B-Cell Epitope Prediction of Dengue Virus NS1 Protein Using Bioinformatics Tools

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Abstract. Reinfection with different DENV serotypes might develop cross immunity that aggravates the illness via antibody-dependent enhancement. Identifying the DENV epitopes can help in the development of a tetravalent vaccine. However, the available prediction technologies (i.e., X-crystallography and NMR) are prohibitively expensive and time-consuming compared to the *in silico*-based methods. Hence, the main purpose of this study is to predict the linear B-cell epitopes (BCEs) of DENV NS1 protein using multiple prediction tools such as BepiPred 2.0, SVMTrip, and BCPred. BepiPred 2.0 is operated based on the crystal structures which uses a Random Forest algorithm trained on epitopes and non-epitope amino acids. SVMTrip applied the SVM model to improve prediction performance by combining tri-peptide similarity and propensity scores. BCPred predicts fixed length of linear BCEs using SVM models with the subsequence kernel. Forty-eight sequences from each serotype, representing four countries in Southeast Asia, were aligned using ClustalW. Further analysis using conservancy tools of the predicted epitopes were then analyzed using WebLogo 3.0 and Epitope conservancy Analysis tool. Finally, the predicted epitopes were evaluated by comparing the epitopes among the prediction tools and the data of the immunogenic epitopes from other studies. There were 24 linear BCEs on the NS1 protein obtained whereby most of them have nearly identical regions predicted as epitopes by three different prediction tools, indicating high agreement across all the prediction tools. EP12 (^{182}RLMSAAIKDSKAVHADMGYW^{201}) and EP15 (^{217}FIEVKTCIWPKSHTLWSNGV^{236}) were predicted showing more than 85% conservancy across all four DENV serotypes, indicating their potential as a vaccine target or biomarker for the detection of four DENV groups. The epitopes also found overlapping or partially constituting immunogenic regions on the NS1 protein which had previously been identified by reference [1]. NS1 protein epitopes with therapeutic potential can be determined *in silico* due to its simplicity, cost-effectiveness, and speed.

Keywords: B-cell epitopes, bioinformatics, dengue, NS1 protein, biomarkers.

S. Gandaseca et al. (eds.), *Proceedings of the International Conference on Science, Technology and Social Sciences – Biology Track (ICONSTAS-BIO 2023)*, Advances in Biological Sciences Research 43,
https://doi.org/10.2991/978-94-6463-536-2_18

1 Introduction

The B-cell epitope (BCE) is a portion of antigen that can be identified by the B-cell lymphocyte and elicit adaptive humoral immune response [2]. The paratope region of the B-cell receptor (BCR) can recognize epitopes either in linear or conformational structure [3,4]. Binding of the epitope to the paratope region of the B-cell receptor induces differentiation of the B-cell into effector plasma cells and memory B cells [2,3]. Effector plasma cells operate as a factory for antibody production. Each plasma cell descended from a unique B cell that manufactures and distributes millions of identical antibody molecules into the blood circulatory system. Neutralizing antibodies can combat the pathogen by attaching to the invader, stopping it from entering the cell or replicating once inside. The memory B-cell contributes to the B-cell response against similar infections in the future [5].

Dengue virus (DENV) is a member of the Flavivirus genus. Dengue virus serotypes 1-4 are all transmissible by the bite of female Aedes mosquitos and are the causal agent of Dengue disease. Dengue viruses can cause conventional Dengue fever (DF), Dengue shock syndrome (DSS), and Dengue hemorrhagic fever (DHF) [6]. Dengue virus non-structural protein 1 (NS1) is a 352 amino acid glycoprotein [7] that can be present in three forms in the host cell: monomer, dimer, or hexamer [1,8,9]. The NS1 protein is required for viral replication and has been demonstrated to have immunological significance. Two proteins, NS2A [6,10-12] and NS2B [10,13], are both encoded by the NS2 genes and are involved in viral production and virions assembly.

BepiPred 2.0, SVMTrip, and BCPred are the bioinformatic tools that can be used to predict the linear BCES of Dengue virus NS1 protein [14,15]. BepiPred 2.0 is operated based on the crystal structures which uses a Random Forest algorithm trained on epitopes and non-epitope amino acids [14]. SVMTrip applied the SVM model to improve prediction performance by combining tripeptide similarity and propensity scores [1,14,16]. BCPred predicts fixed length of linear BCES using SVM models with the subsequence kernel [14,17].

Reinfection with different DENV serotypes might develop cross immunity that aggravates the illness via antibody-dependent enhancement (ADE) [18]. Numerous studies have been undertaken and are continuously being conducted to create an effective dengue vaccine and treatment medications. Nonetheless, there is no antiviral medicine or vaccine available that is really effective against all the DENV serotypes [6]. For example, the currently available tetravalent vaccination called *Dengvaxia* is only 80% effective and is only efficacious in prior patients with laboratory-confirmed Dengue disease. Predicting the virus's epitope can be a first step in developing a tetravalent vaccine. However, the available prediction technologies (X-ray crystallography and nuclear magnetic resonance (NMR)) are prohibitively expensive and time consuming in comparison to the computational approach [15,19]. This demonstrates why the computational technique is the most often used method for *in silico* epitope prediction.

