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A NOVEL TETRAVALENT RECOMBINANT ENVELOPE DOMAIN III VACCINE AGAINST DENGUE: AN *IN SILICO* APPROACH

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ABSTRACT: The global rise in dengue cases is a major public health concern in terms of morbidity and mortality. The recent study reports 390 million dengue infections annually of which 96 million infections becomes clinically or subclinically severe. Therefore, development of an effective tetravalent vaccine against dengue is a top priority. Dengue envelope domain III is a surface exposed protein; involved in host cell binding and containing multiple, serotype-specific and subcomplex-specific neutralizing epitopes, thus becomes an ideal target for vaccine development. The rapid growth in bioinformatics or immunoinformatics area in terms of development of sophisticated tools assists researchers to predict immunodominant epitopes and study various characteristics of the predicted vaccine model. The combination of computer-aided or *in silico* methods and experimental methods are useful tools to address complex problems such as deciphering immune responses and vaccine design. In the present study we aim to develop a recombinant tetravalent vaccine model using bioinformatics tools of our vaccine candidate containing envelope domain III of all four dengue serotypes (GenBank ID: KF 855114) and study its role and characteristics with its sequence and structure based features. *In silico* approach showed that our vaccine is stable, properly folded, antigenic and having multiple predicted B and T cell epitopes that are known to be immunogenic. Also the docking studies using a mouse monoclonal antibody (4E11), which neutralizes all four DENV serotypes, predicted a favourable and stable protein-protein interaction model. Further studies are underway to test its immunogenicity and efficacy in mice.

INTRODUCTION: Dengue virus (DENV) is a flavivirus causing major threat to health in tropical countries around the world. DENV is endemic in more than 125 countries [1]. Annually 390 million people get infected by dengue of which 96 million cases have clinical or subclinical severity [2]. DENV are maintained in nature in two cycles namely a sylvatic cycle and an urban cycle.

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A sylvatic cycle is exist between non-human primates and arboreal *Aedes* mosquitoes, while an urban cycle is maintained between humans and domestic, peridomestic *Aedes aegypti* and *Aedes albopictus* mosquito vectors [3].

Four DENV serotypes (DENV-1 to 4) are capable of causing self-limited dengue fever (DF) or even life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The host immune system plays a significant role in dengue infection as well as in protection. The primary dengue infection provides lifelong protection to the homologous serotype, while secondary dengue infection with heterologous serotype causes severe

complications like DHF/ DSS [4]. Controlling severe life-threatening DENV infections (DHF/ DSS) are presently depends on modern supportive intensive care as there is no specific treatment (antivirals) or licensed vaccine present in the market to date [5].

Immunity to DENV infection is primarily mediated by neutralizing antibodies [6, 7]. The role of T cells in protection as well as in pathogenesis of dengue has also been documented [8, 9]. Envelope protein is the major protective antigen in DENV infection as it is exposed to the immune system and most of the neutralizing antibodies are directed against it [6]. Most of the vaccine strategies focus on inducing neutralizing antibodies against this antigen[10-13].

It has been well documented that most of the epitopes that are multiple, serotype-specific and subcomplex-specific elicit only virus-neutralizing monoclonal antibodies, having low potential for inducing cross-reactive antibodies to heterologous dengue serotypes located in domain III of envelope protein (EDIII) [10]; also it is exposed to the surface and thus becomes the primary target for antibody-mediated neutralization. It is also involved in host cell binding [14]. So neutralizing antibodies produced against EDIII may block the entry of the virus into the cell, thus become the ideal target for vaccine development [15].

Development of safe and effective dengue vaccine is a challenging task and has been hampered mainly because of the concern that cross reactive immunological memory elicited by a vaccine candidate could increase the risk of DHF and DSS as secondary heterologous DENV infection could lead to antibody dependant enhancement (ADE) and cytokine storm/ Tsunami that is known to accelerate DENV pathogenesis [8-9]. Therefore, a safe and effective DENV vaccine must be tetravalent and induce balanced protective immune response against all four serotypes.

Bioinformatics or immunoinformatics is an interdisciplinary area involving chemical, biological and computational sciences. The bioinformatics and immunoinformatics fields are emerging rapidly in terms of development of various sophisticated bioinformatics tools that facilitate the process of designing vaccine candidate by assisting researchers in identifying the immunodominant T-cell and B-cell epitopes or immunological 'hot-spots', the most crucial step in vaccine design. *In silico* methods uses variety of statistical and machine learning approaches to study the various characteristics of predicted vaccine model. Experimental methods in combination with *in silico* methods are useful tools to address complex problems such as deciphering immune responses and vaccine design[16, 17].

