% of the amino acids in the generously allowed region. No outlier was found in the HV-1 vaccine. HV-2 had 85.2% of the amino acids in the most favored regions and 10.9% of the amino acids in the additional allowed regions. It also had 2.4% of the amino acids in the generously allowed regions and 1.5% of the amino acids in the disallowed regions. However, HV-3 showed a very good result with 87.5% of the amino acids in the most favored regions and 9.4% of the amino acids in the additional allowed regions (Fig. 8a and b).

3.11. Protein disulfide engineering

In disulfide engineering, disulfide bonds were generated using the 3D structures of the vaccine constructs. The DbD2 server identifies the pairs that have the capability to form disulfide bonds based on the given selection criteria; however, in this experiment, we selected only those pairs whose bond energy value were less than 2.2 kcal/mol. For this reason, only two pairs of residues were selected for disulfide bond formation for the DV-1: 21 gly-47 arg and 282 thr-287 cys. Three pairs of residues were selected for disulfide bond formation for the DV-2 vaccine: 212 ser-222 pro, 398 trp-401 lys and 417 pro-422 lys. And four residue pairs were also selected for the DV-3: 180 ala-189 leu, 396 thr-401 cys, 435 lys-441 ile and 446 pro-451 lys.

Table 6

The antigenicity, allergenicity, toxicity and conservancy analysis of MHC class-II epitopes of DENV-1 envelope protein E and HPV-16 major capsid protein L1. AN; antigenicity, AG; allergenicity, MI; minimum identity, CV; conservancy.

Dengue virus-1							HPV-16						
Epitope	AN	AG	Toxicity	CV	MI	Remarks	Epitope	AN	AG	Toxicity	CV	MI	Remarks
LLKTEVTNPABL	Non-antigen	Non-allergen	Non-toxic	75%	41.67%	Not-selected	PPLLEINTYQD	Antigen	Non-allergen	Non-toxic	1.00%	58.33%	Selected
WFLDLPLPWTSG	Antigen	Allergen	Non-toxic	100%	83.33%	Not-selected	YRVRHHLPDPN	Antigen	Non-allergen	Non-toxic	80%	41.67%	Not-selected
VQVKFESTGIDAPC	Antigen	Non-allergen	Non-toxic	50.00%	41.67%	Not-selected	YIHSMNNTSILED	Antigen	Non-allergen	Non-toxic	1.00%	58.33%	Selected
VGNESTHEGTTA	Antigen	Allergen	Non-toxic	50%	41.67%	Not-selected	YPDYTKMVSEPY	Antigen	Non-allergen	Non-toxic	1.00%	50.00%	Not-selected
IOTSGTTIFAG	Non-antigen	Allergen	Non-toxic	100%	91.67%	Selected	MDDFTLQANKSE	Antigen	Allergen	Non-toxic	80%	41.67%	Not-selected
VVLGLSQEGAMH	Antigen	Non-allergen	Non-toxic	100%	50.00%	Not-selected	RKFILQAGLIKAK	Non-antigen	Allergen	Non-toxic	1.00%	58.33%	Not-selected
YGVLFSGVSWTM	Non-antigen	Allergen	Non-toxin	100%	58.33%	Not-selected	TNYYHAGTSRL	Antigen	Non-allergen	Non-toxic	1.00%	66.67%	Selected
TLDIELLKTEVT	Non-antigen	Allergen	Non-toxin	75%	41.67%	Not-selected	YWLQRAQGHNNNG	Antigen	Allergen	Non-toxic	1.00%	91.67%	Not-selected
YSVIVTVHTGDQ	Antigen	Non-allergen	Non-toxin	100%	50.00%	Selected	VRHFVNRAAGAV	Antigen	Non-allergen	Non-toxic	1.00%	58.33%	Selected
RLKMDKLTLKGM	Antigen	Allergen	Non-toxin	75%	41.67%	Not-selected	LQFIFQLCKTIL	Antigen	Allergen	Non-toxic	1.00%	58.33%	Not-selected
VIVTVHGTQHQ	Antigen	Allergen	Non-toxin	100%	83.33%	Not-selected	WLPEATVYLPP	Non-antigen	Non-allergen	Non-toxic	1.00%	66.67%	Not-selected
WVDDVILEHGSCV	Antigen	Allergen	Non-toxin	100%	75.00%	Selected	YFPPTSGSMVTS	Antigen	Allergen	Non-toxic	1.00%	66.67%	Not-selected
WFKRGSTIGKMF	Non-antigen	Allergen	Non-toxin	100%	50.00%	Not-selected	TLQANKSEVPLD	Non-antigen	Non-allergen	Non-toxic	1.00%	50.00%	Not-selected
VIFSGVSWTMKI	Non-antigen	Non-allergen	Non-toxin	100%	50.00%	Not-selected	SMVTSDAQFNK	Antigen	Non-allergen	Non-toxic	1.00%	58.33%	Selected
YENLKYSVITV	Non-antigen	Allergen	Non-toxin	75%	41.67%	Not-selected	LKKYTFWEVNLK	Antigen	Allergen	Non-toxic	80%	41.67%	Not-selected
WTSGATTSOETW	Antigen	Allergen	Non-toxin	50%	33.33%	Not-selected	YDLQFIFQLCKI	Antigen	Allergen	Non-toxic	1.00%	58.33%	Not-selected
QETWNQDILVT	Antigen	Non-allergen	Non-toxin	100%	58.33%	Selected	YVARTNIIYHAG	Non-antigen	Allergen	Non-toxic	1.00%	66.67%	Not-selected
WLGJNSRSTSLS	Antigen	Allergen	Non-toxin	100%	66.67%	Not-selected	WNFGIQLPPPGT	Antigen	Allergen	Non-toxic	80%	33.33%	Not-selected
KISNTTDSRCP	Antigen	Non-allergen	Non-toxin	100%	50.00%	Not-selected	FVRHLFNRAVAV	Antigen	Non-allergen	Non-toxic	1.00%	58.33%	Selected

Table 7

Results of the population coverage analysis of the MHC alleles of the selected proteins.

Name of the region	Dengue virus-1 envelope protein E		MHC class-II coverage (%)	HPV-16 major capsid protein L1	MHC class-II coverage (%)	MHC class-II coverage (%)
	-	MHC class-I coverage (%)				
East Asia		90%	83%	85%	91%	91%
Northeast Asia		92%	84%	82%	96%	96%
Southwest Asia		87%	87%	66%	93%	93%
Southeast Asia		95%	82%	81%	91%	91%
Europe		82%	66%	92%	98%	98%
Central Africa		84%	73%	61%	95%	95%
South America		88%	70%	70%	97%	97%
North America		91%	77%	87%	96%	96%
Australia		90%	89%	78%	88%	88%
China		88%	75%	73%	90%	90%
India		91%	84%	68%	91%	91%

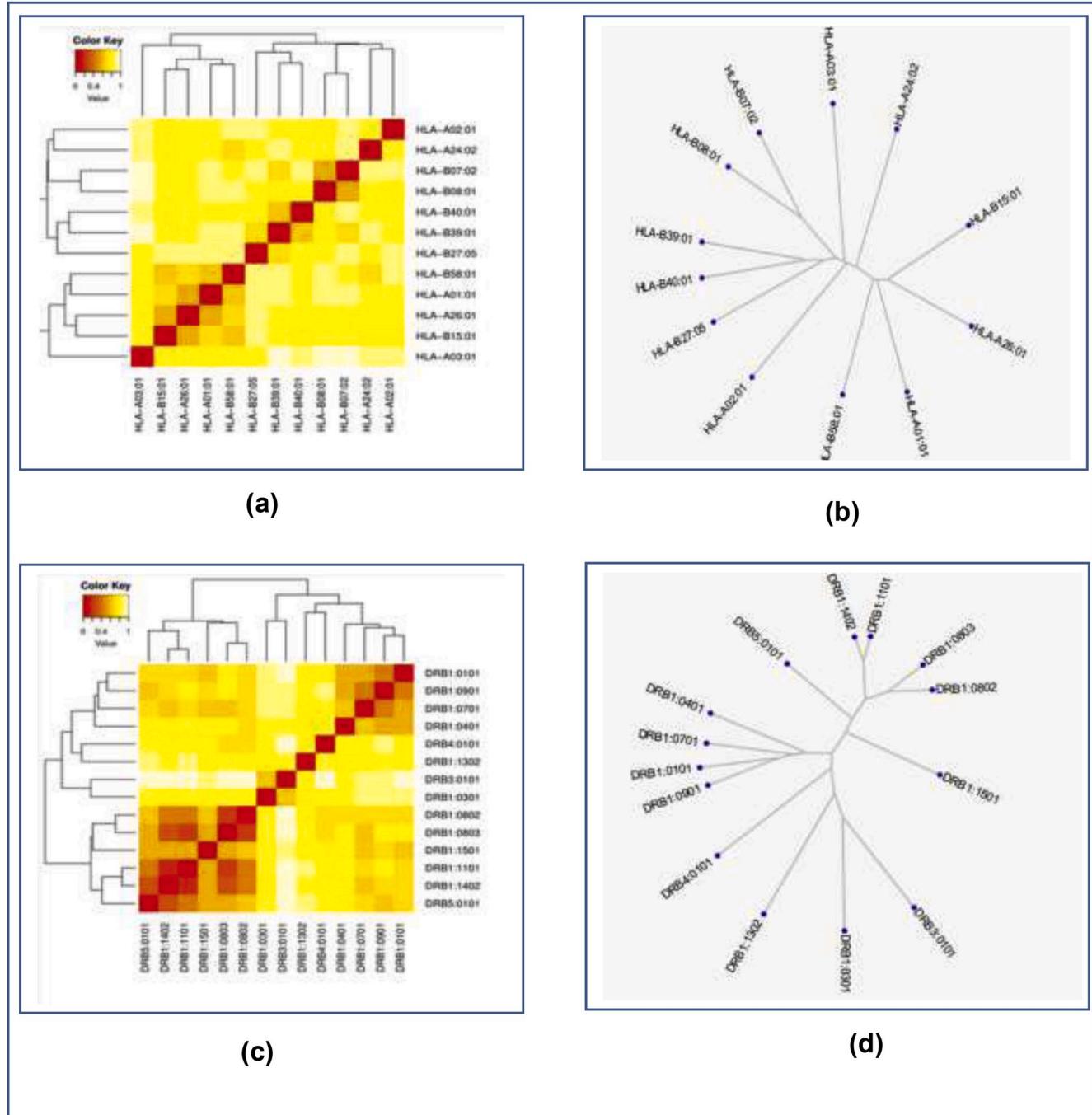


Fig. 3. The results of the MHC cluster analysis. Here, (a) is the heat map of MHC class-I cluster analysis, (b) is the tree map of MHC class-I cluster analysis, (c) is the heat map of MHC class-II cluster analysis, (d) is the tree map of MHC class-II cluster analysis.

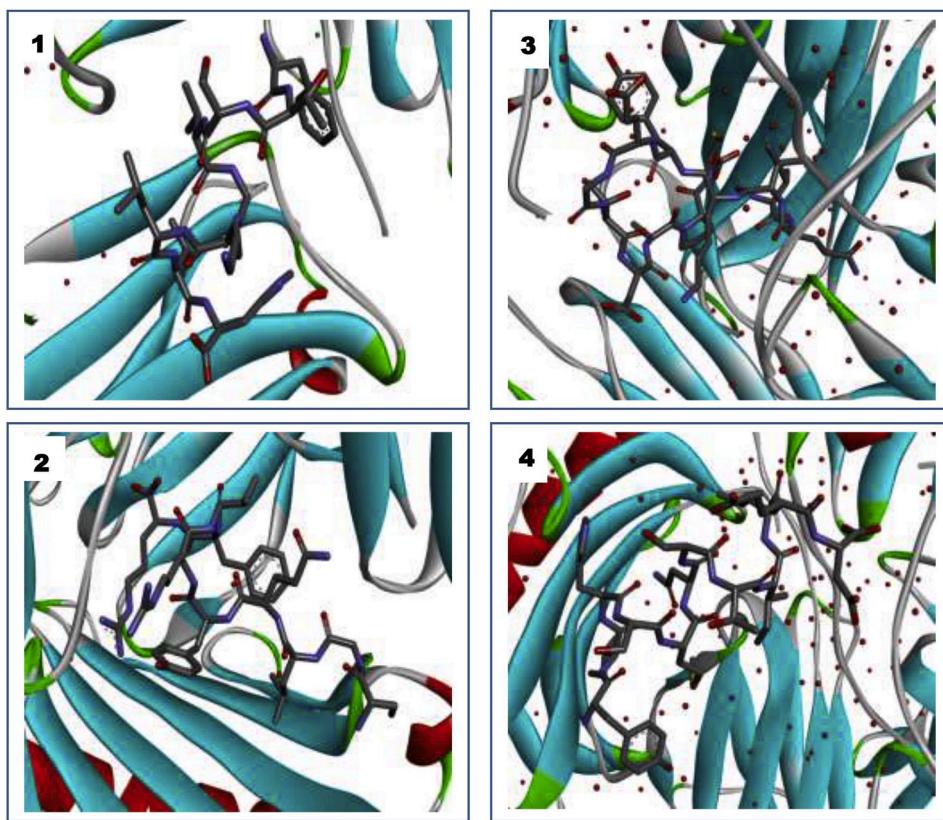


Fig. 4. The interaction between the four epitopes with their respective target proteins, that gave the best results. Here, 1. Interaction between FTSVGKLVH and MHC class-I allele, 2. Interaction between SGLQYRVFR and MHC class-I allele, 3. Interaction between VQVKYEGTDAPC and MHC class-II allele, 4. Interaction between YIHSMNSTILED and MHC class-II allele.

