IMMUNOINFORMATICS-DRIVEN MULTI-EPITOPE VACCINE APPROACHES FOR DENGUE VIRUS

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Approximately, there have been over 1.4 crore cases with more than 10,000 deaths reported globally in 2024 alone, making Dengue virus (DENV) a major global health threat. DENV is structurally 11kb long, consisting of a capsid, a pre-membrane, and an envelope protein. This virus exists as four different serotypes ranging from DENV-1 to 4. Antibody-dependent enhancement (ADE) is a phenomenon that stands as a formidable challenge in managing dengue infection that escalates mild dengue fever into more severe forms, such as dengue hemorrhagic fever and dengue shock syndrome. This happens due to an increased risk of severity as a result of secondary infections with a different serotype, thus making the vaccine development process against this virus more complicated. Current vaccine candidates such as Dengvaxia and TV003/TV005 face limitations in safety and effectuality due to the risk of secondary infections. My review emphasizes the major significance and potential of multi-epitope vaccines against all DENV serotypes, and how it is primarily designed using immunoinformatics by targeting both T-cell and B-cell epitopes, which has a huge potential to provide cross-protective immunity. Immunoinformatics offers an efficient and cost-effective approach that has already demonstrated successful results in developing mRNA vaccines against SARS-CoV-2. It reinforces the potential of multi-epitope vaccines against DENV, which can also overcome the limitations of traditional vaccine development, protecting any future genetic variations and therefore offering a promising solution globally.

Keywords: Immunoinformatics, DENV, Multi-epitope vaccine, Databases, Epitope prediction, Molecular Docking.

INTRODUCTION

Due to the high prevalence of dengue virus infections across several tropical and subtropical regions of the world, substantial health-related burdens are imposed globally. Approximately, there have been indications of over 14 million dengue cases and over 10,000 deaths worldwide, with 32,000 dengue cases in India alone (Dengue Worldwide Overview, 2024), making Dengue virus (DENV) a major global health threat. Dengue Virus (DENV) is primarily transmitted by the female mosquitoes of *Aedes aegypti* belonging to the *Flaviviridae* family. It is mainly identified by its 11 kb long, single-stranded, positive-sense RNA genome and it exists in four closely related serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), which exhibit approximately 30% nucleotide variability between serotypes (Morgan *et al*., 2024).

DENV serotype infections can range in severity from mild flu-like disease in Dengue fever (DF) to severe manifestations that lead to Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) (Martina *et al.*, 2009). Secondary infection with a novel serotype of DENV may acquire cross-immunity, which, in turn, can enhance the disease by a mechanism referred to as ADE (Rohim & Kambol., 2024). The severity of the illness can be measured by symptoms of coagulopathy and changes in vascular permeability, which can lead to hypovolemic shock with mortality of as much as 44%. (Martina *et al.*, 2009). Despite efforts for many decades, effective prevention of dengue has not yet been entirely successful, making it a global threat (Saha *et al.*, 2024).

There is neither an antiviral drug nor a vaccine that can be efficacious against all serotypes of dengue virus (DENV). Despite many vaccine candidates in the trial phase, only Dengvaxia and TV003/TV005 are WHO-approved but are insufficient in terms of safety as well as efficacy. Several obstacles have been faced, while administering Dengvaxia and TV003/TV005, especially concerning decreased efficacy as well as the increased risks of antibody-dependent enhancement (ADE). For instance, Dengvaxia has been demonstrated to have limited efficacy against DENV-1 and DENV-2 as well as offers utility only against individuals with prior laboratory-verified dengue infection (Rohim & Kambol, 2024). TV003/TV005, on the other hand, was authorized only for children aged from 6 to 16 years (Whitehead, 2015). The stochastic co-emergence of four highly correlated serotypes of the DENV in humans contributes to antibody-dependent enhancement and presents a major challenge to the effectiveness of

vaccines. Identification of the conserved epitopes is central to the development of tetravalent subunit vaccines, which can induce broad immunity against all the serotypes of the dengue virus (Rohim & Kambol, 2024).

