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Design of Multi-Epitope Subunit Vaccine for Salivary Proteins of *Aedes Aegypti*: An Immuno-Informatics Approach

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Abstract

Vector borne diseases such as dengue cause significant morbidity in developing tropical regions including the Indian sub-continent. Dengue is caused by dengue virus (DENV) that is transmitted within the human race by its vector i.e. female *Aedes aegypti* mosquito. The salivary proteins of vectors help in blood feeding and have the ability to elicit an immune response in humans. Therefore, salivary proteins may be an effective target for design of vaccines to prevent the transmission of the disease causing pathogens into the human host. The present study was undertaken to identify candidate T-cell and B-cell epitopes for design of an effective multi–epitope subunit vaccine against salivary proteins of *Aedes aegypti* using an immuno-informatics based approach. The sequences of previously reported immunogenic salivary proteins of *Aedes aegypti* were retrieved from UniProtKB and analyzed using various *in-silico* tools such as NetCTL, IEDB resource server, AntigenPro and AllerTOP for identification of T-cell and B-cell epitopes. The identified 9-mer and 15-mer peptide sequences of TC and TH cell epitopes were combined with suitable TLR-4 agonist, linkers and adjuvant for design of the 714 aa multi-epitope subunit vaccine construct whose tertiary structure was predicted using I-TASSER server followed by refinement and validation using GalaxyRefine and RAMPAGE servers. Finally protein-protein docking of the vaccine construct and TLR-4, which is involved in anti-parasitic immune response, was performed using ClusPro. The binding pose and binding energy of the docked structure indicated significant interactions and that the multi-epitope subunit vaccine construct would be able to elicit an immune response.

Keywords: Immuno-informatics; T-cell epitope; B-cell epitope; Vaccine; *In silico*; *Aedes aegypti*; Dengue.

1. Introduction

Immunological relevant data generated from sequencing studies on humans and other disease causing organisms has lead to the emergence of important fields such as Immuno-informatics also known as computational immunology, which acts as an interface between computer science and experimental immunology [1]. Sequencing data generated from with technologies such as proteomics, bioinformatics and microarray has lead to the revolution of vaccine design [2]. This integration of immuno-informatics with immunogenomics has lead to a novel approach known as vaccinomics. *In-silico* approaches can identify reliable lymphocytes (B-lymphocytes or T-lymphocytes) that can elicit immunogenic responses in the human body upon activation by virus or infection making them perfect candidate for vaccine design [3]. Vaccinomics has gained much importance in recent years in view of failure of conventional approaches of vaccine design which are based on pathogen dissection, by using various immunological, biochemical and microbiological methods, for some serious infections [3].

Dengue virus (DENV) is a member of Flaviviridae family (DENV-1, 2, 3 and 4) which includes causative agents of number of diseases such as yellow fever, hepatitis, Japanese encephalitis and West Nile fever [4]. The Flaviviridae family is characterized by the presence of single-stranded RNA viruses and enveloped membranes [5].

Dengue is arthropod borne disease transmitted through genus *Aedes* mainly by species *aegypti* and to lesser extent by species *albopictus* [6]. It's causative agent can be any of the 4 serotypes of DENV through its transmitting vector – the female of *sp. Aedes aegypti* mosquito within human race [7].

Dengue is associated with high fever including some symptoms which lasts for 2-7 days: nausea, severe headache, muscle and joint pains, pain behind the eyes, vomiting and rash [8]. Severe complications which can occur during dengue include fluid accumulation, plasma leaking, severe bleeding, respiratory distress and can even lead to organ impairment. The fever duration in DENV-4 is much shorter than DENV-1 and DENV-1 shows more clinical symptoms and severe illness as compared to DENV-4 which shows mild-illness [9].

Critical stage in dengue epidemiology occurs between 28-48 hours and can be lethal if not medically treated. Infection due to DENV has been reported to evoke abrasions in the CD4/CD8 activation with overproduction of cytokines. The infected endothelial and hepatocytes becomes dysfunctional resulting in apoptosis [10]. Dengue fever can affect children and adults and even infants, and rarely leads to death [11].

The DENV virus replicates in the mosquito mid-gut only after feeding on DENV-infected person [12]. During the incubation period of 8-12 days, the DENV virus replicates in the mid-gut and virus infects other body tissue such

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as salivary gland, body fat and trachea through the haemolymph making the mosquito capable of transmitting the infection [13]. The peak level of virus in salivary glands *of Aedes* mosquito is after a week of feeding [14]. The EIP (extrinsic incubation period) is very relevant epidemiologically and is the time between intake of the meal by the mosquito to the time when it become fully infectious and ready to pass the virus to next host [15]. *Aedes aegypti* mosquito breeds mostly in man-made containers and is at its peak biting period early in the morning and in evening there by being day feeder.