Table 8
Results of molecular docking analysis of the selected epitopes of DENV-1 and.

Name of the virus	Epitope	MHC allele	Global energy	Hydrogen bond energy	Epitope	MHC allele	Global energy	Hydrogen bond energy
Dengue virus type 1	KALKLSWFK	HLA-A*11-01 allele (PDB ID: 5WJL)	-11.76	-1.08	VQVKYEGTDAPC	HLA DRB1*04-01 (PDB ID: 5JLZ)	-49.55	-2.58
	MVLLTMKEK		-19.52	-1.43	VVVLGSEQEAMH		-1.11	0.00
	ALKLSWFKK		-22.93	-0.89	RLKMDKLTLKGM		-5.59	-1.44
	ISNTTTDSR		-21.40	-0.94	WFKKGSTIGKMF		-10.89	-0.88
	FTSVGKLVH		-38.88	-0.94	WLGLNRSRTSLS		-16.66	-2.46
	SGLQYRVFR		-49.76	-2.45	FVRHLFNRAAGAV		-3.26	-6.25
	FVTVVDTTR		-24.16	-3.09	PPLEINTVIQD		-2.57	0.00
	DSLFFYLRR		-24.98	-2.81	VFRHLPDPNKF		-5.11	-0.52
	-		-	-	YIHSMNSTILED		-18.54	-0.65
	-		-	-	TNIYYHAGTSRL		-0.08	0.00
	-		-	-	VRHLFNRAAGAVG		-1.66	0.00
	-		-	-	SMVTSDAQIFNK		-1.03	-1.19

The disulfide engineering of the three HPV-16 vaccine constructs were performed using the DbD2 server in the same way as the DENV vaccines. The HV-1 vaccine had no pair with bond energy less than 2.5 kcal/mol. HV-2 had 2 pairs of amino acids within the selection criteria: 68 phe-118 ala and 150 lys-185 ser and HV-3 had 3 pairs of amino acids that had bond energy of less than 2.2 kcal/mol; 230 his-252 val, 345 ile-388 thr and 347 glu-386 lys. All the vaccines were converted to mutant form for analysis (Fig. 9 and Fig. 10).

3.12. Protein-protein docking

The protein-protein docking study of the vaccine constructs and the MHC alleles revealed that among the three dengue vaccines, DV-1 generated the best performances in the docking analysis. It showed the lowest binding free energies while binding with all the MHC alleles

in the MM-GBSA study. Moreover, the lowest HawkDock scores of DV-1, while binding with MHC alleles DRB3*0202 (-5442.91), DRB5*0101 (-6920.47), DRB1*0101 (-7739.52) and DRB3*0101 (-6455.32), were also predicted by the HawkDock server. On the other hand, according to the FireDock server, DV-1 showed best performances while binding with DRB5*0101 (-6.33) and DRB1*0101 (-29.13). And the ClusPro 2.0 server also predicted DV-1 as the best vaccine construct where it was found to generate the best energy scores with all of its targets. None of the other two DENV-1 vaccine constructs showed such good results as DV-1. For these reasons, DV-1 was considered as the best vaccine construct among the three vaccines of DENV-1.

Among the three HPV-16 vaccines, HV-1 vaccine construct should be considered as the best vaccine construct. HV-1 generated the best result in the MM-GBSA study, with the lowest binding free energies while binding with all the receptor proteins. Again, when the docking was

Table 9

List of the vaccines constructed for DENV-1 (DV) and HPV-16 (HV). Here, the bolded words represent the linker sequences.

Name of the vaccines	Vaccine constructs	Number of amino acids
Dengue vaccine-1 (DV-1)	EAAAKGIIINTLQKYCCRVRGGRCAVLSCPKEEIQIKCSTRGRKCCRKEAAAKAKFVAAWTLKAAAGGGSS KALKLSWFKGGSVLLTMKEGGGSALKLSWFKKGGGSISNTTDSRGGSFTSVGKLVHGPGPVG QVKYEGTDAPCGPGPGVVLGSQEGAMHGPGPGRLKMDKLTLMGMPGPFWFKGSTIGKMFPGPGP WLGLNSRSTSLSKKTGDHQVGNESTEHGTATITPQAPTSEIQLKKYEGTDAPCKIPFSTQDEKGVTQNKSNT TTDSRCTQGEATLVEEKPKWTSGATTSQETWNKK	354
Dengue vaccine-2 (DV-2)	EAAAKMAKLSTDDELLAFKEMTLLSDFVKKFEETFEVTAAPVAVAAGAAPAGAAVEAE QSEFDVILEAGDKKIGVVKVREIVSGLKEAKDLDGAPKPLLEKVAKEADEAKAKLEAGATVTVK EAAAAKAKFVAAWTLKAAAGGGSKALKLSWFKGGSVLLTMKEGGGSALKLSWFKKGGGSISNTTDSRGGS FTSVGKLVHGPGPVGQVVKYEGTDAPCGPGPGVVLGSQEGAMHGPGPGRLKMDKLTLMGMPGP WFKKGSTIGKMFPGPGWGLNSRSTSLSKKTGDHQVGNESTEHGTATITPQAPTSEIQLKK YEGTDAPCKIPFSTQDEKGVTQNKSNTTDSRCTQGEATLVEEKPKWTSGATTSQETWNKK IVTDKEKPVNIEAEPFFGEKAKFVAAWTLKAAAGGGSS	439
Dengue vaccine-3 (DV-3)	EAAAKMAENPNIDDLPLAPLALGAADLALATVNDLIANLRERAETRAETRTRVEERRARLTQFQEDLPEQ FIELRDKFTEELRKAAEGYLEAATNRYNELVERGEAALQRRLRSQTAFEDASARAEGYVDQAELTQEALGT VASQTRAVGERAAKLVGLIEAAAKAKFVAAWTLKAAAGGGSKALKLSWFKGGSVLLTMKEK GGGSALKLSWFKGGSISNTTDSRGGSFTSVGKLVHGPGPVGQVVKYEGTDAPCGPGPGVVLGS QEGAMHGPGPGRLKMDKLTLMGMPGPFWFKGSTIGKMFPGPGWGLNSRSTSLSKKTGDHQVGNESTEHGTATITPQ APTSEIQLKKYEGTDAPCKIPFSTQDEKGVTQNKSNTTDSRCTQGEATLVEEKPKWTSGATTSQETWNKK KKVITDKEKPVNIEAEPFFGEKAKFVAAWTLKAAAGGGSS	468
HPV vaccine-1 (HV-1)	EAAAKGIIINTLQKYCCRVRGGRCAVLSCPKEEIQIKCSTRGRKCCRKEAAAK AKFVAAWTLKAAAGGGSSGLQYRVRFGGSFTVVDTTRGGSDSLFFYLRGPGPFWHLNRAGAVGPGP PPLEINTVIQDGPGPGVFRILHPDPNPKPGPGPYIHSMNSTILEDGPGPGTNIYYHAGTSRLGPGPVGVRHLNRAGAVG GPGPMSMVTSDAQIFNKKKPIGEHWGKGSPCTNVAVNPDCPPKKDDTENASAYAANAGVD KTKTVLPPVPVKVSKKSNYFPPTPSGMVTKKYIKGSGSTANLAKKAKFVAAWTLKAAAGGGSS	335
HPV vaccine-2 (HV-2)	EAAAKMAKLSTDDELLAFKEMTLLSDFVKKFEETFEVTAAPVAVAAGAAPAGAAVEAE SEFDVILEAAGDKKIGVVKVREIVSGLKEAKDLDGAPKPLLEKVAKEADEAKAKLEAGATVTVK EAAAAKAKFVAAWTLKAAAGGGSSGLQYRVRFGGSFTVVDTTRGGSDSLFFYLRGPGPFWHLNRAGAVG GPGPMSMVTSDAQIFNKKKPIGEHWGKGSPCTNVAVNPDCPPKKDDTENASAYAANAGVD AGVDKKTVLPVPVKVSKKSNYFPPTPSGMVTKKYIKGSGSTANLAKKAKFVAAWTLKAAAGGGSS	420
HPV vaccine-3 (HV-3)	EAAAKMAENPNIDDLPLAPLALGAADLALATVNDLIANLRERAETRAETRTRVEERRARLTQFQEDLPEQ TKFQEDLPEQFIELRDKFTEELRKAAEGYLEAATNRYNELVERGEAALQRRLRSQTAFEDA SARAEGYVDQAVELTQEALGTVASQTRAVGERAAKLVGLIEAAAKAKFVAAWTLKAAAGGGSS GGGSSGLQYRVRFGGSFTVVDTTRGGSDSLFFYLRGPGPFWHLNRAGAVGPGPFWHLNRAGAVGP VFRIHLPDPNPKFGPGPYIHSMNSTILEDGPGPFWHLNRAGAVGPGPFWHLNRAGAVGP PPIGEHWGKGSPCTNVAVNPDCPPKKDDTENASAYAANAGVDKKTVLPVPVKVSKKSNYFPPTPSGMVTKKYIKGSG STANLAKKAKFVAAWTLKAAAGGGSS	449

Table 10

The results of the allergenicity, antigenicity and physicochemical property analysis of the constructed vaccines of DENV-1 (DV) and HPV-16 (HV). AG; allergenicity, AN; antigenicity, AI; aliphatic index, GRAVY; grand average of hydropathicity.

Vaccine constructs	AG	AN (Threshold 0.4)	No of amino acids	Molecular weight	Ext. coefficient (in $\text{M}^{-1} \text{cm}^{-1}$)	Instability index	AI	GRAVY	Predicted half-life (in human/mammal)
DV-1	Non-allergen	Antigenic	354	37035.36	50,460	27.13 (stable)	56.27	-0.582	1 h
DV-2	Non-allergen	Antigenic	439	45314.65	47,105	23.26 (stable)	69.23	-0.344	1 h
DV-3	Non-allergen	Antigenic	468	49502.81	51,575	31.15 (stable)	67.05	-0.541	1 h
HV-1	Non-allergen	Antigenic	335	34841.93	33,390	30.62 (stable)	64.39	-0.351	1 h
HV-2	Non-allergen	Antigenic	420	43121.21	30,035	25.88 (stable)	76.29	-0.149	1 h
HV-3	Non-allergen	Antigenic	449	47309.38	34505	33.92 (stable)	73.56	-0.367	1 h

performed by the HawkDock server, HV-1 also generated the lowest scores while binding with DRB3*0202 (-5678.68), DRB5*0101 (-5599.06), DRB1*0101 (-5413.34), DRB1*0401 (-6330.38) and DRB1*0301 (-5640.72). Furthermore, HV-1 showed best performances by the PatchDock server (later refined by FireDock server) while docking with DRB3*0202 (-24.99), DRB1*0401 (-17.25) and TLR-3 (-3.45) and the ClusPro 2.0 server also declared HV-1 as the best vaccine construct, where it was found to generate best scores with all the receptors except the DRB5*0101. Like the DV-1 vaccine, HV-1 gave far better performances than the other two HPV-16 vaccines in the docking analysis. Hence, HV-1 was considered as the best vaccine construct for further analysis. The results of the docking study for all the DENV-1 and HPV-16 vaccine constructs are listed in Table 13. The interactions of DV-

1 with TLR-8 and HV-1 with TLR-3 are illustrated with Fig. 11.