The primary aim of this review is to highlight the stratagem to develop multi-epitope-based vaccines, using immunoinformatics that can provide eminent protection against all four serotypes of DENV. These vaccines are capable of targeting both T-cell and B-cell epitopes simultaneously, thus creating more enhanced cellular and humoral immune responses. These vaccines have the potential to target T-cell and B-cell epitopes at the same time, hence inducing stronger cellular and humoral immune responses. Multi-epitope vaccines are not affected by genetic mutation, retain efficacy against emerging diseases, and have improved safety and stability, hence lowering the incidence of side effects (Saha *et al.*, 2024). Thus, by exploiting immunoinformatics, a tetravalent subunit vaccine designed based on epitopes can be developed to protect against all serotypes of DENV (Ferdousy, 2024). Immunoinformatics have already been effectively utilized in designing multi-epitope vaccines such as Pfizer-BioNTech and Moderna to combat viruses such as SARS-CoV-2 (Noor, 2021). The advantages of using in-silico approaches can be further also highlighted by comparing them with the traditional vaccine development methods such as X-ray crystallography and NMR are expensive and time-consuming (Rahman & Rahman, 2024).

LITERATURE REVIEW:

Structure and Pathogenesis of Dengue Virus

Since 2010, the incidence of dengue has risen to approximately 15 cases per million people annually in various states of India (Mutheneni *et al.*, 2017). In several Indian districts, infections have also been discovered to occur with different serotypes simultaneously, indicating that the virus has well established itself and is prevalent across the nation (Gupta *et al.*, 2012). The mosquito *Aedes aegypti* is the primary vector for DENV transmission which is endophilic, usually residing indoors, and extensively breeds in water-filled containers. The transmission cycle of DENV consists of a sylvatic cycle utilizing wild animals such as non-human primates and a human cycle. The DENV is structurally made up of a single-stranded RNA genome of approximately 11 kilobases in length. The genetic relationship among the four DENV serotypes

is estimated at 75% (Fadaka *et al.*, 2021). It consists of several non-structural proteins that are essential during virus replication and immune evasion. The NS1 protein plays a role in viral replication and modulates the immune response of the host, whereas NS2A and NS2B proteins are involved in viral RNA synthesis and NS3 activity. Additionally, NS4A and NS4B contribute to the formation of protective structures in infected cells and are advantageous to the virus through the inhibition of the interferon response of the host, which is generally counterproductive to infections. Among the proteins, NS5 is the most conserved and plays the critical function of replicating the viral genetic material and suppressing immune signaling in the host (Harris *et al.*, 2006). The viral structure consists of a capsid, a pre-membrane (prM), and an envelope (E) protein. The envelope (E) protein is necessary for host cell receptor recognition and virus entry. It gets reorganized at the low pH in endosomes and facilitates membrane fusion through a cascade of domain-specific processes. The prM protein caps the E protein's fusion loop, preventing premature fusion during virus maturation in the acidic trans-Golgi network (Nanaware *et al.*, 2021).

Host factors such as age and genetics also play important roles in susceptibility and disease severity in DENV infections. Young children are at higher risk of developing severe disease with increased vascular permeability (Bhatt *et al.*, 2020). Genetic mutations of NS1 and NS4B non-structural proteins also mediate endothelial permeability and immunopathy to result in severe disease (Khanam *et al.*, 2022). Diversity among DENV serotypes also increases the risks for the severity of the infection. Moreover, excessive production of proinflammatory cytokines during secondary infections may lead to a "cytokine storm," which is the etiology of vascular leakage and dengue shock syndrome (DSS) (Mangione *et al.*, 2014). Genome-wide association studies have implicated loci such as MICB and PLCE1 linked with severe disease, suggesting the important role of immune regulation in disease pathogenesis (Simmons *et al.*, 2012).

Dengue virus infection has a wide range of clinical manifestations that vary from mild to severe. Primary infection with a specific serotype usually results in asymptomatic illness or mild dengue fever (DF) with signs and symptoms like fever, myalgia, headache, arthralgia, and thrombocytopenia. DF occurs in a three-stage progression: febrile, critical, and convalescent. The febrile stage is marked by high fever and dehydration for 2 to 7 days, while the convalescent stage is marked by recovery, development of a rash, and increased appetite (Roy &

Bhattacharjee, 2021). Severe forms of the disease, such as Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), are characterized by heightened vascular permeability, hemorrhage, and hypovolemic shock, which can ultimately lead to multi-organ failure (Martina *et al.*, 2009). Antibody response to primary infection includes an early increase in IgM 3-5 days after the start of fever followed by a conversion to IgG antibodies that last a lifetime. Secondary infections show a rapid increase in IgG with decreased production of IgM. This is the basis for diagnostic tests wherein an IgM: IgG ratio >1.8 represents a primary infection and <1.8 represents a secondary infection (Iglesias *et al.*, 2011).