In the present study we aim to develop a recombinant tetravalent vaccine model using bioinformatics tools of our vaccine candidate containing EDIII of all four dengue serotypes (GenBank ID: KF 855114)[18] and study its role and characteristics with its sequence and structure based features.

MATERIALS AND METHODS:

Recombinant tetravalent protein sequence:

We used the protein sequence of our recombinant tetravalent EDIII based dengue vaccine construct (GenBank ID: KF 855114)[18] to predict the structure, and study various characteristics using bioinformatics tools see **Fig. 1**.

Immuno-informatics analysis with B and T cell epitope prediction:

We used IEDB sources to screen known epitopes against the tetravalent sequence to get maximum number of antigenic epitopes that are able to induce both the B-cell and T-cell response. B cell and T cell prediction tools from IEDB (www.iedb.org)[19] were used to screen all reported epitopes in literature and further all the epitopes were manually inspected with respect to its presence in desire region, then aligned and confirmed using local Perl scripts and Emboss utilities see **Table 1 and 2**.

Primary sequence analysis and Biological activity prediction:

Various physico-chemical parameters like amino acid composition, theoretical pI, instability index, *in vitro* half-life, aliphatic index, grand average of hydropathicity (GRAVY) and molecular weight were evaluated using BioPerl scripts see **Table 3 and Fig.2**. Sequence directed biological activity and molecular function ontology predicted with Predict Protein (https://www.predictprotein.org/) [20] **s**ee **Fig.3**.

Antigenicity and allergenicity evaluation:
ANTIGENpro (http://scratch. proteomics.ics.uci. edu/), and VaxiJen v2.0 server were used to predict protein antigenicity. These are alignment independent approaches based on statistical approaches between principal amino acid properties. We used AlgPred web server (http://www.imtech.res.in/raghava/algpred/) in order to predict protein allergenicity [21] see **Table 3**.

Vaccine features:
Secondary structure prediction:
Secondary structure of recombinant tetravalent EDIII protein was predicted using secondary structure prediction utility at I-TASSER (zhanglab.ccmb.med.umich.edu/I-TASSER)[22] and ProCheck (www.ebi.ac.uk/thornton-srv/ software/ PROCHECK) see **Fig. 4**.

Protein structure modeling:
Recombinant tetravalent sequence was submitted to I-TASSER. It generates full length model of proteins by excising continuous fragments from threading alignments and then reassembling them using replica-exchanged Monte Carlo simulations[23] see **Fig.5A**.

Tertiary structure refinement:
As the sequence of tetravalent vaccine is the product of EDIII from different DENV serotypes, we selected homology and threading approach for protein tertiary structure modeling. The critical steps of structure refinement was specified and modeled by GalaxyLoop (http://galaxy.seoklab. org/) [22]. The structure optimization of the model was performed using stepwise and direct energy minimization of knowledge based potential of mean force and stereochemistry correction see **Fig. 5B**.

Tertiary structure validation:
In order to find the potential errors in initial 3D models, ProSA-web at (https://prosa.services.came. sbg.ac.at/prosa.php) was used [24]. The residue-by-residue stereochemical qualities of models were validated by Ramachandran plot obtained from RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) see **Fig. 5C and D** and **Table 4**.

Ligand binding site prediction and protein-protein interaction study:
Protein-protein interaction was studied using Zdock server (http: //zdock. umassmed.edu/) [25]. Interpolated partial charge surfaces and hydrophobic patches of vaccine were assessed by stand alone softwares viz. Accelerys Discovery Studio 4.5 (Accelrys Inc) see **Fig. 6**.

Data validation:
To predict potential B-cell and T-cell epitopes several servers were used. IEDB sources are using data from more than 15 locations and given more than 1000 epitope sequences as hits from all DENV serotypes. All the hits were then manually inspected with local Perl scripts and using Emboss services with different thresholds and scores. The shortlisted data is provided in **Table 1** and **2**.

RESULTS AND DISCUSSION:
Vaccination is an important global strategy for controlling the number of clinically significant DENV infections. A recombinant DNA vaccine against flaviviruses becomes an attractive and promising approach in order to understand the important immunodominant epitopes involved in protection. Furthermore, several advantages like simplicity of production, safety, target specificity, induction of both humoral and cellular immune responses and success in preclinical models has attracted global attention[26-29].

Recombinant tetravalent protein sequence:
In the present study we analyzed various parameters of our dengue vaccine construct using bioinformatics tools. The protein sequence of our ED III based recombinant tetravalent dengue vaccine construct (GenBank Accession Number: KF855114)[18] has been shown in **Fig. 1**.