3.13. Molecular dynamics simulation study

Fig. 12 and Fig. 13 illustrate the molecular dynamics simulation and normal mode analysis (NMA) of DV-1-TLR-8 docked complex and HV-1-TLR-3 docked complex. The deformability graphs of the two complexes illustrate the peaks in the graphs which correspond to the regions of the proteins with deformability (Figs. 12b and 13b). The B-factor graphs of the complexes provide an easy understanding and visualization of the comparison between the NMA and the PDB field of the docked complexes (Figs. 12c and 13c). The eigenvalues of the complexes are illustrated in Figs. 12d and 13d. DV-1 and TLR8 docked complex generated

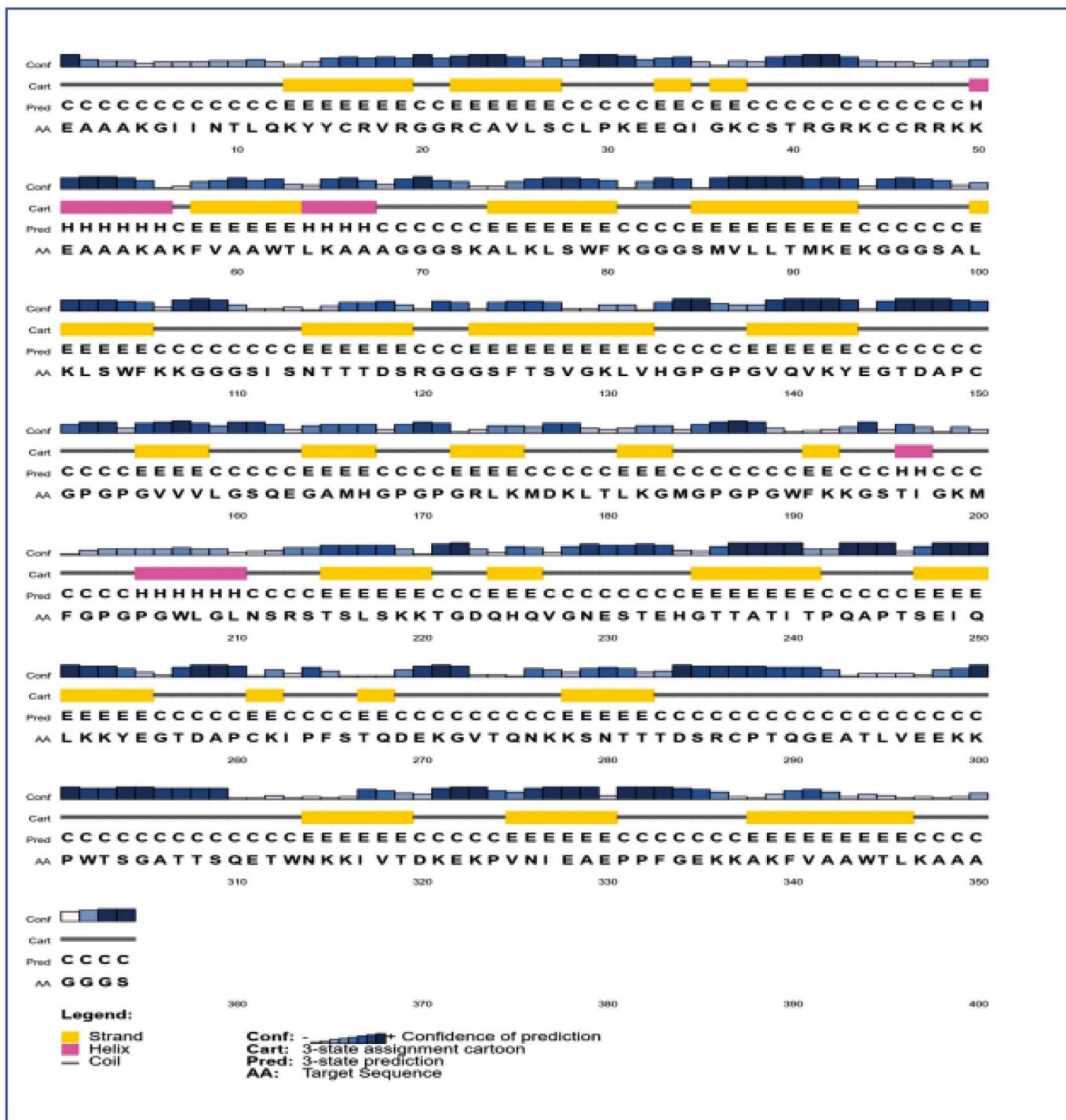


Fig. 5a. The results of the secondary structure prediction of the constructed DV-1 vaccine.

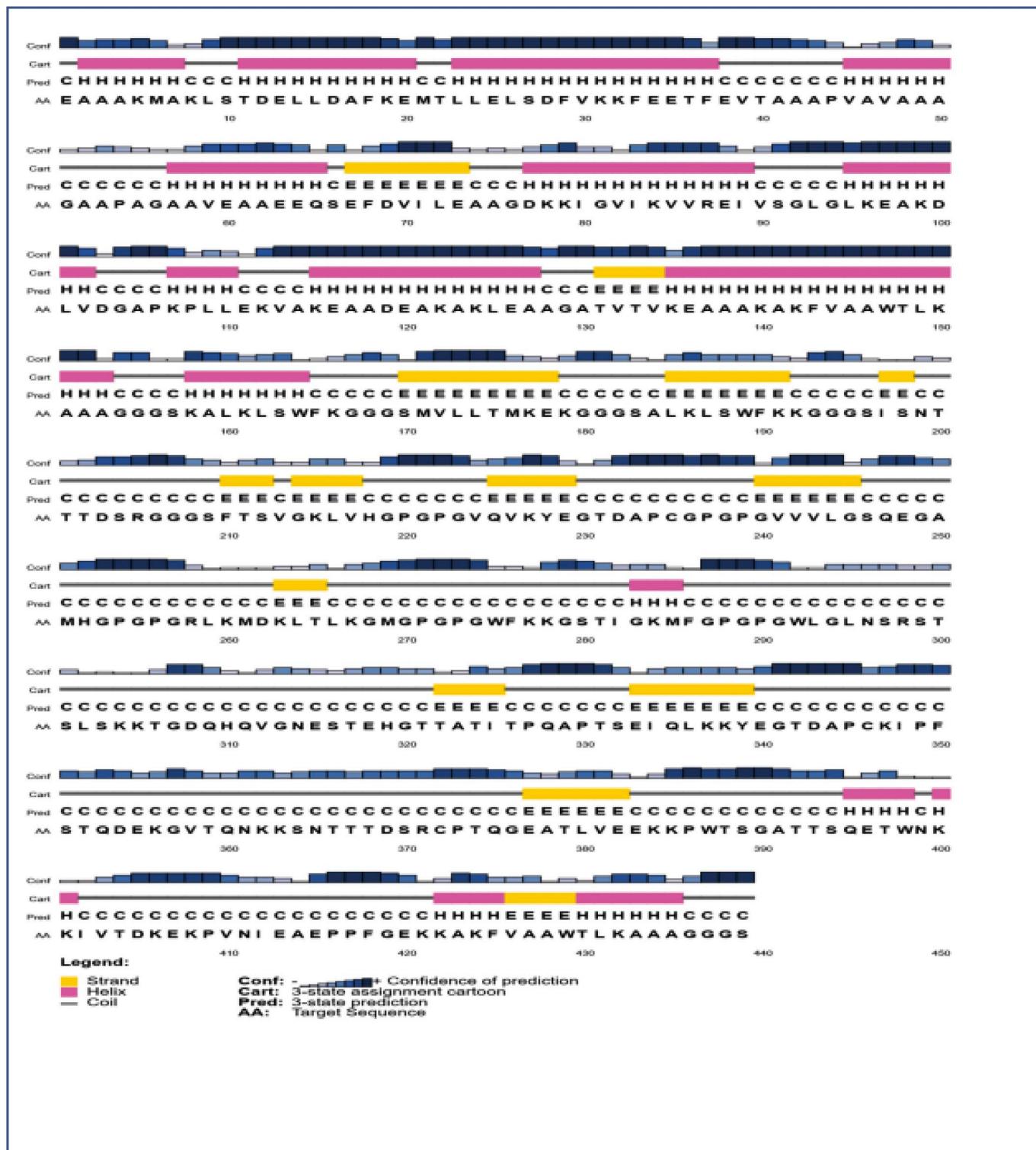


Fig. 5b. The results of the secondary structure prediction of the constructed dengue vaccine- DV-2.

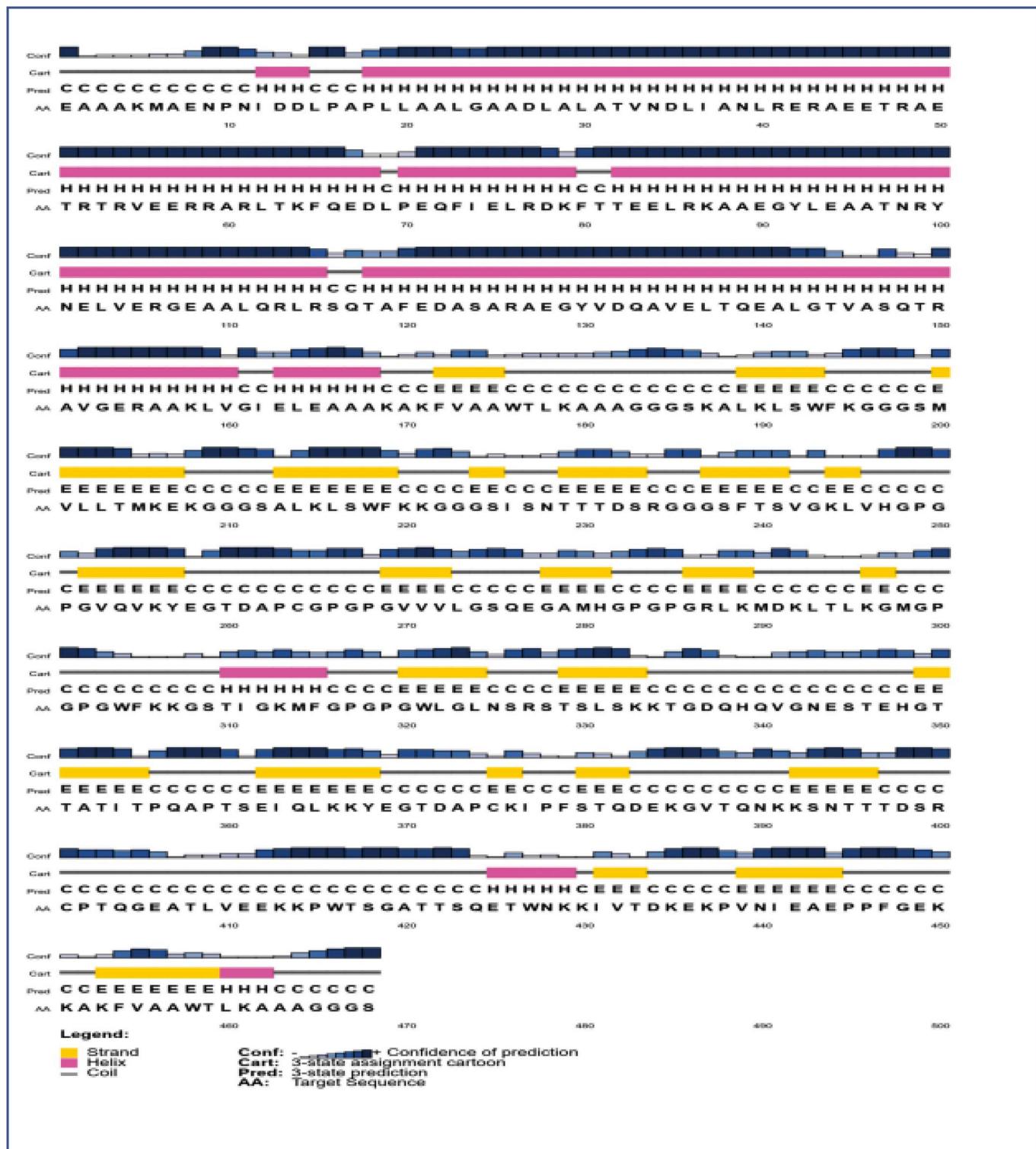
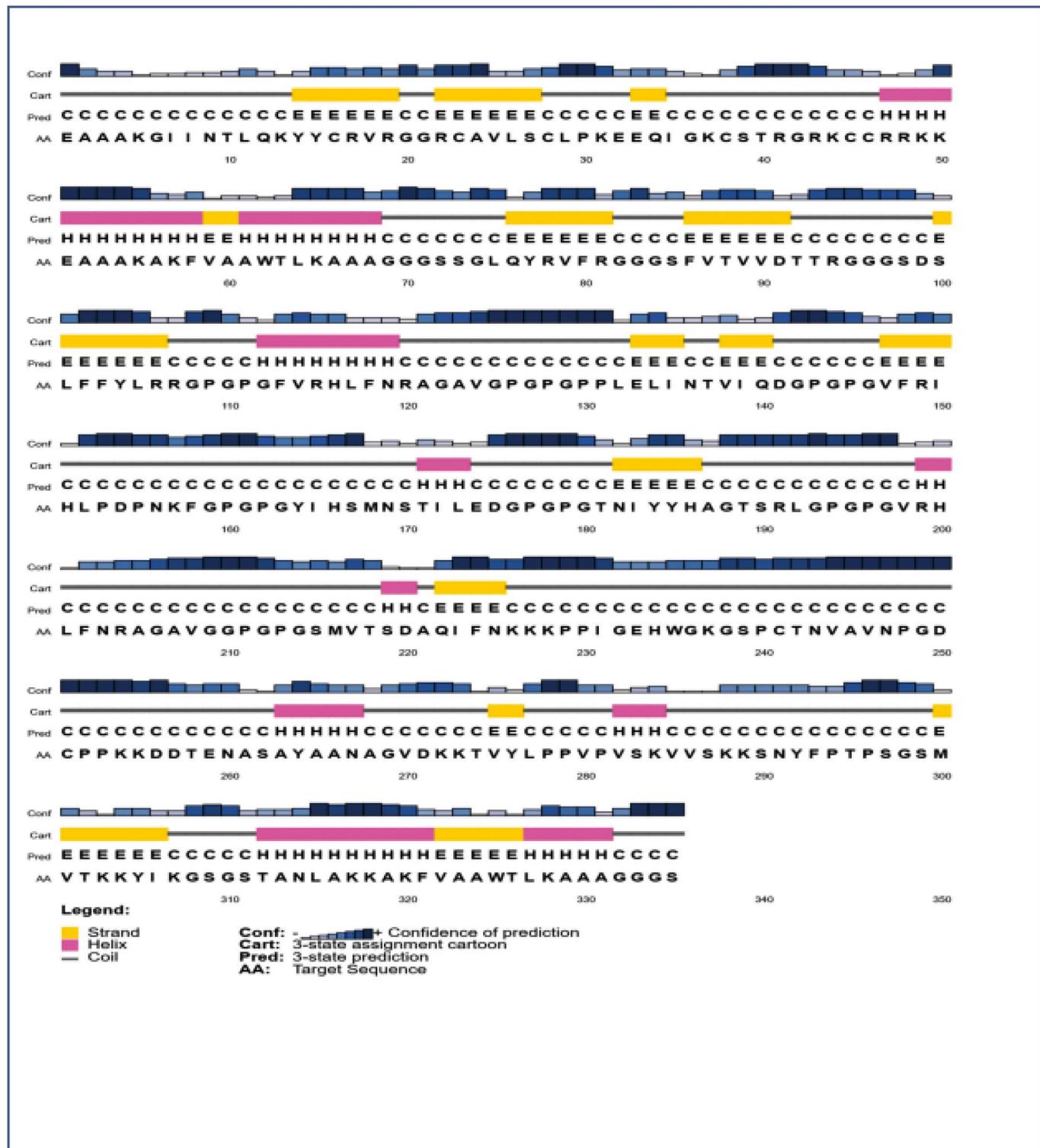