Furthermore, while the activation of the complement system limits DENV by triggering adaptive immune responses and neutralizing the virus, activation of FcγR by antibodies surprisingly enhances viral replication, especially in myeloid cells. Pathogenesis in DENV is defined by the virus hijacking host cellular machinery for replication while evading immune detection (Roy & Bhattacharjee, 2021).

Computational Workflow for Dengue Vaccine Designing

Predicting whether the conserved areas of the DENV genome could be the target for a broad-spectrum tetravalent vaccine depends heavily on the computational tools that are utilized in the process of vaccine development. Sequence retrieval from GenBank, VIPR, or PubMed is the initial stage of vaccine development. This helps in obtaining the complete collection of the genetic sequences of DENV serotypes required. Following that is the prediction of B-cell and T-cell epitopes using algorithms using sophisticated computational tools like BepiPred and NETCTL 1.2. which allows the identification of both linear and conformational epitopes. The IEDB Linear Epitope Prediction Tool v2.0 can further be used for epitope analysis that enables comparing the experimentally validated epitopes against epitopes predicted by the researcher, thereby filtering out the candidates to the best possible epitopes for vaccine development.

The epitope conservancy is the next step towards the development of a vaccine that aids in predicting peptides and is checked using the Epitope Conservancy Analysis Tool. It allows the analysis of the conservancy of the selected epitopes, a key consideration in the development of a tetravalent vaccine. Subsequent to this, the antigenicity, toxicity, and allergenicity of the epitopes

are evaluated with the help of VaxiJen, AllerTOP, and ToxinPred software. Only epitopes having high antigenicity scores coupled with low toxicity and allergenicity are selected further.

To forecast the immunogenicity of the designed vaccine candidates, scientists employ the C-IMMSIM server. This computational tool mimics the immune response, yielding valuable information about the responses involving both the primary and secondary immune systems. This gives a detailed simulation of the large-scale immune activation, reassuring the vaccine's potential effectiveness. Molecular dynamics (MD) simulations are then carried out to help provide insight into the behaviour of the vaccine constructs and their stability within the body's given conditions. MD simulations involve tools such as HADDOCK, GROMACS, and CHARMM, to validate the stability of protein-ligand interactions. It also further confirms the flexibility and stability of the vaccine construct in the simulated biological environment, which can further facilitate optimization before advancing to experimental trials. Lastly, the in-silico cloning and expression optimization are considered to achieve a high yield of expressed protein using software such as EMBOSS Backtranseq, JCat, and SnapGene.