Gubler [16], reported that the evolutionary history of DENV is quite recent and may have emerged in humans within the last nine decades. It was further reported that dengue was a sylvatic disease mostly occurring in jungle species such as monkeys of *Presbyti* & *Macaca* in Asia with sporadic outbreaks in humans [16]. However, the prominent interference and encroachment of humans in forest habitat with presence of sufficient number of susceptible human host has resulted in sustained transmission to humans and *Aedes aegypti* lives in close association to humans. Increase in population size has provided en-numerous hosts for the virus to replicate and to transmit disease to cause major outbreaks, in recent years [4].

Geographical distribution of dengue is throughout the tropics, and is affected by various factors such as temperature, humidity and rainfall rapid growing urbanization [17]. Globally the number of the dengue cases reported by WHO has shown an increase from 505,430 cases in 2000, to over 2.4 million in 2010, and 4.2 million in 2019 [18]. India is one of the few nations alongside Bangladesh, Indonesia, Brazil, Sri Lanka, Singapore and others that have reported an increase in number of dengue cases in recent years. The Indian national statistical data of dengue shows 188401, 101192, and 136422 numbers of suspected and confirmed cases in years 2017, 2018, and 2019 [19].

Till date there is no vaccine or immunization for dengue which is being prevented and controlled through various precautions such as removing stagnant to prevent the breeding of mosquitoes and cleaning the water retaining bodies [20]. Existence of different 4 type of DENV virus causing dengue is an obstacle in the vaccine development, as each one of them is able to induce cross –reactive responses against each other. As per a report of 2016 an attenuated live vaccine against dengue is being evaluated in persons of age parameter of 9 to 45 years in Brazil, Philippines and Mexico population [11].

In recent years there is a trend of designing vaccine against the salivary proteins of vectors that are the carriers of various pathogens so that transmission of vector born diseases that are carried by same vectors can be controlled. Ribeiro, *et al.* [21] reported that the salivary gland of the mosquitoes plays an important part in vector survival and influence their feeding behavior by the production of the anti-inflammatory, immune-modulatory and anti-coagulant biochemical molecules [21]. Furthermore the infectivity of the pathogen carried by the vector is enhanced by the immunogenic and the antigenic nature of the salivary proteins of vector host. Therefore, the present *in-silico* immunoformatics based study was undertaken for the design of multi-epitope subunit vaccine containing T_H and T_C cell epitopes against the salivary proteins of the vector *Aedes aegypti* that transmits DENV and other pathogens.

2. Research Method

2.1. Retrieval of Immunogenic Protein Sequences

Sequences of nineteen salivary proteins of *Aedes aegypti* mosquito reported to be immunogenic by Oktarianti, *et al.* [22] were retrieved from UniProt Knowledgebase [23] in FASTA format.

T_C-cell epitope prediction and MHC-I allele analysis:

Prediction of T-cell epitopes was done based on the combinatorial score with threshold value of 0.75 using NetCTL server 1.2. Epitope prediction is based on the combined score of major histo-compatibility complex class I (MHC-I) binding, TAP transport efficiency scores and proteasomal C terminal cleavage [24]. Immunogenicity of the predicted epitopes was examined using MHC-I binding tool of IEDB server (Immune Epitope Database) as immunogenic epitopes act as efficient response activators in the body [25].

2.2. T_H-cell Epitope Prediction

The prediction of T_H-cell epitopes was done using MHC -II tool of IEDB resource server. Anticipated epitopes were arranged based on their percentile rank (<1) of MHC-I molecules binding peptides, that are determined using SMM (stabilized matrix base method), a network based technique [26].

2.3. IFN-Gamma Induced Epitope Analysis

T_H-cell epitopes were further examined for their ability to elicit the IFN-gamma production using INfepitope server. This server gives predictions of epitopes specific for activation of T_H immune response based on three models *i.e* motif-based, hybrid approach and SVM (Support Vector Machine) based model [27].

Allergenicity and Antigenicity assessment: AllerTOP server was used to analyze the allergenicity of T_C and T_H-cell epitopes and the final vaccine construct. It gives prediction of peptide sequences as either allergen or non-allergen based on the physio-chemical properties of the amino acids [28].