Fig. 5c. The results of the secondary structure prediction of the constructed dengue vaccine- DV-3.

Table 11

Results of the secondary structure analysis of the vaccine constructs.

Name of the vaccine	Alpha helix (percentage of amino acids)	Beta sheet (percentage of amino acids)	Coil structure (percentage of amino acids)
DV-1	5.3%	42.6%	51.9%
DV-2	29.1%	16.2%	54.6%
DV-3	42.1%	23.5%	42.1%
HV-1	17.3%	20.2%	62.3%
HV-2	34.5%	12.1%	53.3%
HV-3	42.7%	9.5%	47.6%

**Fig. 6a.** The results of the secondary structure prediction of the constructed HPV-16 vaccine-HV-1.

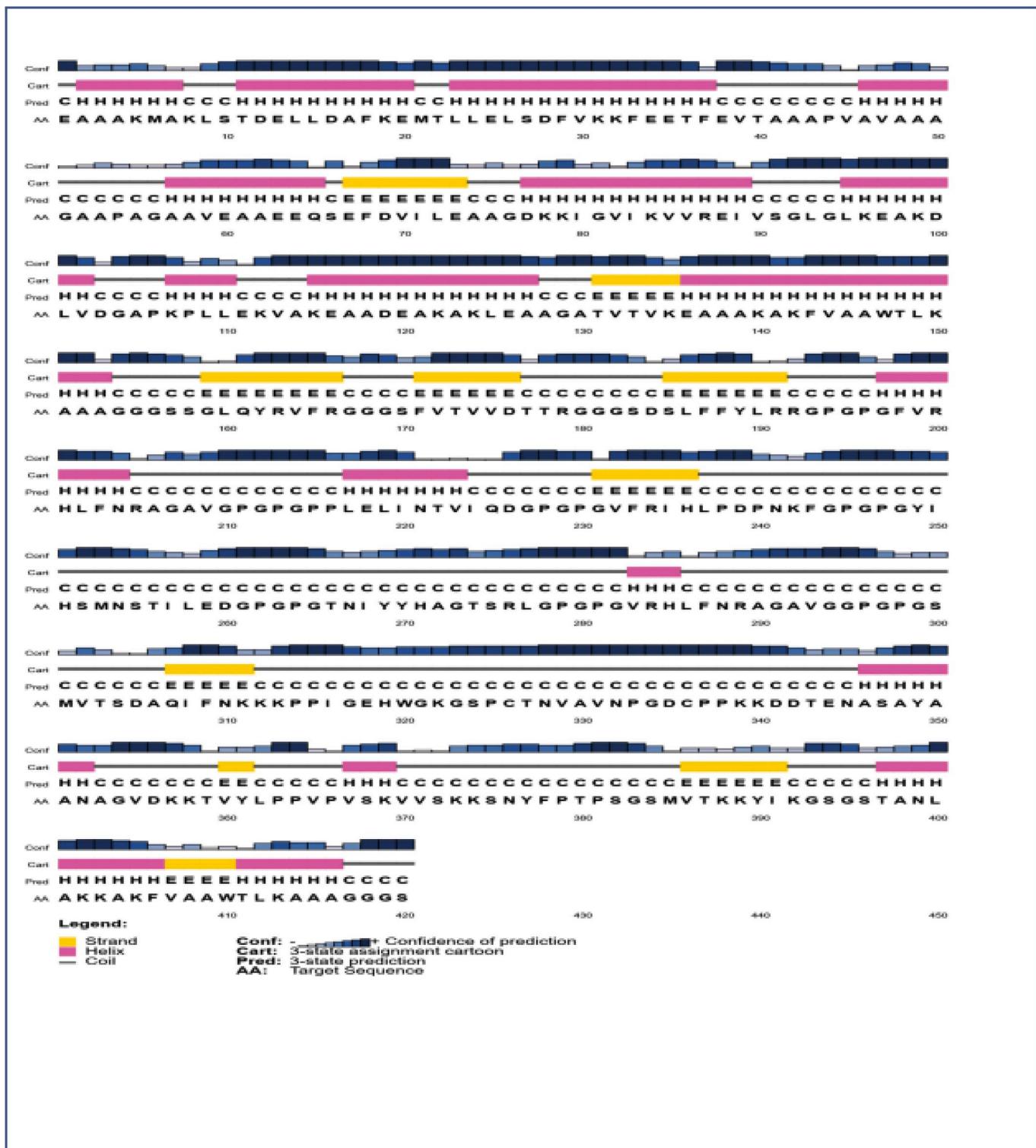


Fig. 6b. The results of the secondary structure prediction of the constructed HPV-16 vaccine-HV-2.

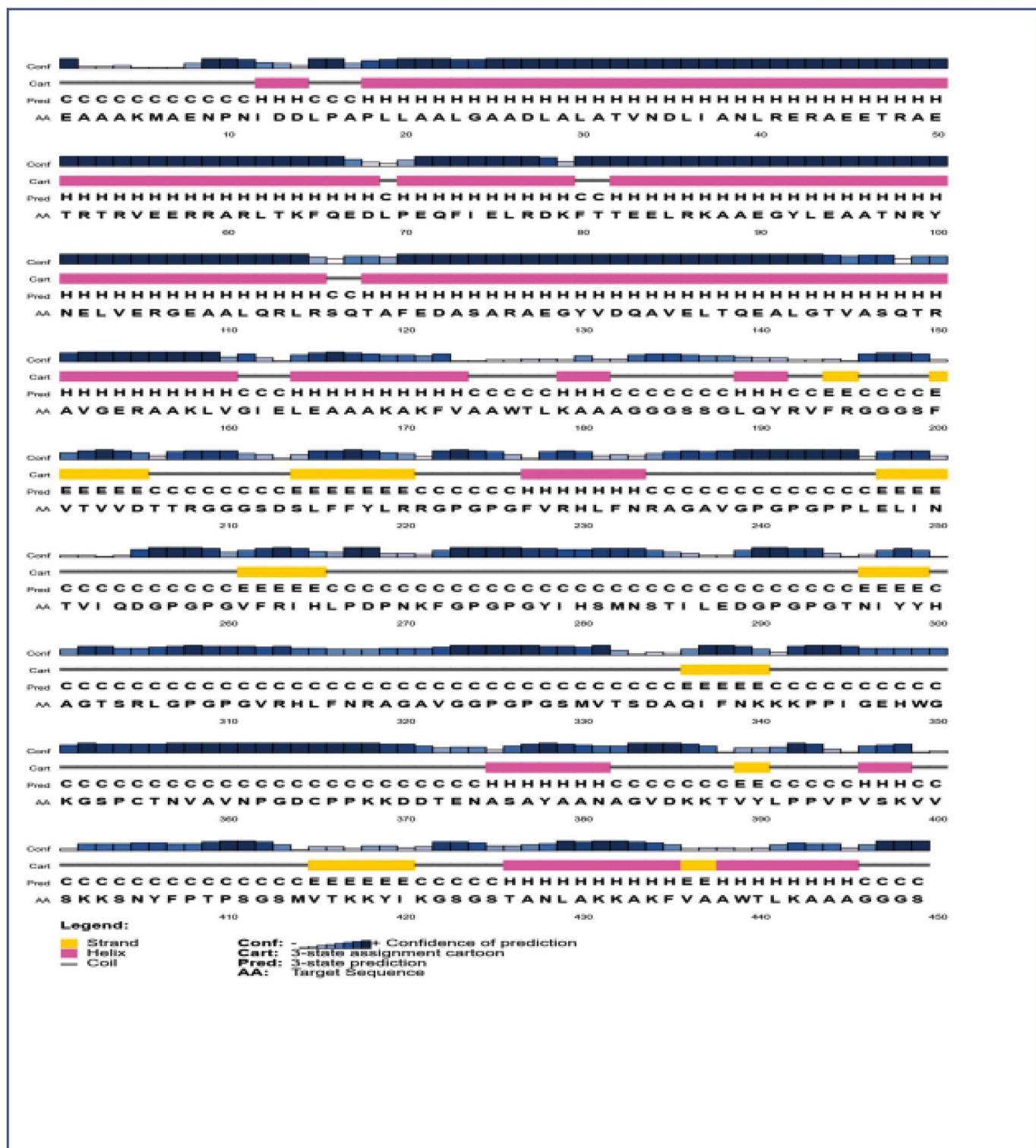


Fig. 6c. The results of the secondary structure prediction of the constructed HPV-16 vaccine HV-3.

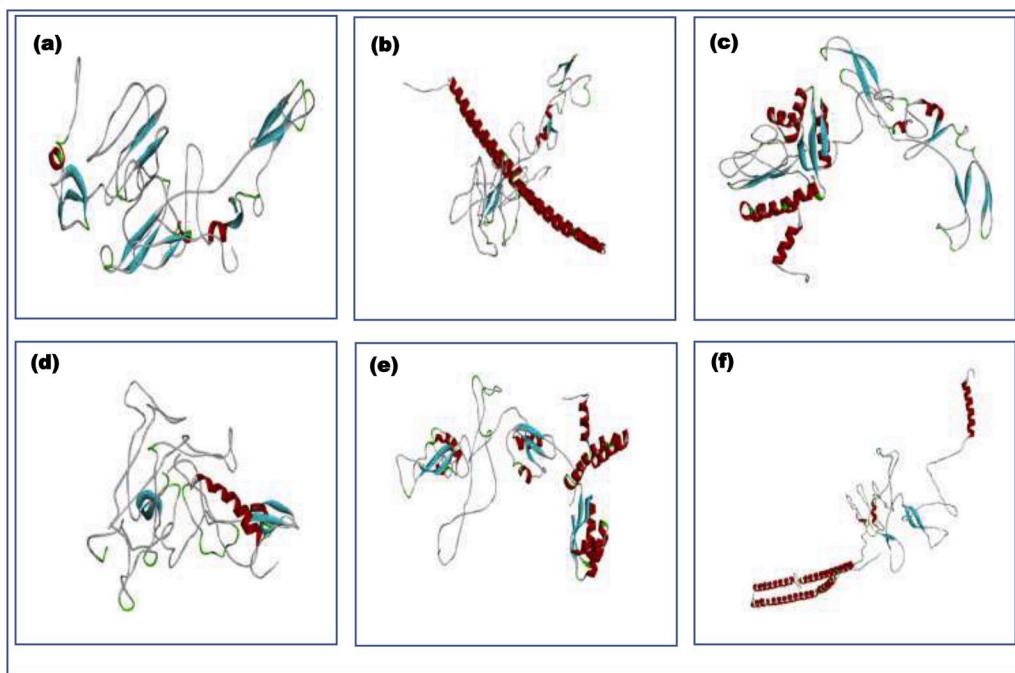


Fig. 7. The tertiary structures of the constructed vaccines. Here, (a) is DV-1, (b) is DV-2, (c) is DV-3, (d) is HV-1, (e) is HV-2, (f) is HV-3. The tertiary structures were visualized by Discovery Studio Visualizer.

eigenvalue of 3.949835e-05 and HV-1 and TLR3 complex generated eigenvalue of 4.474326e-05. The variance graphs indicate the individual variance by red colored bars and cumulative variance by green colored bars (Figs. 12e and 13e). Figs. 12f and 13f illustrate the covariance map of the complexes, where the correlated motion between a pair of residues is indicated by red colour, uncorrelated motion is indicated by white colour, and anti-correlated motion is indicated by blue colour. The elastic maps of the complexes show the connection between the atoms and darker gray regions indicate stiffer regions (Figs. 12g and 13g) [84–86].