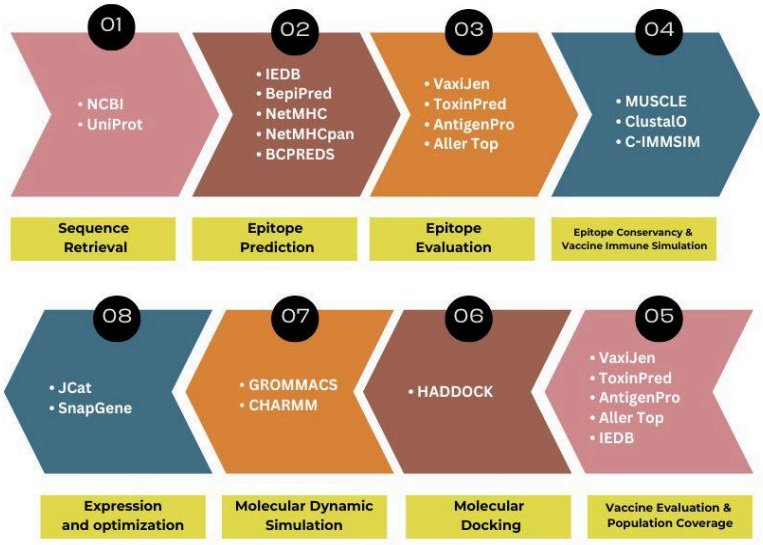

Fig. 1 A diagram illustrating in-silico DENV vaccine design steps

Databases for Sequence Retrieval

Retrieval of the complete collection of the genetic sequences of DENV is the initial step towards vaccine development. The NCBI (National Center for Biotechnology Information) is a major repository, consisting of databases such as GenBank that possess detailed information on nucleotide sequences of more than 300,000 organisms, and it has also largely contributed to the

availability of genetic data for DENV, which is imperative for the first steps of vaccine design. The VIPR (Virus Pathogen Database and Analysis Resource) offers a very rich database of nucleotide sequences and functional information of a variety of viral structures, which are quite important in understanding the potential antigenic proteins of DENV. For instance, Ullah *et al.* (2024) obtained full-length polyprotein sequences of DENV serotypes 1 to 4 from 10 representative countries and four reference strains using such databases.

Sequence Analysis and Epitope Conservancy

Analysis by conservancy can help to identify epitopes that are highly conserved across the four serotypes of DENV, to broaden the protection of the immunization against the virus. In-silico tools such as the Epitope Conservancy Analysis Tool for conservancy analysis, are very essential since they can help researchers identify epitopes that have high conservation which thus require high priority in vaccine constructs. The epitope conservancy analysis performed in the research by Rohim & Kambol (2024), aided in isolating epitopes EP12 and EP15 which were thus expected to exhibit more than 85% conservancy across all four DENV serotypes, making them perfect candidates for vaccine development. Within each serotype, conserved fragments are further identified by performing multiple sequence alignments (MSA). The primary alignment is performed with MUSCLE with subsequent verification through CLUSTALW version 2.0 and MAFFT. Multiple sequence comparison by log-expectation (MUSCLE) is one of the tools used in the study for multiple alignments. MUSCLE has been the most preferable to use in the study for its very high speed and good accuracy, whereby it makes it easier for one to find the conservancy level among the sequences in DENV serotypes.

B-cell epitope prediction

Predictions of conformational B-cell epitopes using in-silico methods have the potential to significantly advance disease diagnosis, drug design, and vaccine development. BepiPred is one of the potential bioinformatics tools that use sequence-based information to improve linear B-cell epitope prediction (Jespersen *et al.*, 2017). For instance, Ullah *et al.* (2024) predicted linear B-cell epitopes in conserved sequences of dengue serotypes 1 to 4, based on the Bepipred2.0 method in the IEDB database. This method predicts the part of the regions in a given amino acid

sequence of a protein that is likely to act as linear B-cell epitopes. In another research conducted by Fadaka *et al.* (2021), BCPREDS was used to evaluate 47 B-cell epitopes from structural and non-structural proteins.

T-cell epitope prediction

The NetMHC and NetMHCpan are some of the most recent computer-based algorithms that predict the binding of peptide sequences with MHC class-I and -II molecules. This is very critical since a linear correlation exists between binding and the potentiality of the peptide to trigger T-cell-mediated immunity. NetMHCIIpan version 4.0 has been reported as a powerful tool for predicting MHC II binding CD4+ T-cell epitopes on parameters like Binding Affinity and Eluted Ligand mass spectrometry score. (Kaushik *et al.*, 2022).

Epitope evaluation

The epitopes of vaccine constructs for DENV are further tested for antigenicity, allergenicity, and safety. This stage goes beyond the theoretical level and involves practical confirmation of the vaccine's ability to produce the intended immune response. AllerTOP, ToxinPred, and VaxiJen are such tools to look for insights into the antigenic potential profile of the constructs, meant to be part of a vaccine application. VaxiJen is a software that does not rely on sequence similarity and alignment, unlike most other bioinformatics tools but rather bases its prediction on the antigenicity of certain physicochemical properties of amino acids and their distribution within the protein. From there, it goes on to predict potential antigenicity in the absence of homologous sequences. The VaxiJen could then be applied for the comparative analysis of antigenic potentials between wild-type and mutant proteins, a very interesting application in the study of pathogen evolution and vaccine escape mutants. Tools like ToxinPred predict high complexity for toxicity and immune response. Lastly, AllerTop is one of the allergenicity-predicting software that helps in scoring the allergenicity of the designed vaccine against DENV to come up with hypoallergenic DENV vaccine candidates.