The predicted sequence shows an extracellular involvement. This feature has importance in terms of exposure of epitopes to immune system to induce an immune response as EDIII contains multiple, serotype-specific and subcomplex-specific epitopes that are dominant neutralizing

determinants having low potential for inducing cross-reactive antibodies to heterologous dengue serotypes. Also it is exposed and accessible on virion surface, and involved in host cell receptor binding.[10, 14, 15]

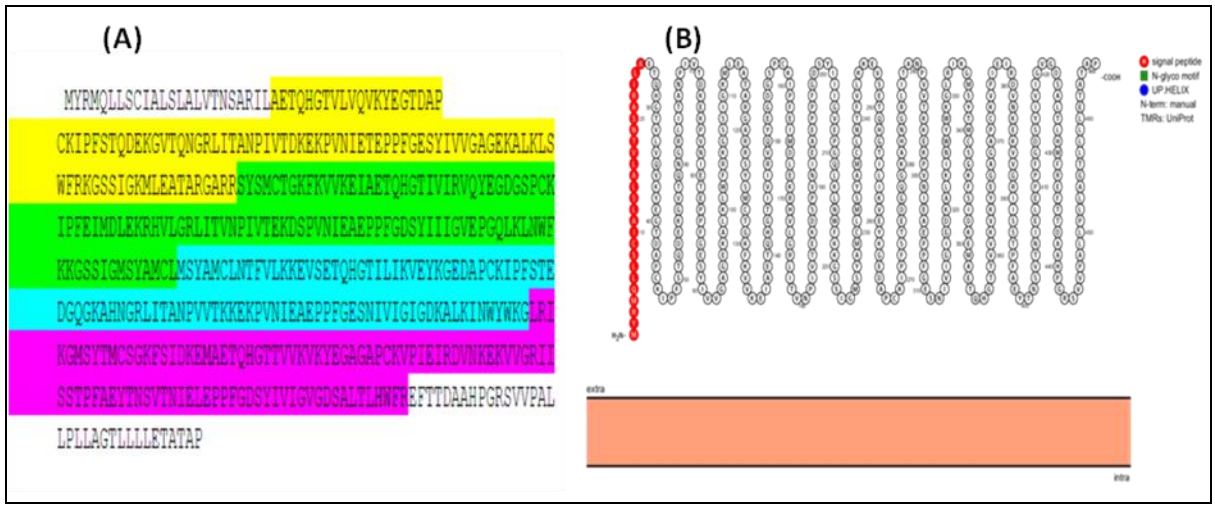

FIGURE 1: (A) SEQUENCE OF RECOMBINANT TETRAVALENT EDIII PROTEIN- CONSTRUCTED USING CLONING OF EDIII FROM DENV-1 TO 4 INTO A PVAC1-MCS MAMMALIAN EXPRESSION VECTOR (RESIDUES SHOWN YELLOW ARE FROM DENV-1, GREEN FROM DENV-2, BLUE FROM DENV-3, PINK FROM DENV-4, AND NON HIGHLIGHTED SEQUENCES ARE VECTOR SEQUENCES) (B) RECOMBINANT TETRAVALENT EDIII PREDICTED TO BE HAVING EXTRACELLULAR INVOLVEMENT AND HAVING SIGNAL PEPTIDE FROM 1 TO 25 AMINO ACIDS

B and T cell epitopes prediction:

B and T cell epitopes were predicted using bioinformatics tools in our novel recombinant tetravalent EDIII based dengue vaccine with known published B cell (neutralizing) and T cell (CD4+, CD8+ CTL) epitope data. The predicted epitopes were restricted to EDIII as our vaccine construct is based on EDIII of DENV-1 to 4 serotypes. Also the prediction is based on the known available data which is mostly focused on DENV-2, and the information regarding B cell (neutralizing) and T cell (CD4+, CD8+ CTL) epitopes present in EDIII of other DENV serotypes is limited. **Table 1** and **2** summarizes the predicted B and T cell epitopes that are known to be neutralizing and CD4+ or CD8+ CTL epitopes respectively.