3.14. Codon adaptation and in silico cloning

For *in silico* cloning and plasmid construction, the protein sequences of the best selected vaccines were adapted by the JCcat server.

Since the DV-1 protein had 354 amino acids and HV-1 had 335 amino acids, therefore, after reverse translation, the number nucleotides of the probable DNA sequence of DV-1 would be 1062 nucleotides long and HV-1 would be 1005 nucleotides long. The codon adaptation index (CAI) value of 0.994 of DV-1 and 0.953 of HV-1 indicated that the DNA sequences had a higher proportion of the codons that were most likely to be present and used in the cellular machinery of the target organism *E. coli* strain K12 (codon bias), so that the production of the DV-1 and HV-1 vaccines would be carried out efficiently [90,91]. The GC content of the improved sequences were 51.32% (DV-1) and 53.73% (HV-1), which reflected the good percentages of the GC content of the sequences. The predicted DNA sequences of DV-1 and HV-1 were inserted into two separate pET-19b vector plasmids between the SgrAI and SphI restriction sites, separately. Since the DNA sequences did not have restriction sites for SgrAI and SphI restriction enzymes, SgrAI and SphI restriction sites were conjugated at the N-terminal and C-terminal sites, respectively, before inserting the sequences into the plasmid pET-19b vector. The newly constructed cloned plasmids were found to be 6634 (DV-1)

and 6577 (HV-1) base pairs long, including the constructed DNA sequence of the DV-1 and HV-1 vaccines (Fig. 14 and Fig. 15).

4. Discussions

Vaccines are one of the most important pharmaceutical products, but their development and production processes are costly and sometimes it takes many years to achieve a proper vaccine candidate against a particular pathogen. In modern times, different methods and tools of bioinformatics, immunoinformatics, and vaccinomics are employed for vaccine development, which save both time and cost of the vaccine development process [92]. Conventional vaccines are developed based on inactivated or killed antigens or pathogens. Such processes may take up to 15 years or more to develop a successful vaccine [28]. Moreover, difficulties with the proper cultivation of the microbes and adverse immune responses resulting from the improper attenuation are also potential drawbacks of conventional vaccine development processes. Such complications in vaccine development have led the way to develop many computer software and pre-screening programs in recent years, which can be effectively utilized in designing and developing potential vaccines rapidly. These software and database based methods have acquired great acceptance among the scientific community [27,93,94]. For this reason, the rapidly accepted *in silico* based methods were exploited in this study to design potential vaccines against DENV-1 and HPV-16 to combat dengue fever and cervical cancer.

At first, the viral strains against which the vaccines would be designed were identified by reviewing the NCBI database. DENV-1 envelope protein E and HPV-16 major capsid protein L1 were selected as the potential targets for the vaccines. Next, the sequences were retrieved from the UniProt database. The accession numbers of the DENV-1 envelope protein E and HPV-16 major capsid protein L1 were Q8BE390 and Q9WLQ6, respectively.

The antigenicity analysis of the two proteins had predicted both of

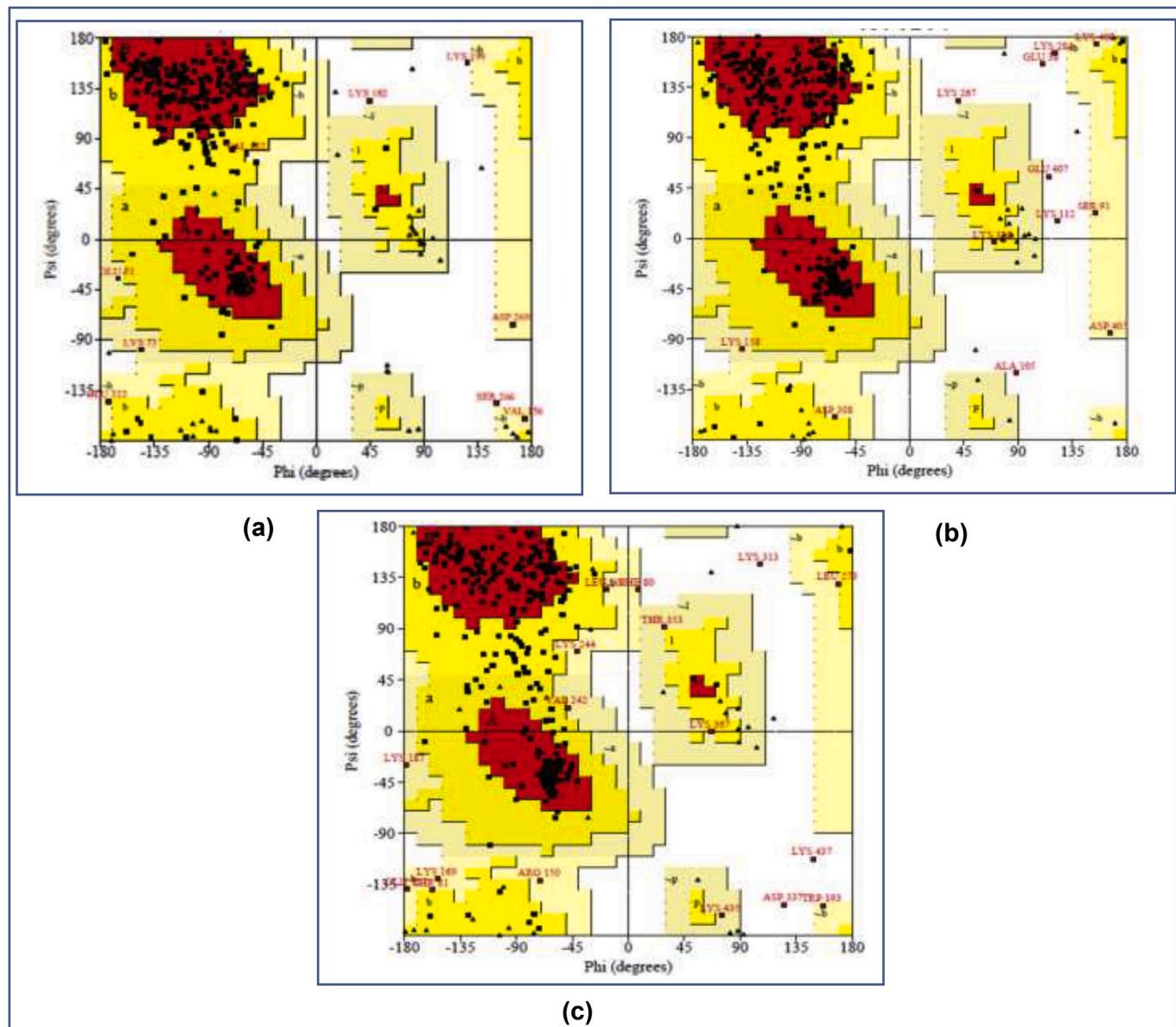


Fig. 8a. The Ramachandran plot analysis of the three dengue vaccine constructs. Here, (a) DV-1, (b) DV-2, (c) DV-3.

Table 12
Results of the tertiary structure analysis of the vaccine constructs.

Name of the vaccine	Number of the domains	p-value
DV-1	3	1.35e-09
DV-2	3	1.78e-09
DV-3	3	1.79e-09
HV-1	3	5.62e-09
HV-2	4	9.06e-12
HV-3	4	4.62e-09

them to be potentially antigenic. The physicochemical properties of the selected proteins like the number of amino acids, molecular weight, theoretical pI, extinction co-efficient, instability index, aliphatic index, GRAVY were determined. The instability index of a compound

corresponds to the probability of the compound to be stable. If a compound has a predicted instability index over 40, then that compound is considered to be unstable [95]. With an instability index of less than 40, both the proteins were predicted to be stable. The extinction coefficient can be defined as the amount of light that is absorbed by a compound at a certain wavelength [96,97]. HPV-16 major capsid protein L1 had the highest extinction coefficient and so it might absorb a higher amount of light than the selected DENV-1 protein at a certain wavelength. The aliphatic index of a protein refers to the relative volume occupied by the aliphatic amino acids in the side chains, for example: alanine, valine etc. [98]. Both the proteins had a high aliphatic index. Moreover, the negative GRAVY values of both proteins reflect that both of them might be hydrophilic in nature [99]. The T lymphocytic cell and B lymphocytic cell are the main cells that function in the immune response process of an individual. Once an antigen is recognized by an antigen presenting cell

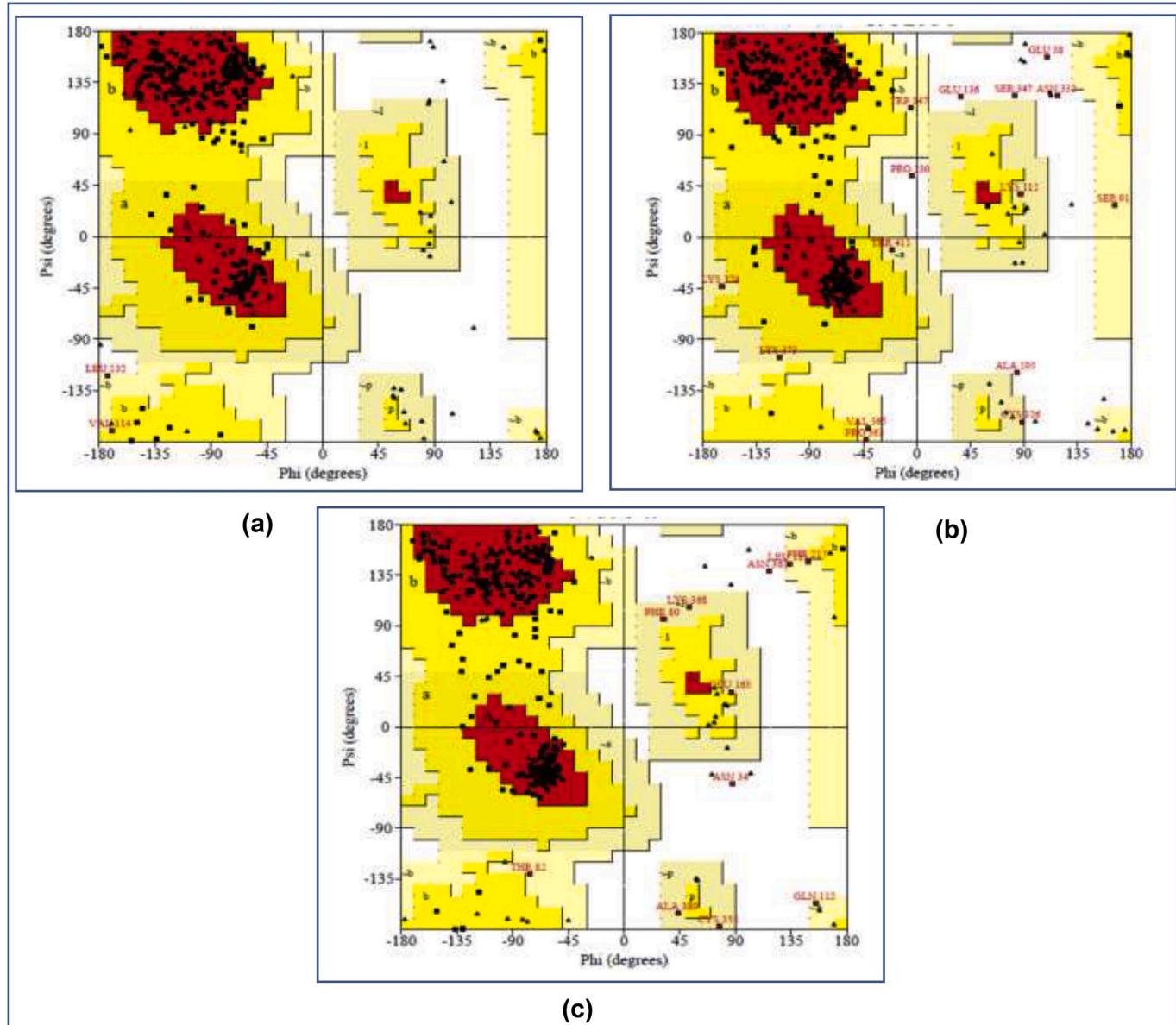


Fig. 8b. The Ramachandran plot analysis of the three HPV-16 vaccine constructs. Here, (a) HV-1, (b) HV-2, (c) HV-3.