AntigenPro is an antigenicity evaluating server that predicts antigenicity of the designed vaccine. It is a sequence-based, alignment-free and pathogen-independent predictor of protein antigenicity that predicts whether the protein is likely to be antigenic or not and also gives the corresponding probability [29].

2.4. Tertiary Structure Prediction; Refinement and Validation

The prediction of the tertiary structure of the vaccine construct was done using I-TASSER server. It is a hierarchical approach for protein structure and function prediction. It identifies structural templates from the PDB using multiple threading approaches [30].

Galaxy Refine was used for structure refinement of the predicted 3-D structure obtained from I-TASSER. It performs repeated structure perturbation and subsequent overall structural relaxation by molecular dynamics simulation [31].

The structure was evaluated for its quality by generating Ramachandran plot using RAMPAGE server that gives information regarding preferred, permitted and denied torsional edges; psi against phi of amino acids deposits based on Van der Waal range of their side chains [32].

2.5. Docking Studies

Molecular docking is a computational based approach to predict the binding affinity between the ligand and the receptor for understanding of cellular and organization function. The binding affinity of the multi-epitope subunit vaccine construct and TLR-4 (PDB ID: 4G84) obtained from RCSB-protein Data Bank was performed using ClusPro protein-protein docking server to check the binding affinity [33].

3. Results and Discussion

The sequences of nineteen immunogenic salivary proteins of *Aedes aegypti* (Table 1) reported by Oktarianti, *et al.* [22] were retrieved from UniProt Consortium [23].

3.1. Immunological Analysis using In-Silico Tools

All these proteins were analyzed using NetCTL server to predict T_H-cell epitopes as cytotoxic T-lymphocytes (CTL or T_C-cells) have the ability to induce cell mediated immune response against antigens in our body and also form memory cells [34]. One hundred and fifty-nine T_C-cell epitopes (9-mer peptide sequences) were obtained with 0.75 as the threshold combinatorial score for reliable epitope identification.

All the 159 predicted T_C-cell epitopes were analysed for immunogenicity using MHC-I binding tool of IEDB server. IEDB server returns positive and negative scores based on the immunogenicity of T_C-cell epitopes. Eighty-two T_C-cell epitopes were obtained that have positive imunogenicity score, which were further analyzed for allergenicity using AllerTop server. The T_C-cell epitopes that were non-allergens were shortlisted and the allergen epitopes were discarded as they may cause harmful and fatal reactions in the body and cannot be used in vaccine design [35]. Therefore, a total of 31 T_C-cell epitopes were obtained that were used for design of the multi-epitope subunit vaccine (Table 2).

An integral part of cell-mediated and humoral immunity are Helper T-lymphocytes (T_H-cell) that are involved in generating an effective immune response [36]. Therefore, T_H-cell epitopes shall form an integral part of the multi-epitope subunit vaccine construct. IEDB server MHC-II epitope binding tool was used to predict T_H-cell epitopes for the 19 reported immunogenic salivary proteins of *Aedes aegypti*. Nominated alleles for *Homo sapiens* namely *H2-IAb, H2-IAd* and *H2-IEd* were selected and 59 T_H-cell epitopes (15-mer peptide sequences) were short-listed based on lower percentile rank (<1) that denotes higher immunogenicity [37]. These T_H-cell epitopes were analyzed using IFNepitope server to assess their efficacy in eliciting the production of IFN-gamma after activating T_H type immune response. Nine T_H-cell epitopes (15-mer peptide sequences) having a positive IFN score that indicates the epitopes to be non-allergens were selected using the AllerTop server that predicts allergenicity (Table 3).

3.2. Design of the Vaccine Construct

For evoking an effective immune response a multi-epitope subunit vaccine should contain both T_H and T_C-cell epitopes so as to activate both innate and adaptive immune responses [35]. Toll-like receptors (TLRs) have a major contribution in production of cytokines and therefore play a crucial role in innate immunity against viral infection [36]. Hence, TLR4 agonist (PDB ID: 4G84) was selected as an adjuvant and the T_H and T_C–cell epitopes identified in this study were linked together using suitable linkers. Linkers play an important part in simulating the vaccine construct to work as an independent immunogen and producing higher antibody titer than that of a single immunogen [35]. In the present study three linkers *i.e* EAAAK, GGGS and GPGPG were used to join thirty one 9-mer T_C-cell epitopes and nine 15-mer T_H-cell epitopes with the adjuvant to form the 714 amino acid (aa) multi-epitope subunit vaccine construct. GGGS and GPGPG linkers were added at the intra-epitope position to link the T_C and T_H epitopes respectively.