TABLE 1: B-CELL EPITOPES PREDICTED USING IEDB RESOURCES CONSIDERING B CELL RESPONSE ASSAYS

Sr. No.	Start-end position	Epitope sequence	Sr. No.	Start-end position	Epitope sequence
1	124-135	SYSMCTGKFKVV	20	176-181	RLITVN
2	128- 159	CTGKFKIVKEIAETQHGTIVIRVQY EGDGSPC	21	177-182	LITVNP
3	135-144	VKEIAETQHG	22	177-185	LITVNPIVT
4	138-146	IAETQHGTI	23	178-194	ITVNPIVTEKDSPVNIE
5	143-148	HGTIVI	24	187-214	KDSPVNIEAEPPFGDSYII IGVEPGQLK
6	144-149	GTIVIR	25	198-203	PFGDSY
7	144-154	GTIVIRVQYEG	26	198-209	PFGDSYIIIGVE
8	145-150	TIVIRV	27	199-204	FGDSYI
9	149-154	RVQYEG	28	200-205	GDSYII
10	150-161	VQYEGDGSPCKI	29	201-206	DSYIII
11	159-177	CKIPFEIMDLEKRHVLGRL	30	202-207	SYIIIG
12	170-175	KRHVLG	31	204-209	IIIGVE
13	171-176	RHVLGR	32	212-217	QLKLNW
14	171-182	RHVLGRLITVNP	33	212-218	QLKLNWF
15	171-185	RHVLGRLITVNPIVT	34	212-223	QLKLNWFKKGSS
16	172-177	HVLGRL	35	213-218	LKLNWF
17	174-179	LGRLIT	36	214-225	KLNWFKKGSSIGQ
18	175-182	GRLITVNP	37	219-226	KKGSSIGM
19	175-185	GRLITVNPIVT			

TABLE 2: T-CELL EPITOPES PREDICTED USING IEDB RESOURCES CONSIDERING T CELL RESPONSE ASSAYS

Sr. No.	Start-end position	Epitope sequence
1.	108-120	SSIGKMFEATARG
2.	157-172	SPCKIPFEIMDLEKRH
3.	159-177	CKIPFEIMDLEKRHVLGRL
4.	163-185	FEIMDLEKRHVLGRLITVNPIVT
5.	178-194	ITVNPIVTEKDSPVNIE
6.	188-194	ITVNPIVTEKDSPVNIE
7.	234-248	SYAMCTNTFVLKKEV
8.	239-253	TNTFVLKKEVSETQH
9.	244-258	LKKEVSETQHGTILV
10.	254-268	GTILVKVEYKGEDAP
11.	304-318	EAEPPFGESNIVIGI

Thus our predicted vaccine model shall induce both B cell and T cell immune responses, which further need to be evaluated for immunogenicity and efficacy studies in laboratory animals.

Analysis of various physico-chemical parameters of recombinant tetravalent dengue vaccine:

Various physico-chemical parameters of recombinant tetravalent dengue vaccine are given in **Table 3**.

TABLE 3: PHYSICO-CHEMICAL PARAMETERS OF RECOMBINANT TETRAVALENT DENGUE VACCINE

Results: Property		Value
No. of amino acids		466
Molecular weight (Da)		5 1 0 4 5. 1
Theoretical pI		7.95
Negatively charged residuse (Asp+Glu)		52
Positively residue (Arg+lys)		54
Instability index		35.98
Extinction coefficient (M-1cm-1) at 280nm		0.947
Grand Average of hydropathicity (GRAVY)		- 0.114
Half Life in mammalian reticulocytes (*in vitro*)		30 hours
Vaccine antigenicity	ANTIGENpro	0.73
	VaxiJen	0.64

Negatively charged residue (Asp+Glu) and positively charged residue (Arg+lys) charged were equally distributed in the recombinant vaccine suggesting its stability with respect to its electrical charge distribution. The instability index is used to determine the stability of protein and it was found to be 35.98 describing its probable stability. Extinction coefficient found to be 0.947 which is closer to 1 showing the greatest extent of purity which is a very important aspect in commercial vaccine production.

The Window position values shown on the x-axis of the graph reflect the average hydropathy of the entire window, with the corresponding amino acid as the middle element of that window peaks with scores greater than 1.8 (red line) indicated possible transmembrane and surface protein regions. The transmembrane regions were found be at 10-18, 223-240, and 438-458 amino acid positions **(**see **Fig.2**. Also, GRAVY value found to be -0.114 indicating the hydrophilicity of the vaccine for its suitability intended for vaccine route selection where hydrophilicity is preferred. Half-life was estimated to be 30 h in mammalian reticulocytes showing its increasing bioavailability and slow enzymatic degradation during systemic circulation.

The antigenicity of vaccine found to be 0.73 and 0.64 with ANTIGENpro and VaxiJen to servers suggesting the binding specificity with a group of certain products that have adaptive immunity (T and B cell receptors). The peptide composition was also predicted to be non-allergen using Hybrid Approach (SVMc+IgE epitope+ARPs BLAST+MAST) of Alg Pred. The biological role of recombinant was predicted to be in viral life cycle, viral genome replication and RNA-dependant transcription. Also, molecular function ontology predicted its activities in protein binding and other activities see **Fig. 3**. These activities are very essential in predicting the activities of recombinant construct as a vaccine.