Molecular docking

HADDOCK, PatchDock, Hawkdock, and ClusPro v2.0 are the most widely used molecular docking tools. High Ambiguity Driven Docking (HADDOCK) is a docking server based on information, for the study of biomolecular structures. It models the relationship between proteins and other biomolecules and can be used in research. HADDOCK is an integrative approach driven both by experimental data e.g., NMR or other means, obtained from other techniques with predefined secondary-structure elements. Molecular docking is potent in predicting the interaction of vaccine epitopes with immune system elements, e.g., adjuvants, MHC molecules, T cell receptor (TCR), and B cell receptor (BCR), which determine immune recognition and reactions. For instance, the molecular docking simulations by Ferdousy *et al.* (2024) investigated the stability and dynamics of two epitopes, TLR-4 and TLR-2 complexes. Saha *et al.* (2023) has used ClusPro v2.0 to validate their findings on the significance of TLR-4 complexes for vaccine development.

MD and Immune simulation

For Molecular Dynamic simulations, servers such as GROMACS and iModS are used majorly. Fadaka *et al.* (2021) have used Maestro v12.4 for the MD simulations to validate his findings on the three isolated antigenic, non-toxic, and non-allergenic epitopes. The C-IMMSIM server is an immune simulation tool to model how the mammalian immune system responds to various immunological challenges; these could be vaccines, pathogens, or autoimmune diseases. This approach could aid in the identification of the best antigen-adjuvant combination to elicit robust and long-lasting immunity. This method operates on sets of mathematical equations and algorithms that model complex interactions between a wide variety of immune cells e.g., T-cells, B-cells, macrophages, dendritic cells, signaling molecules (cytokines, chemokines), and antigens. This helps in the prediction of the immunogenicity of such a vaccine and the likelihood of being able to raise a protective immune response by simulating an immune response toward a vaccine construct.

Epitope expression and cloning optimization

In-silico cloning of the nucleotide sequence into a plasmid vector is the first step for the optimization of vaccines. This process leads to increasing the efficacy of expression of recombinant vaccines, immunogenicity, and stability. Choosing a suitable vector and host, identifying the optimal cloning sites, and selecting the effective promoters are critical steps in in-silico optimization. Also copy number and recombination rates, which lead to optimizing both expression and stability, are two important properties of vectors that should be considered in expression optimization. One method of codon optimization involves altering the genetic code within vaccine constructs to increase protein expression to a level that contributes to enhancing immunogenicity. A study by Rao *et al.* (2024) shows a synthetic multi-epitope protein called rDME-BR that was cloned into the pET-28a(+) expression vector and expressed in E. coli BL21 (DE3) cells using JCat to ensure expression optimization.