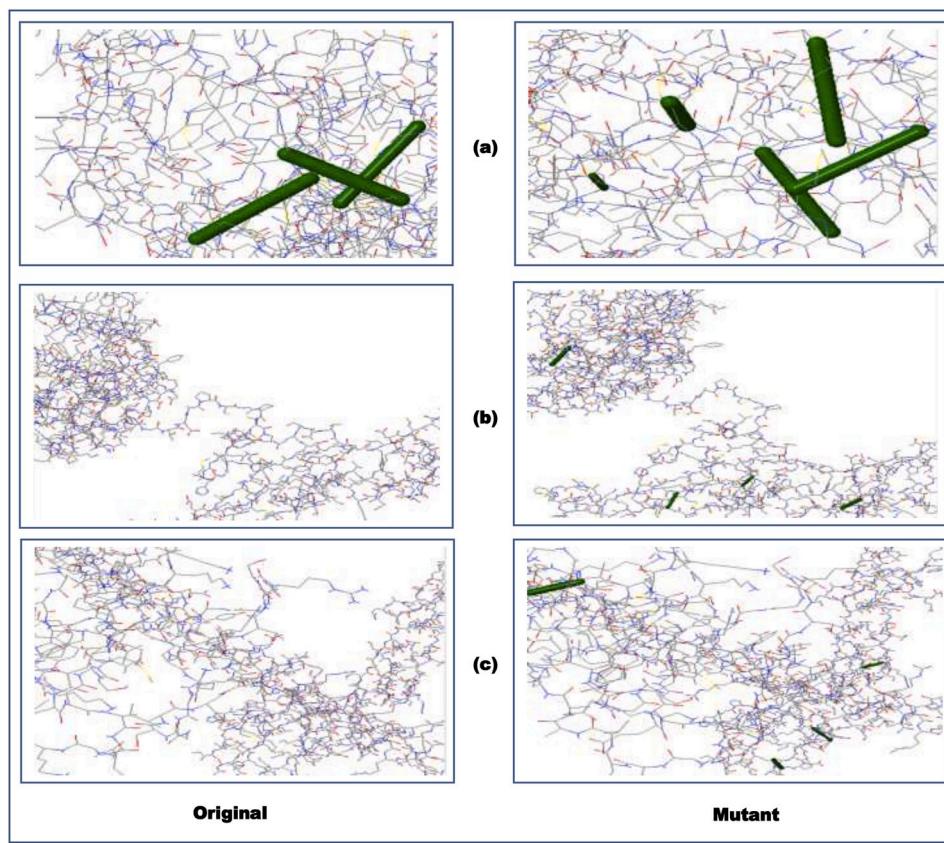


Fig. 9. The disulfide engineering of the three DENV-1 vaccine constructs; both the original (left) and mutant (right) forms are shown. Here, (a) DV-1, (b) DV-2, (c) DV-3. The disulfide bonds are represented by green colored tube or rod shaped structures.

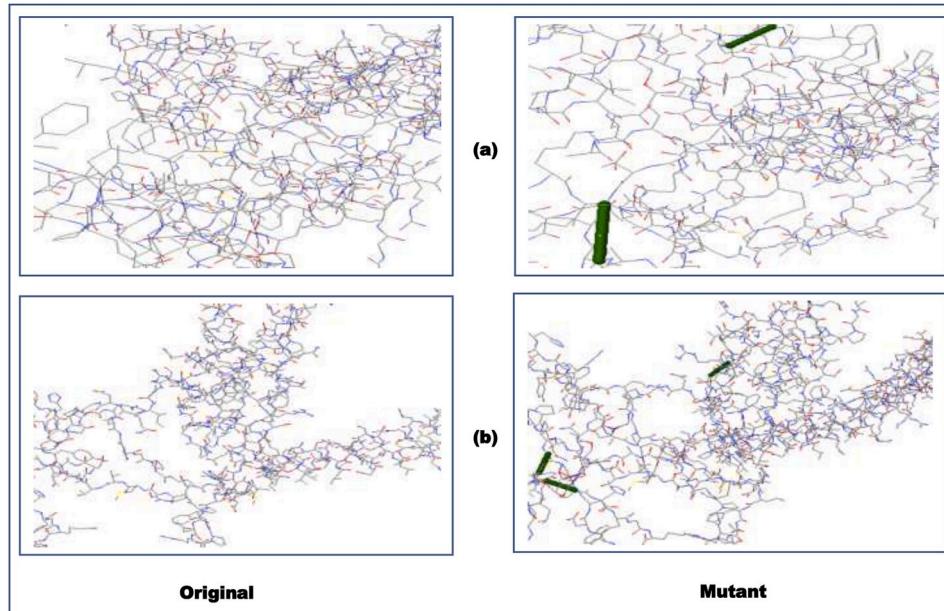


Fig. 10. The disulfide engineering of the two HPV-16 vaccine constructs, both the original (left) and mutant (right) forms are shown. Here, (a) HV-2 and (b) HV-3. The HV-1 didn't meet the criteria and therefore, is not shown here. The disulfide bonds are represented by green colored tube or rod shaped structures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 13
Results of the docking study of all the vaccine constructs.

Name of the vaccines	Name of the Targets	PDB IDs of the targets	Energy score (ClusPro 2.0 server)	Global energy	HawkDock score (the lowest score)	MM-GBSA (binding free energy, in kcal mol ⁻¹)
DV-1	DRB3*0202	1A6A	-1356.45	3.42	-5442.91	-41.16
	DRB5*0101	1H15	-1098.51	-6.33	-6920.47	-130.75
	DRB1*0101	2FSE	-1134.25	-29.13	-7739.52	-129.02
	DRB3*0101	2Q6W	-1260.90	4.36	-6455.32	-57.08
	DRB1*0401	2SEB	-922.47	9.29	-5421.15	-66.48
	DRB1*0301	3C5J	-1109.58	1.49	-6630.52	-62.16
	TLR8	3W3M	-1013.47	0.26	-5267.87	-31.56
	DRB3*0202	1A6A	-1167.40	-19.68	-5410.31	-32.89
DV-2	DRB5*0101	1H15	-923.41	2.64	-5396.50	-46.08
	DRB1*0101	2FSE	-957.52	4.53	-6043.14	-17.95
	DRB3*0101	2Q6W	-1008.95	3.43	-5506.47	-40.73
	DRB1*0401	2SEB	-981.35	-10.53	-6104.58	-24.01
	DRB1*0301	3C5J	-1092.32	5.69	-5548.24	-4.54
	TLR8	3W3M	-980.42	-9.81	-5188.25	11.9
	DRB3*0202	1A6A	-945.34	-16.37	-3944.00	-16.39
	DRB5*0101	1H15	-874.17	5.20	-5175.63	-37.69
DV-3	DRB1*0101	2FSE	-1002.49	1.03	-5768.78	-56.55
	DRB3*0101	2Q6W	-996.81	-27.25	-4272.11	-28.60
	DRB1*0401	2SEB	-872.95	2.19	-4504.73	-21.40
	DRB1*0301	3C5J	-986.12	-10.40	-4437.37	0.48
	TLR8	3W3M	-822.10	7.25	-5341.80	-26.22
	DRB3*0202	1A6A	-1204.50	-24.99	-5678.68	-65.25
	DRB5*0101	1H15	-977.32	-4.67	-5599.06	-120.88
	DRB1*0101	2FSE	-1057.65	-3.16	-5413.34	-131.41
HV-1	DRB3*0101	2Q6W	-1224.09	7.93	-3603.78	-87.29
	DRB1*0401	2SEB	-1033.80	-17.25	-6330.38	-93.27
	DRB1*0301	3C5J	-1017.94	3.57	-5640.72	-58.18
	TLR3	2A0Z	-1155.62	-3.45	-3509.95	-51.26
	DRB3*0202	1A6A	-1119.48	-17.14	-4337.25	-29.91
	DRB5*0101	1H15	-1089.42	-20.16	-5061.65	-46.66
	DRB1*0101	2FSE	-881.97	-7.10	-5069.31	-60.28
	DRB3*0101	2Q6W	-944.78	-0.96	-5017.47	-12.72
HV-2	DRB1*0401	2SEB	-1017.42	-11.27	-4813.92	-30.23
	DRB1*0301	3C5J	-922.95	-7.92	-4213.13	-28.58
	TLR3	2A0Z	-1089.69	-1.75	-4083.48	-20.80
	DRB3*0202	1A6A	-856.98	-21.16	-3396.57	-31.35
	DRB5*0101	1H15	-942.23	-19.11	-3733.68	-74.73
	DRB1*0101	2FSE	-956.20	-10.55	-3415.00	-64.19
	DRB3*0101	2Q6W	-913.44	6.52	-3101.06	-35.74
	DRB1*0401	2SEB	-990.40	3.12	-3380.51	-40.65
HV-3	DRB1*0301	3C5J	-945.23	1.10	-3285.64	-45.86
	TLR3	2A0Z	-1067.86	-2.56	-3292.74	-10.63

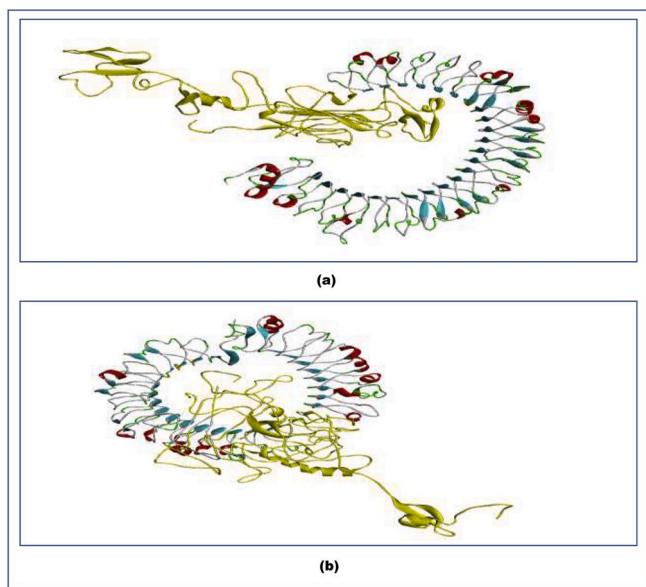


Fig. 11. The interactions between the ligand proteins (vaccine) and receptor proteins. The ligand proteins are indicated by yellow color. Here, (a) is the interaction between the TLR-8 and DV-1 and (b) is the interaction between the TLR-3 and HV-1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

or APC (such as macrophage, dendritic cell etc.), the APC presents the antigen through its MHC class-II molecule, present on its surface, to the helper T cell. The helper T cell contains a CD4⁺ molecule on its surface; therefore, it is also known as a CD4⁺ T cell. The T-helper cell then activates the B cell, causing the production of memory B cell and antibody producing plasma B cell which produces a large number of antibodies. However, the helper T-cell also activates macrophage and CD8⁺ cytotoxic T cell which destroy the target antigen [100–104]. The T cell and B cell epitopes of the selected viral proteins were determined by the IEDB server. These T cell and B cell epitopes would be able to induce the immune responses against the vaccines. Twenty MHC class-I epitopes and twenty MHC class-II epitopes were selected for further analysis. Moreover, the transmembrane topology of the epitopes were determined to identify whether the epitopes would be present inside or outside of the cell membrane.