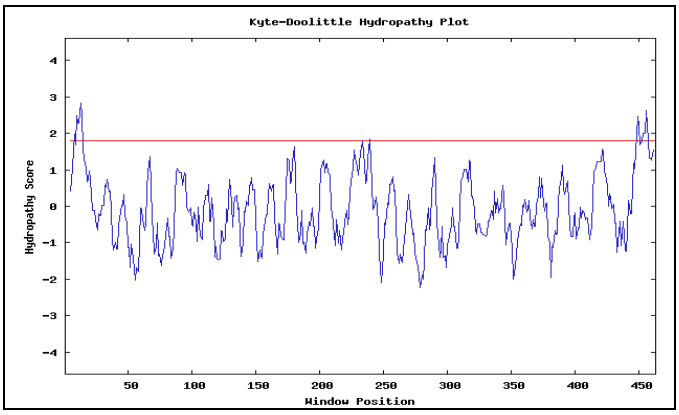

FIG. 2: KYTE DOOLITTLE HYDROPATHY PLOT SHOWING PEAKS WITH SCORES NEARER AND GREATER THAN 1.8 (RED LINE) INDICATE POSSIBLE TRANSMEMBRANE REGIONS FOUND TO BE AT 10-18, 223-240, 438-458. (THE WINDOW POSITION VALUES SHOWN ON THE X-AXIS OF THE GRAPH REFLECT THE AVERAGE HYDROPATHY OF THE ENTIRE WINDOW, WITH THE CORRESPONDING AMINO ACID AS THE MIDDLE ELEMENT OF THAT WINDOW)

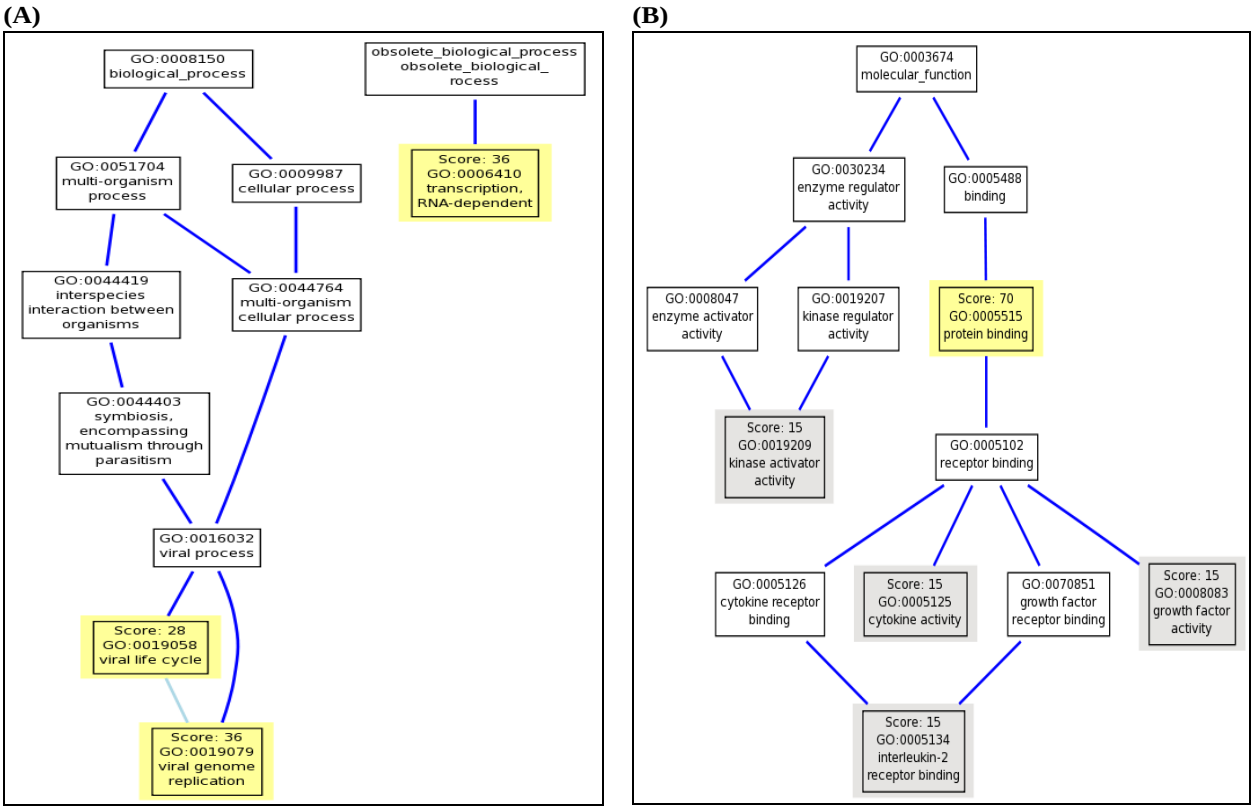

FIG.3: CONNECTOGRAM OF CONSERVED ACTIVITIES FOR TETRAVALENT DENGUE VACCINE SHOWING (A) BIOLOGICAL ACTIVITY (B) MOLECULAR FUNCTION ONTOLOGY PREDICTED WITH PREDICTPROTEIN

Secondary structure of recombinant tetravalent vaccine was predicted using PSIPRED. It showed around 43% of amino acids involved in formation of beta sheets, 48% of amino acids involved in coil formation and remaining amino acids involved in formation of alpha helix, confirming the ability of recombinant tetravalent vaccine in its structure formation see **Fig. 4**.