Antigenicity analysis of the vaccine construct was done using ANTIGENpro. A probability score of 0.4682 was obtained for the vaccine construct which indicates a good antigenic nature [34] and furthermore analysis with AllerTop server the vaccine construct was found to be non-allergen therefore being safe for human administration.

3.3. Tertiary Structure Prediction Refinement and Validation

Tertiary structure of multi-epitope vaccine construct was predicted using I-TASSER server that models the 3-D structure using homology based threading approach. All the 714 amino acid residues of the vaccine construct were modeled as a single domain with 1% disorder and five 3-D models were obtained from I-TASSER server. The structure with the highest C-score of -1.24 was selected for further refinement, as a model with a high C-score signifies higher confidence and vice-versa. The selected secondary structure contained 20% helical, 7% beta-strands

and 72% coils. This structure was refined using Galaxy Refine to increase the number of amino acid residues in the Rama favored residues. Initially the number of Rama favored residues was 64% which improved to 85% after refinement. The refined 3-D structure was validated by plotting Ramachandran plot in which only 3.9% residues were present in disallowed region (Fig. 1 A and B).

3.4. Protein-Protein Docking

Molecular docking of the vaccine-construct and TLR-4 (PDB ID: 4G84) was performed using ClusPro server and total 30 models were generated. From amongst these, the model having lowest binding energy of -1178 KJ/mole and in which the vaccine construct was properly occupying the Toll-like receptor was selected Fig 2.

In this study an immuno-informatics approach was used to design an effective vaccine against the salivary proteins of *Aedes aegypti* and needs experimental validation by the wet lab researcher.

4. Conclusion

Vector borne diseases account for significant morbidity and mortality and there is a need to search for options to tackle them. The vector of dengue, Zika, West Nile, is the mosquito *Aedes aegypti* which is involved in the transmission of parasite to humans. Literature mining indicates that salivary proteins of *Aedes* mosquito effect pathogenesis and immunogenicity. Therefore, in this immuno-informatics study a vaccine construct was designed against the immunogenic salivary proteins of *Aedes aegypti*, having T_C and T_H epitopes joined with suitable linkers and TLR-4 agonist. The multi-epitope subunit vaccine may be effective to control VDB spread by *Aedes* mosquito, however needs experimental validation.

Table-1. Immunogenic salivary proteins present in *Aedes aegypti* mosquito

S.No	Protein Name	UniProt Id	S.No	Protein Name	UniProt Id
1	AAEL004338-PA	Q17D51	11	AAEL004739-PA	Q17BX4
2	37 kDa salivary gland allergen	P18153	12	AAEL003600-PA	Q1HRF7
3	Long form D7Bclu1 salivary protein	Q95V90	13	Putative 34 kDa secreted protein	Q8T9V1
4	Angiopoietin-like protein variant	Q1HRV2	14	30 kDa salivary gland allergen	O01949
5	Annexin	Q17A53	15	AAEL006417-PA	Q0IF93
6	AAEL003107-PA	Q17GF0	16	AAEL007776-PA	Q8T9T9
7	Malate dehydrogenase	Q171B2	17	Apyrase	P50635
8	AAEL000732-PA	Q17NC2	18	5-nucleotidase	Q1HQJ1
9	AAEL009524-PA	Q16VL4	19	Putative secreted protein	Q8T9U9
10	AAEL017349-PA	Q17BX4			

Table-2. Predicted T_C-cell epitopes using NetCTL, IEDB MHC-I binding tool and Allertop

FVALTGIQY	YSEVIFLYV	TLPDIAVTY	FSTYDNDDF	GYSEVIFLY
YAKGGWWYY	LTCCLTFLI	GTEAVALGL	YSSIVWVKF	CIAGIARVY
GSDLGNLLA	KFDRTWAEY	KCSNTAEVY	HSETIRAIL	LTNIFRGEV
ISATTTLAL	HAVVTVPAY	FASESAWYV	CTDLIMADI	TTNPENTVF
LLGEEVAQY	NQDADSEDY	FVDPVTRFF	HSQCFGAWY	LLDVNPLTM
HLDAPVWRV	WLDRGVSGF	LPDIAVTYY	CEDDNYLFY	CSNTAEVYF
ATADALWEL				