For selecting the final T cell epitopes for vaccine construction, some special criteria i.e., high antigenicity, non-allergenicity, non-toxicity and high conservancy were followed. The T cell epitopes following these criteria were selected for vaccine construction. However, for selecting the final B cell epitopes, only the non-allergenicity of the epitopes were considered. Antigenicity refers to the ability of a foreign material to act as antigen and induce the T cell and B cell responses, through their antigenic determinant portions or epitopes [105]. For this reason, the epitopes with high antigenicity were selected for vaccine construction because they would be able to induce high immunogenic responses. The allergenicity of a substance can be defined as the ability of that substance to act as allergen and induce allergic reactions within the body [106]. As a result, the non-allergenic epitopes were selected for vaccine construction so that they would not be able to cause any unwanted allergic reaction within the body. The conserved epitopes of a protein or foreign substance were given much emphasis during vaccine development rather than the highly variable genomic regions because the conserved epitopes provide broader protection across various strains and sometimes, across species [107]. The epitopes with 100% conservancy and over 50% minimum identity were selected for vaccine construction. The vaccines with these conserved epitopes might also provide immunity towards other strains of DENV and HPV along with the DENV-1 and HPV-16. Furthermore, the epitopes that were found to

be non-toxic, were used to design the vaccines. From the DENV-1 envelope protein E, five MHC class-I epitopes (KALKLSWFK, MVLLTMKEK, ALKLSWFKK, ISNTTTDSR and FTSVGKLVH) and five MHC class-II epitopes (VQVKYEGTDAPC, VVVLGSQEGAMH, RLKMDKLTLKGM, WFKKGSTIGKMF and WLGLNSRSTSLS) met the criteria and were selected for constructing the vaccines. On the other hand, from the HPV-16 major capsid protein L1, three MHC class-I epitopes (SGLQYRVFR, FTVVVDTTR and DSLFFYLRR) and seven MHC class-II epitopes (FVRHLFNRAGAV, PPYLEINTVIQD, VFRIHLPDPNKF, YIHSMNSTILED, TNYYHAGTSRL, VRHLFNRAGAVG and SMVTSDA-QIFNK) were found to be within the selection criteria. Again, all the five selected B cell epitopes of DENV-1 and five of the seven initially selected B cell epitopes of HPV-16 were predicted to be non-allergenic (within the selection criteria for B-cell epitopes). For this reason, these T cell and B cell epitopes (that followed the mentioned criteria) from both viruses were considered as the best selected epitopes (good fit) and used in the vaccine construction. The cluster analysis of the MHC class-I alleles and MHC class-II alleles were also conducted to determine their relationship and cluster them functionally based on their predicted binding specificity [38].

In the next step, the peptide-protein docking was carried out between the selected T cell epitopes and their respective targets, HLA-A*11-01 allele (PDB ID: 5WJL) and HLA DRB1*04-01 (PDB ID: 5JLZ), to determine their capability to bind with their respective targets. Therefore, the 3D structures of the selected epitopes were generated by the PEP-FOLD3 server. Then the generated 3D structures were docked with HLA-A*11-01 allele (PDB ID: 5WJL) and HLA DRB1*04-01 (PDB ID: 5JLZ). By analyzing the docking results, it could be concluded that all the T cell epitopes had the ability to bind with their targets. FTSVGKLVH, VQVKYEGTDAPC, SGLQYRVFR and YIHSMNSTILED generated the best global scores among the best selected epitopes.

The best selected T cell and B cell epitopes were used for the vaccine construction. Three different adjuvants i.e., beta defensin, L7/L12 ribosomal protein and HABA protein, were used to construct three different vaccines for the two viruses. The vaccines were constructed maintaining the sequence: adjuvant, PADRE sequence, MHC class-I epitopes, MHC class-II epitopes and B cell epitopes, using appropriate linkers (EAAAK, GGGG, GPGPG, KK) at the appropriate positions. The DENV vaccines were designated as 'DV' and the HPV-16 vaccines were designated as 'HV' vaccines.

After the vaccine construction was done, the antigenicity and allergenicity of the constructed vaccines were determined. All the vaccines were shown to be safe with no possible allergenicity, and had the capability to induce immunity with high antigenicity. The physico-chemical properties of the vaccines were also determined, where the number of amino acids, molecular weight, extinction co-efficient, instability index, aliphatic index, GRAVY value and half-life of the vaccines were predicted. The aliphatic index of a protein corresponds to the protein's thermal stability, and a higher the aliphatic index represents greater thermostability of the protein [108]. Since all the vaccine constructs were predicted to have quite high aliphatic indexes, all of them were considered to be quite thermostable. Furthermore, all the vaccine constructs were found to be stable *in vivo* and had a half-life in the mammals of 1 h. Again, the negative GRAVY value of the vaccine constructs revealed that all of them might be hydrophilic in nature [99]. All of the vaccines exhibited sound results in the physicochemical property analysis.

In the secondary structure prediction, among the dengue vaccines, DV-3 had the highest percentage of the amino acids in the alpha-helix structure (42.1%) and hence the lowest percentage of amino acids in the coil structure (42.1%). DV-1 had the highest percentage of amino acids in the beta-sheet structure of 42.6%. On the other hand, among the HPV-16 vaccines, HV-3 had the highest percentage of amino acids in the alpha-helix structure (42.7%) as well as the highest percentage of amino acids (47.6%) in the coil structure. HV-1 had the highest percentage of amino acids in the beta-sheet structure of 20.2%. In the 3D structure

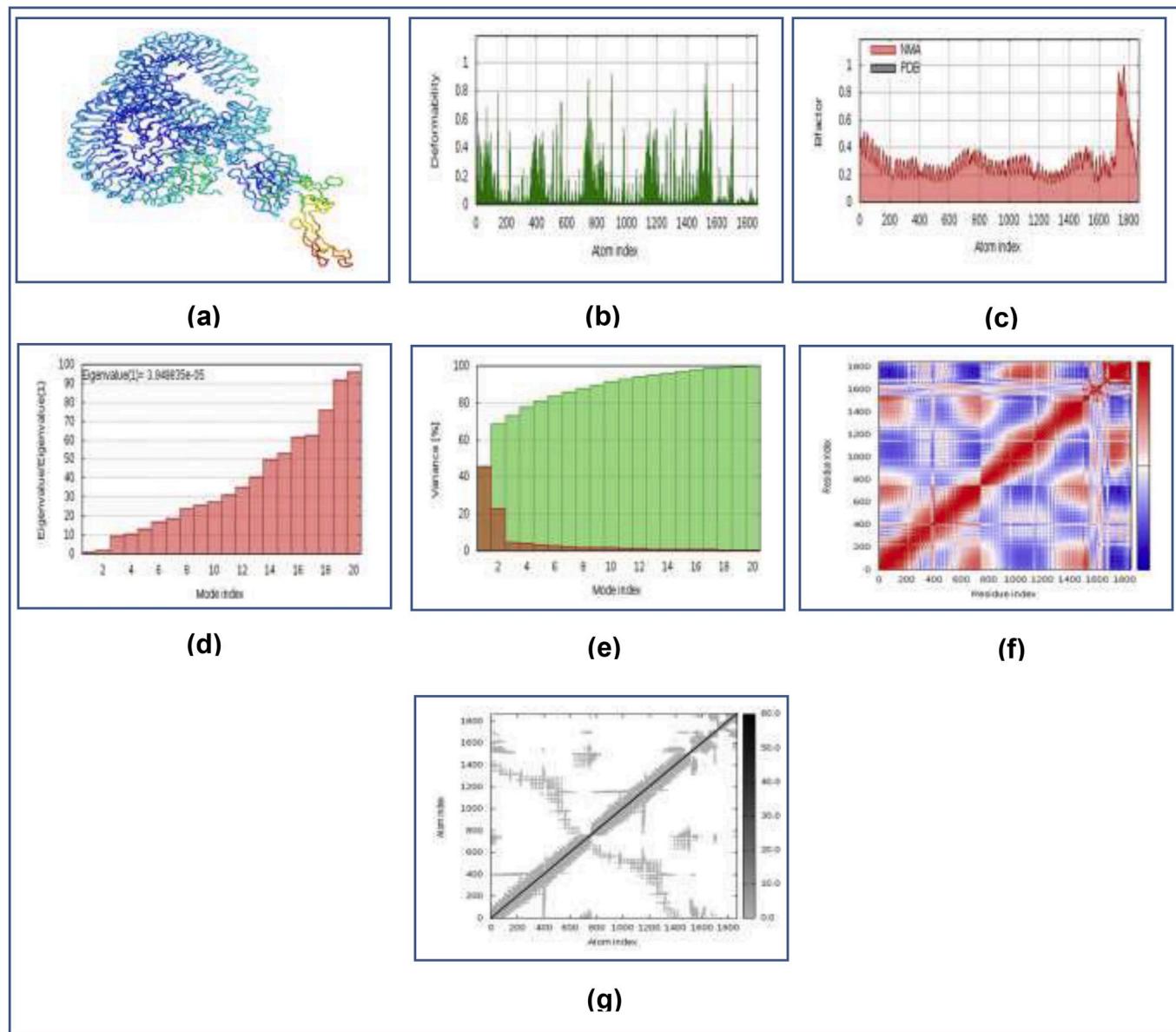


Fig. 12. Figure displaying the results of molecular dynamics simulation study of DV-1 and TLR-8 docked complex. Here, (a) NMA mobility, (b) deformability, (c) B-factor, (d) eigenvalues, (e) variance (red color indicates individual variances and green color indicates cumulative variances), (f) co-variance map (correlated (red), uncorrelated (white) or anti-correlated (blue) motions) and (g) elastic network (darker gray regions indicate more stiffer regions). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

prediction, among the DENV vaccine constructs, DV-1 had the lowest p-value of 1.35e-09 (better quality) and among the HPV-16 vaccines, HV-2 had the lowest p-value of 9.06e-12, which indicated that HV-2 had better quality 3D structure than the other HPV-16 vaccines, because according to the RaptorX server, the lower p-value corresponds to better quality of the predicted 3D model and vice versa [61,109–111].

The 3D structures of the vaccine constructs were later refined and validated by the 3Drefine and PROCHECK servers, respectively. Among the DENV vaccines, DV-2 showed the best result in the validation study of 78.4% of the amino acids in the most favorable regions of the Ramachandran plot and among the HPV-16 vaccines, HV-3 showed the best result with 87.5% of the amino acids in the most favorable regions in the

Ramachandran plot. After validation, the disulfide engineering of the vaccine constructs were conducted and the amino acid pairs having bond energy value less than 2.2 kcal/mol were selected for disulfide bond formation.

For enhancing the accuracy of our prediction, three different online servers, ClusPro 2.0, PatchDock, FireDock, and HawkDock servers were used for the docking study. From the protein-protein docking, it was clear that DV-1 from DENV vaccines and HV-1 from HPV-16 vaccines showed the best results in the docking study with most of their receptors. All of the docking servers declared DV-1 and HV-1 vaccine constructs as the best vaccine constructs. For this reason, these two vaccine constructs were used for the molecular dynamics simulation study as well as codon

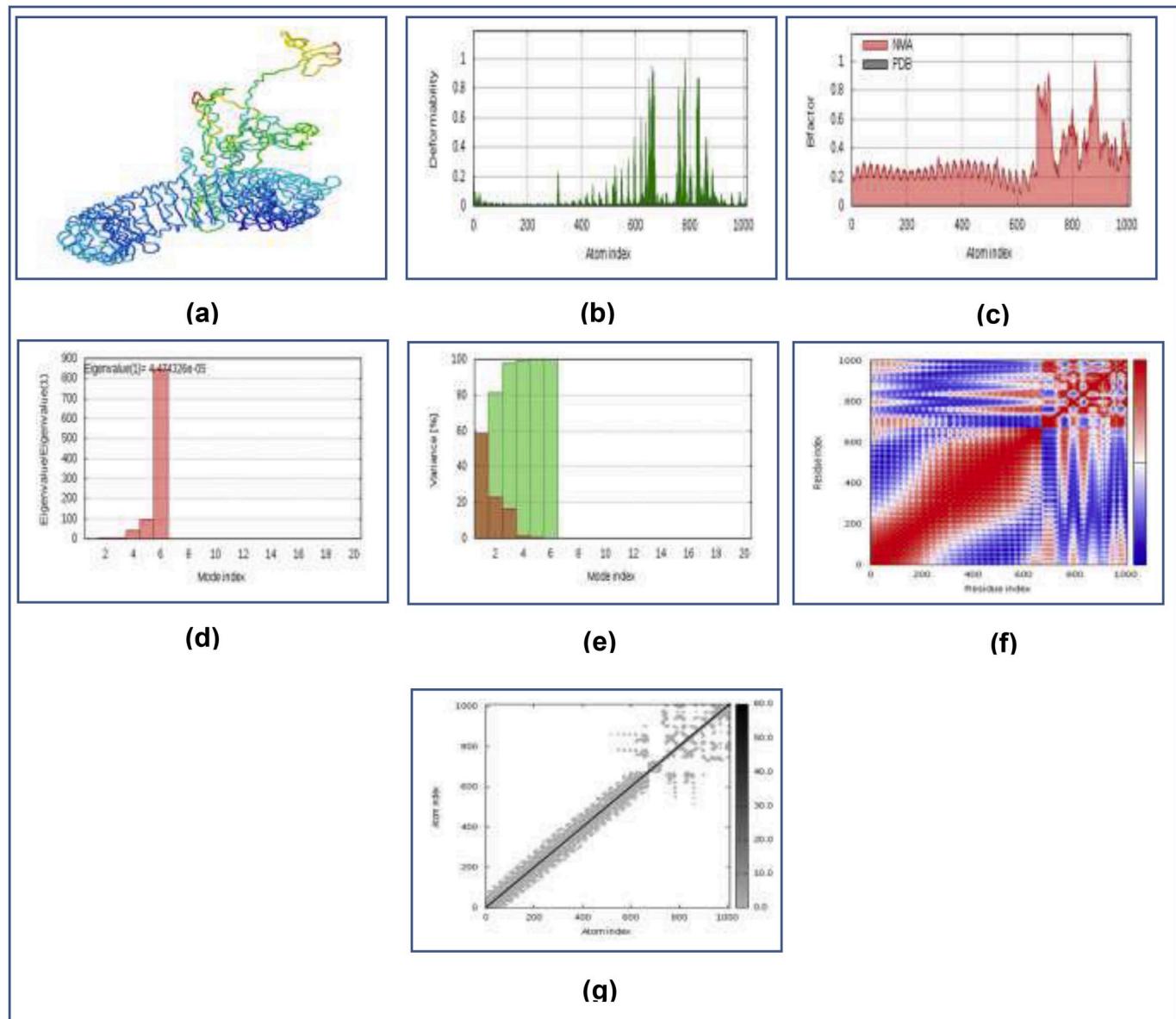


Fig. 13. Figure displaying the results of molecular dynamics simulation study of HV-1 and TLR-3 docked complex. Here, (a) NMA mobility, (b) deformability, (c) B-factor, (d) eigenvalues, (e) variance (red color indicates individual variances and green color indicates cumulative variances), (f) co-variance map (correlated (red), uncorrelated (white) or anti-correlated (blue) motions) and (g) elastic network (darker gray regions indicate more stiffer regions). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