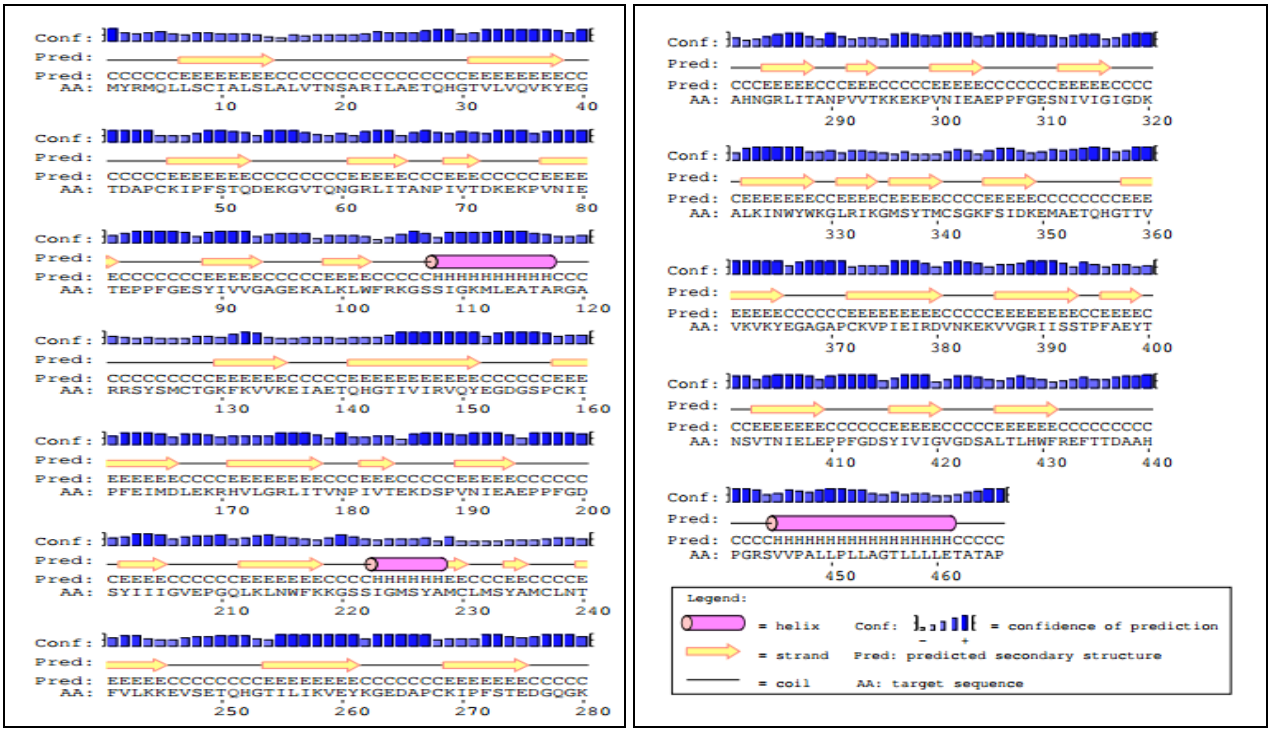

FIG. 4: GRAPHICAL VIEW FOR SECONDARY STRUCTURE OF RECOMBINANT TETRAVALENT EDIII DENGUE VACCINE SHOWING RESIDUES PREDICTED TO BE INVOLVED IN C: COILS, E: SHEET, H: HELIX REGIONS PREDICTED USING PSIPRED

Tertiary structure of protein for recombinant tetravalent vaccine was modeled using knowledge based threading approach where whole stretch of sequence was taken into consideration with secondary and tertiary structure based similarity approaches. The initial model structure was refined with utilities of energy minimizations. Structure had been resolved where all hydrogen atoms have been projected from the backbone and optimized in terms of packing. It was also confirmed that all the amino acid residues were taking part in the structure formation and proper folding patterns were observed where maximum residues were in allowed region of Ramchandran plot see **Fig. 5** and **Table 4**.