Years	Author	Sequence analysis	Epitope prediction	Molecular Docking	Molecular dynamics simulation	Immunogenicity simulation	Cloning and Codon Optimization	Main findings
2017	Ali *et al.*	UniProt	NetCTL 1.2, IEDB, BCPreds, ElliPro	PatchDock 4.0	iModS	C-IMMSIM	Codon Usage Wrangler	The vaccine construct showed stable interactions between ligand and receptor molecule using docking.
2021	Fadaka *et al.*	NCBI, Uniprot	IEDB, BCPreds, CTLPred, BepiPred	PATCHDOCK, FIREDOCK	Maestro v12.4	C-IMMSIM	JCat	3 Antigenic, non toxic, and non-allergenic epitopes were predicted for the B-cells, while 10 antigenic and non-allergenic epitopes for each of the two MHC classes.
2022	Mukhtar *et al.*	NCBI	IEDB, ElliPro, BepiPred	GalaxyPepdock, Discovery Studio Visualizer	iModS	C-IMMSIM	SnapGene	Antigens NS1 and prM were slightly unstable and acidic in nature, while the EIII protein was stable and showed a basic nature.
2022	Kaushik *et al.*	UniProt	NetMHCIIpan	HADDOCK	GROMACS	C-IMMSIM	JCat	MD Simulations Predicted a Stable TLR-5 and Multi-Epitope Vaccine Interaction
2023	Saha *et al.*	NCBI	IEDB	ClusPro v2.0	GROMACS	C-IMMSIM	JCat	A strong interaction network was observed between vaccine models and TLR-4 receptors
2024	Rahman & Rahman	IEDB	Bepipred, NetMHCpan 4.1	ClusPro, HADDOCK, Chimera X	iModS	C-IMMSIM	EMBOSS	2 Vaccines were concluded to have a good immune response
2024	Morgan *et al.*	GenBank	NetCTL 1.2, IEDB, ABCPred	PEP-fold 4, HADDOCK, Hawk dock	ZDOCK	C-IMMSIM	JCat	The vaccine candidates 2 and 3 were found to be more immunogenic than vaccine construct 1 due to the presence of more NS1 epitopes which are more immunogenic.
2024	Ullah *et al.*	NCBI, VIPR	Bepipred, NetMHC	AMBER18, Hawk dock	-	C-IMMSIM	JCat	All 3 candidate vaccines were immunogenic without eliciting adverse reactions
2024	Tariq *et al.*	UniProt	IEDB	ClusPro	-	-	-	Four different stable vaccine models were concluded

Fig. 2 A table highlighting the servers and databases used by different researchers for designing in-silico multi-epitope DENV vaccine and their main findings from 2017 onwards

DISCUSSION

The high prevalence of dengue virus infections globally poses substantial health-related. Vaccination is the primary preventive measure to lower the disease's burden, as there is currently no specific treatment for dengue fever. Therefore, the goal of this review was to perform a literature review to understand the significance of multi-epitope-based vaccines against all the DENV serotypes, and the process of designing various epitope-based vaccines was also further evaluated. In the first steps, databases like NCBI, UniProt, and IEDB are indispensable for sequence retrieval and conservancy analysis. Ullah *et al.* (2024) used NCBI and PubMed for comprehensive sequence data retrieval and further used MUSCLE for primary alignment and CLUSTALW v2.0 for further verification. Pinheiro *et al.* (2021) used IEDB for conservancy analysis aiming to find conserved B-cell and T-cell epitopes. IEDB also was used by Ullah *et al.* (2024), Tariq *et al.* (2024), and Ali *et al.* (2017) for the same purpose. B-cell and T-cell epitopes are to be predicted in software such as NetMHCIIpan, a T-cell epitope prediction tool that supports a wide variety of MHC alleles, covering a significant spectrum of worldwide population. Ali *et al.* (2017) used BCPREDS while Rahman & Rahman (2024) used BepiPred 2.0 for B-cell epitope prediction, which is critical in locating linear B-cell epitopes. Saha *et al.* (2024)` employed NetMHC and BepiPred for predicting epitopes with strong immunogenicity and binding affinity to demonstrate the accuracy of the predicted epitopes to robust immune responses. Yang *et al.* (2024) used HADDOCK for molecular docking experiments to analyze the precision of the predicted protein structures. As mentioned previously, MD simulations are critical for evaluating the atomic-level stability and interactions of vaccine components. Dharani *et al.* (2023) evaluated the stability of multi-epitope vaccine designs using MD simulations by C-IMMSIM server while Ferdousy *et al.* (2024) used GROMACS and CHARMM. Lastly, Rahman and Rahman (2024) further analyzed the expression optimization and cloning using EMBOSS.

CONCLUSION

The review study exhaustively analyzes the present status and future perspectives of computational strategies in the design of multi-epitope vaccines against DENV. One intriguing approach to overcoming the difficulties caused by viral diversity and the possibility of

antibody-dependent enhancement (ADE) is the creation of multi-epitope vaccines for the dengue virus (DENV). These vaccines may lessen the severity of the disease, increase cross-serotype immunity, and increase global population coverage by targeting several epitopes across different DENV serotypes. Furthermore, eliciting a complete immune response that can effectively destroy the virus and regulate its reproduction depends on the dual targeting of B-cell and T-cell epitopes. This paper emphasizes the changing power of immunoinformatics and computational vaccinology to lend a solution to the global burden of Dengue.

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