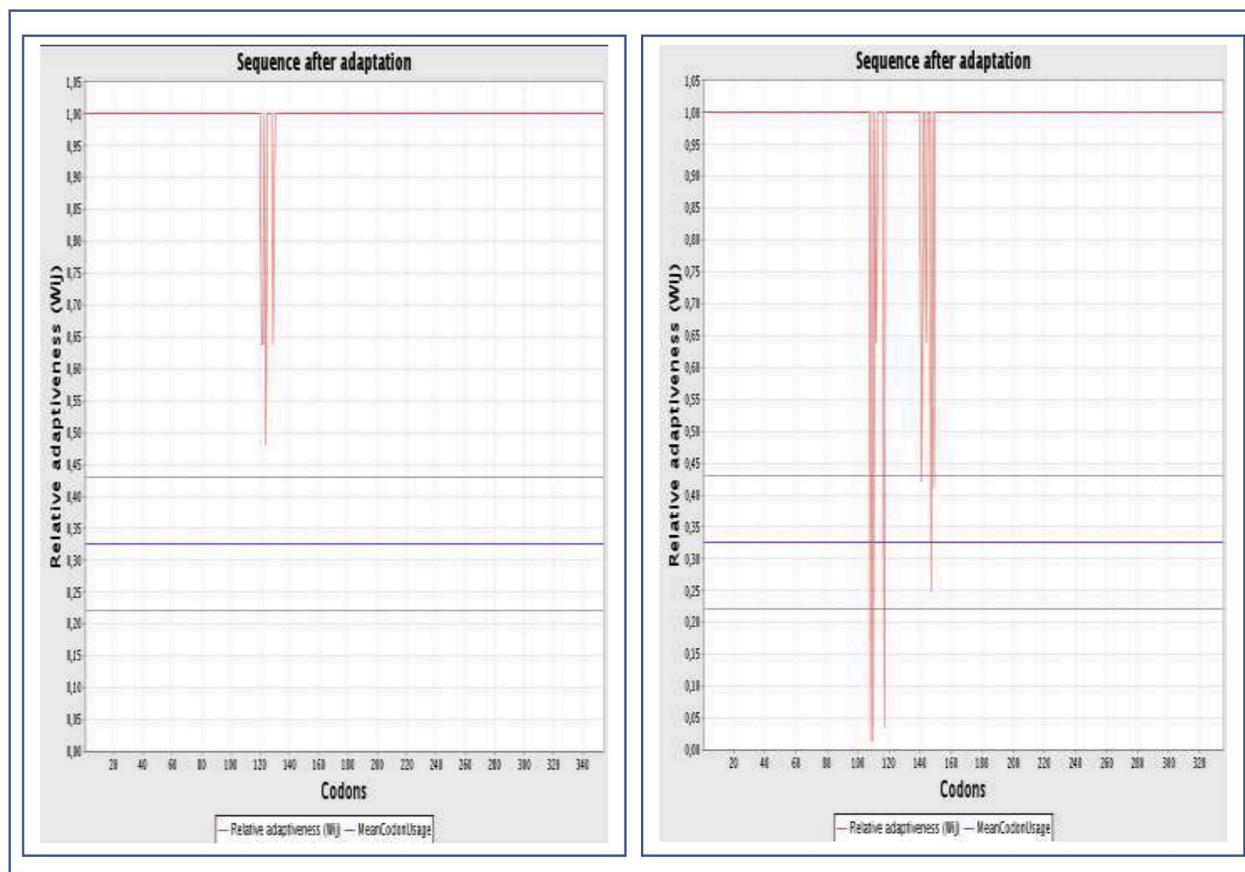


Fig. 14. The results of the codon adaptation graphs of the DV-1 vaccine (left) and HV-1 vaccine (right). The codon adaptation of the vaccine constructs were carried out using the server JCat (<http://www.jcat.de/>).

adaptation and *in silico* cloning.

The molecular dynamics simulation study was conducted for the docked TLR-8 and DV-1 complex and TLR-3 and HV-1 complex using the online tool server iMODS. Both the complexes showed quite similar and sound results in the molecular dynamics simulation study, reflecting good stability of the query complexes. The study showed that both of the complexes had a lesser chance of deformability, with quite high eigenvalues of $3.949835e-05$ and $4.474326e-05$, respectively. The deformability graphs (Figs. 12b and 13b) also predicted that the complexes were quite stable with a lower degree of deforming for each individual residue, since the location of the hinges in the structures were not quite significant. However, both DV-1 and HV-1 had a good amount of correlated amino acids (marked by red colour) as well as a high amount of stiffer regions (marked by darker gray colour) (Fig. 12f, g, Fig. 13f and g). Nevertheless, it can be concluded that both of the complexes showed good results in the molecular dynamics simulation study.

In the final stage, the designed DV-1 and HV-1 vaccines were adapted after reverse translation for cloning in to *E. coli* strain K12. The vaccine DNA sequences were inserted within the SgrAI and SphI restriction sites of two separate pET-19b plasmid vectors for expression and production of the desired vaccine in *E. coli* cells. These insertions produced 6634 (DV-1) and 6577 (HV-1) base pairs long plasmids. These plasmids can be inserted into the *E. coli* cells for efficient vaccine production.

Genome based technologies for vaccine development will continue

to dominate the field of vaccine development in the future because they provide investigators with a great opportunity to develop vaccines by facilitating the optimization of the target antigens. Conventional vaccines like the attenuated vaccines or the inactivated vaccines may fail to provide potential immunity towards a target antigen [112,113]. Moreover, according to some studies, the conventional approaches of vaccine development have raised significant safety concerns in many clinical trials. The epitope-based subunit vaccines, like the vaccines predicted in this study, could overcome such difficulties [114–116].

Overall, this study recommends DV-1 and HPV-1 as the best vaccines to be an effective worldwide treatment based on the strategies employed in the study to be triggered against DENV-1 and HPV-16 infections. However, further *in vivo* and *in vitro* experiments might be required to strengthen the findings of this study.

5. Conclusion

Dengue and cervical cancer are two of the most life-threatening diseases in the world. Currently, research is ongoing to find possible solutions and to cure these diseases; however, no satisfying treatment is currently available. The potentiality of *in silico* methods can be exploited to find desired solutions with relatively lesser amount of trial and error, and thus save both time and costs to scientists. In the current study, possible epitope-based, subunit vaccines against DENV-1 and HPV-16

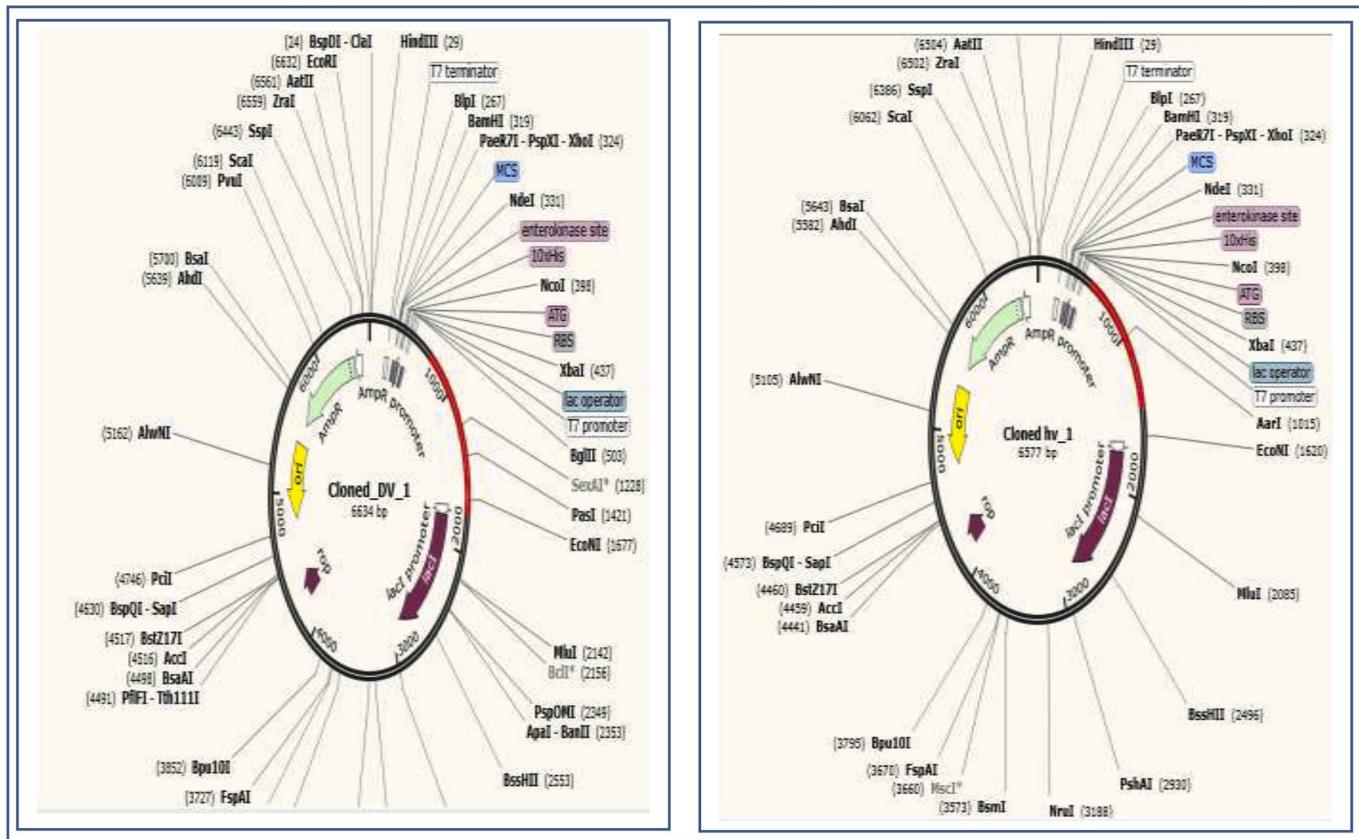


Fig. 15. *In silico* restriction cloning of the DV-1 vaccine sequence (left) and HIV-1 vaccine sequence (right) in the pET-19b plasmid between the SgrAI and SphI restriction enzyme sites. The red colored marked sites contain the DNA inserts of the vaccines. The cloning was carried out using the SnapGene tool. The two newly constructed plasmids can be inserted into *E. coli* strain K12 for efficient vaccine production. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were designed by using the tools of reverse vaccinology and immunoinformatics. To design the vaccines, the highly antigenic and non-allergenic viral proteins as well as epitopes were used. Various robust computational studies on the suggested vaccine constructs revealed that these vaccines might confer good immunogenic responses. For this reason, if satisfactory results are achieved in numerous *in vivo* and *in vitro* tests and trials, these suggested vaccine constructs might be used effectively for vaccination to prevent the infection and spreading of the target viruses. Hopefully, this research should raise interest among the scientists of the respective field.

Ethical statement

The authors declare that they don't have any potential competing interests including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding, regarding the work or project.

Declaration of competing interestCOI

Bishajit Sarkar declares that he has no conflict of interest. Md. Asad Ullah declares that he has no conflict of interest. Yusha Araf declares that he has no conflict of interest.

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