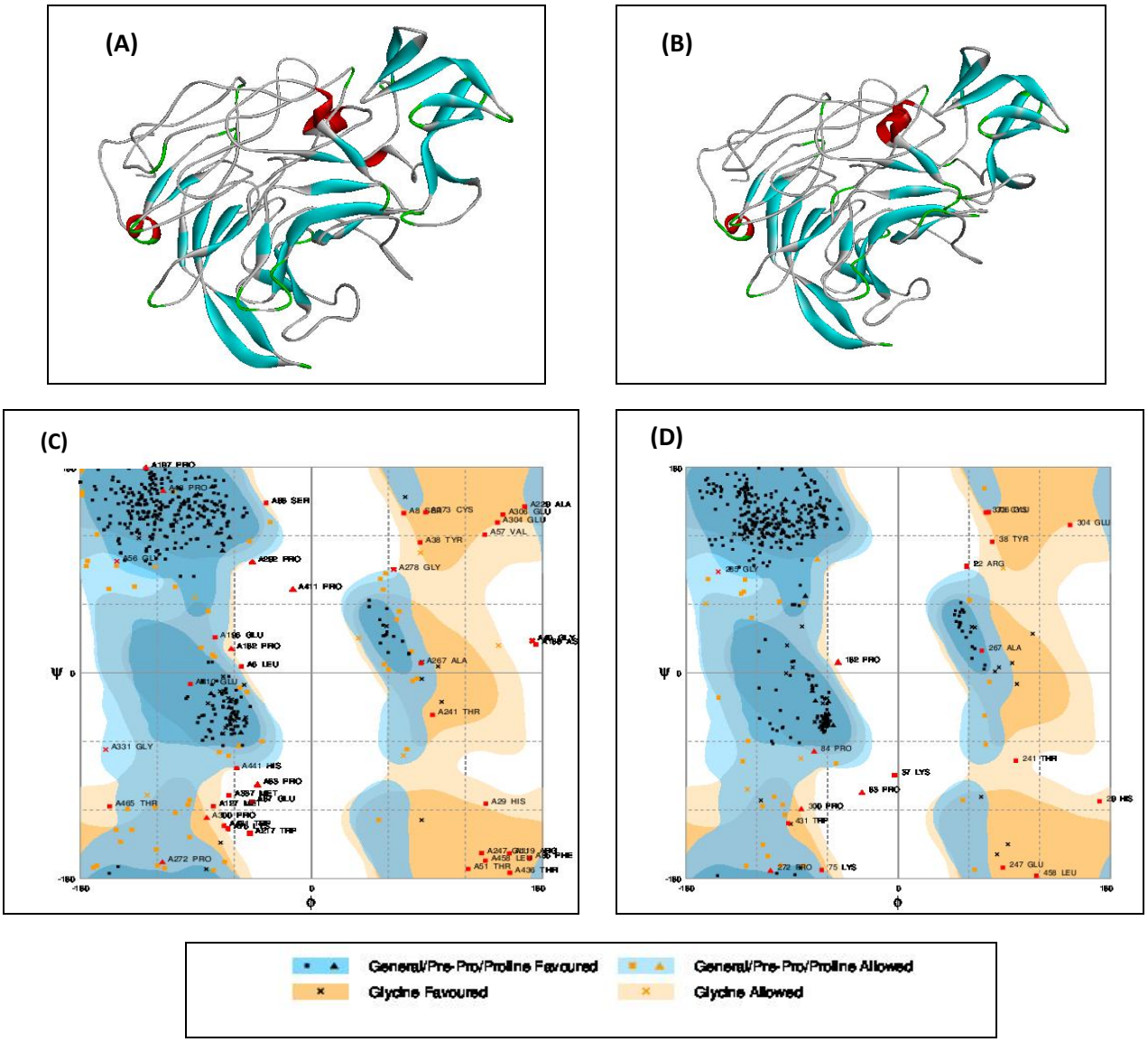

FIG.5: TERTIARY STRUCTURE PREDICTION AND REFINEMENT OF RECOMBINANT TETRAVALENT EDIII (A) INITIAL AND (B) REFINED TERTIARY STRUCTURE ; RAMACHANDRAN PLOT FOR (C) INITIAL (D) REFINED TERTIARY STRUCTURE SHOWING MORE NUMBER OF AMINO ACIDS IN FAVORED REGIONS

TABLE 4: COMPARISON OF RAMACHANDRAN PLOTS STATISTICS FOR INITIAL AND REFINED MODELS

Properties	Initial model		Refined model	
Residues in most favored regions [A, B, L]	303	76.1%	368	92.46%
Residues in additional allowed regions [a, b, l, p]	66	16.6%	15	3.76%
Residues in generously allowed regions [~a,~b,~ l , ~p]	19	4.8%	8	2.01%
Residues in disallowed regions	10	2.5%	7	1.75%