Table-3. Predicted T_H-cell epitopes using IEDB MHC-II, IFNepitope and AllerTop

S. No.	Allele	Peptide
1.	H2-IAb	VKVLVVGNPANTNAL
2.	H2-IAb	LPNMFMQPGVPAVKE
3.	H2-IAb	SLPNMFMQPGVPAVK
4.	H2-IAb	PNMFMQPGVPAVKEC
5.	H2-IAb	NMFMQPGVPAVKECT
6.	H2-IAb	QSLPNMFMQPGVPAV
7.	H2-IEd	ADAYVWHFRSRSNAP
8.	H2-IAb	LIALFVASFTTAEEN
9.	H2-IAd	IAARKMSSAMSADLS

Fig-1. Tertiary structure prediction and validation of vaccine construct. (A) Tertiary structure predicted using I-TASSER server for the multi-epitope subunit vaccine construct showing helixes, sheets and coils. (B) Ramachandran plot to validate the structure showing 85% amino acids in Rama favored regions

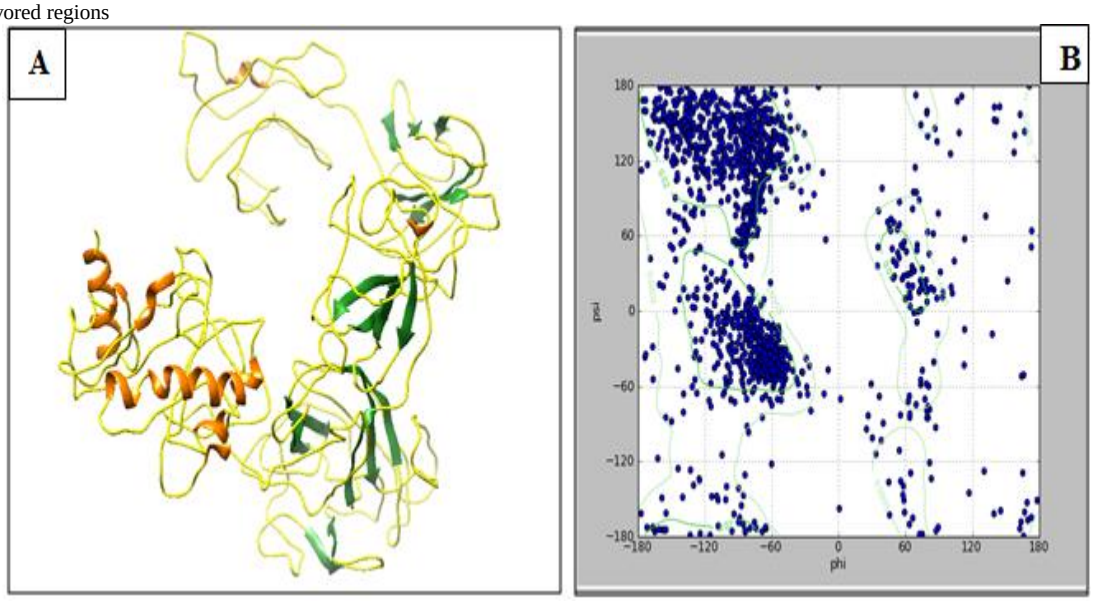
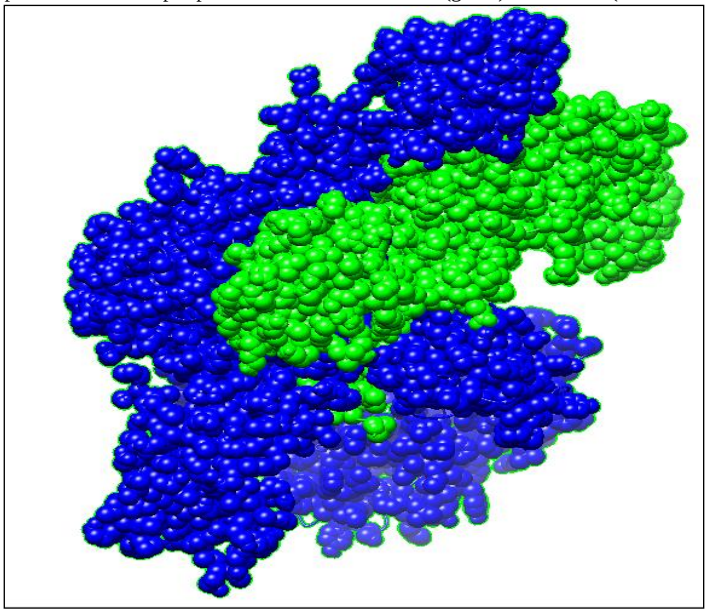


Fig-2. Docked complex of the multi-epitope subunit vaccine construct (green) and TLR-4 (PDB ID: 4G84) ligand (blue)



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