Number of non-glycine and non-proline residues	398	100%	398	100%
Number of end-residues (excl. Gly and Pro)	1		1	
Number of glycine residues	40		40	
Number of proline residues	28		28	
Total number of residues	467		467	

We selected the murine monoclonal antibody 4E11, which neutralizes all four DENV serotypes [30], to check its activity with recombinant tetravalent vaccine. The structure of monoclonal antibody 4E11 was extracted from PDB database with 3UZV identifier. It showed favourable protein-protein interaction with most stable and lowest binding energy amongst see **Fig. 6.A**. The 2D interaction found to be between LYS (55) and VAL (57) amino acid residues of 4E11 and ILE (194) amino acid residues of recombinant vaccine see **Fig.6.B**.

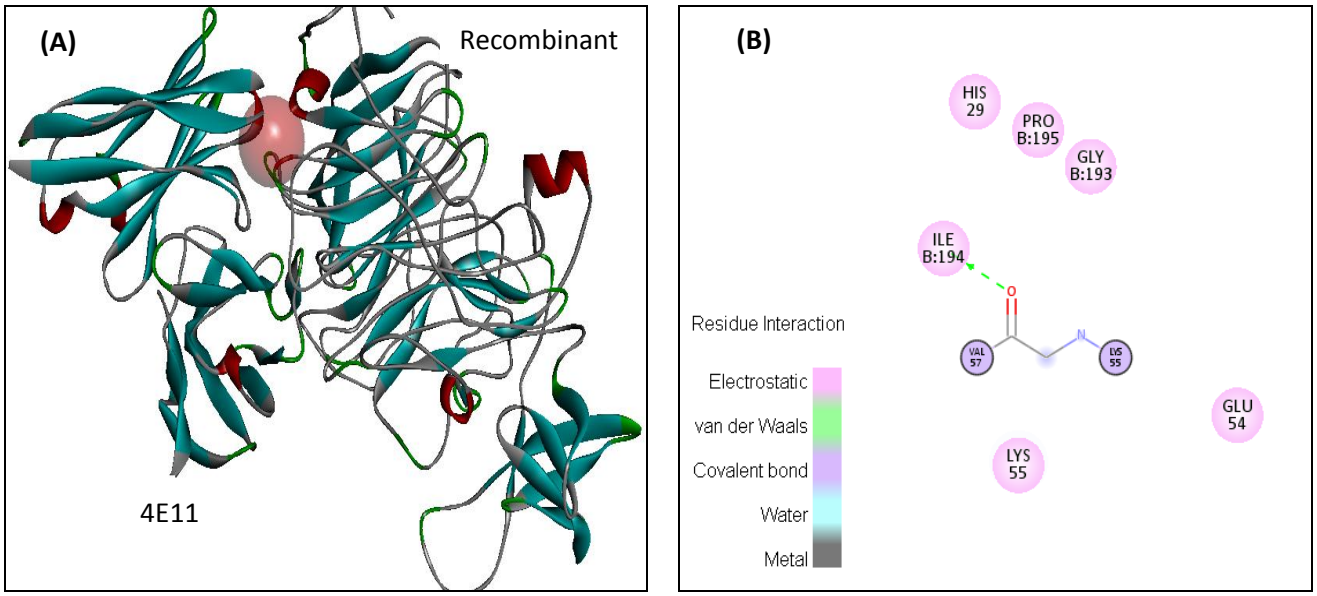

FIG. 6: PROTEIN-PROTEIN INTERACTION BETWEEN MICE MONOCLONAL ANTIBODY 4E11 AND RECOMBINANT VACCINE (A) COMPLETE VIEW WHERE RED BALL SHOWING THE REGION OF INTERACTION (B) 2D INTERACTION DIAGRAM SHOWING ACTUAL AMINO ACID INTERACTION

This finding has been interesting as the ILE (194) amino acid residue of recombinant vaccine has potential to interact with mice monoclonal antibody (4E11) which is known to neutralize all four DENV serotypes. Thus ILE (194) amino acid residue has been predicted to be the critical residue for DENV complex-specific MAb 4E11.

CONCLUSION: *In silico* approach to study various parameters of our dengue vaccine candidate indicates that the vaccine is stable, antigenic, properly folded, with proper binding to a broad cross-neutralizing murine monoclonal antibody against all DENV serotypes. Also multiple B-cell and T-cell epitopes predicted in the vaccine model are known immunogenic epitopes. Thus our predicted vaccine model shall induce both B-cell and T-cell immune response, which further need to be evaluated for immunogenicity and efficacy studies in laboratory animals.

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