The identification of conserved epitopes or biomarkers aids in the development of tetravalent subunit vaccines that are broadly effective against all Dengue serotypes and strains. Applying a computational approach in predicting the epitope is helpful for a

range of applications of epitope mapping, including the discovery of immunological processes, the creation of peptide-based vaccines, the detection of antibody features in various illnesses, and the prediction of epitopes used throughout disease diagnostics [15,20-22]. In comparison to the traditional epitope identification techniques, this *in-silico* based method is significantly cheaper, faster, and more flexible, hence can be a preferred technique in the future for developing peptide vaccines against Dengue viruses. Overall, this study is performed to predict the linear B-cell epitope of DENV NS1 protein using BepiPred 2.0, BCPred, and SVMTrip server, to analyze the conserved epitopes using the Epitope Conservancy Analysis Tool, and to visualize the conserved epitope sequences using WebLogo 3.0 tool.

2 Experimental

2.1 Computer Program and Software

Sample Dengue virus NS1 protein were obtained from the GenBank at (https://www.ncbi.nlm.nih.gov/genbank/). Prediction tools such as BepiPred 2.0 were obtained at http://tools.iedb.org/bcell/), BCPred at http://ailab projects1.ist.psu.edu:8080/bcpred/predict.html), SVMTrip at (http://sysbio.unl.edu/SVMTrip/prediction.php). In addition, Epitope Conservancy Analysis tool was obtained at (http://tools.iedb.org/conservancy/) and sequence alignment tool was downloaded using ClustalWtool at (https://www.genome.jp/tools-bin/clustalw), and WebLogo 3.0 tool at (http://weblogo.threeplusone.com/).

2.2 NS1 Protein Sequence Retrieval from GenBank.

To identify the linear B-cell epitopes of DENV NS1 protein, the sample sequence of dengue virus NS1 protein was first obtained from their polyprotein. The total of 48 samples were successfully selected and retrieved from the National Center for Bioinformatics (NCBI) GenBank at (https://www.ncbi.nlm.nih.gov/genbank/). The samples were isolated from human hosts originating from Malaysia, Singapore, Indonesia, and Thailand. The FASTA and plain format of these NS1 protein sequences were saved into word files. Table 1 below shows the accession number of each Dengue viruses serotype protein obtained from the GenBank:

Table 1. The accession no. of 48 NS1 Protein samples collected from four countries

	Accession No. of NS1 Protein Sequence			
	D1	D2	D3	D4
Malaysia	ARO84722.1	AOE47565.1	AVY51409.1	QBA29684.1
	AOE47566.1	AYH52607.1	QBR34687.1	QBQ58394.1
	ARO84719.1	ARO84686.1	QBQ58392.1	BAR97413.1
Singapore	ANS59155.1	AES93116.1	ADC92353.1	AGE13482.1
	ANS59161.1	ANS59182.1	ADC92354.1	ANN30220.2
	ANS59163.1	ANS59185.1	ANS59200.1	AKL81368.2
Indonesia	QBB90021.1	QBB90022.1	AHG06365.1	QBB90024.1
	AHG06333.1	AHG06353.1	AHG06366.1	AHG06379.1
	AHG06309.1	AHG06346.1	AHG06369.1	AHG06383.1
Thailand	AHI88623.1	QBA57667.1	ANC57610.1	ALL54587.1
	ANC57576.1	ANC57598.1	ANC57606.1	AHG23284.1
	ANC57583.1	ANC57599.1	ALL54587.1	AHG23298.1

2.3 Prediction of B-cell Linear Epitopes of DENV NS1 Protein using various prediction tools.

The NS1 protein sequence of DENV 1 (Accession No. ARO84722.1) was analyzed by using three linear B-cell epitope prediction tools. All these prediction tools can be used either plain or FASTA format as their input data. The prediction tools were run by using their default setting.

The first prediction tool, the BepiPred 2.0 (http://tools.iedb.org/bcell/) uses a Random Forest algorithm trained on epitopes and non-epitope amino acids determined from crystal structures. In the data set, all peptides with lengths less than 5 and greater than 25 residues were removed from the data set, as epitopes are rarely found outside of this range [14]. After protein input data was pasted, the threshold value was set to 0.5 whereby residues with scores above the threshold are predicted to be part of an epitope [23].

The second prediction tool, the SVMTrip (http://sysbio.unl.edu/SVMTrip/prediction.php) predicts epitopes using a representative sequence of a particular protein. SVMTrip applied SVM model to improve prediction performance by combining tri-peptide similarity and propensity scores. The epitope length was set to 20 amino acids.

The third prediction tool, the BCPred (http://ailabprojects1.ist.psu.edu:8080/bcpred/predict.html), predicts the linear BCES using SVM models with the subsequence kernel. This prediction tool was set as default,

with 75% sensitivity, 20 in epitope length and 'only report non-overlapping region' setting was applied.

2.4 Epitope Conservancy Analysis Tool

Determining the conserved epitope is a must due to its advantages in inducing immunogenicity simultaneously against all four DENV strains [1]. Epitope Conservancy analysis tool can be accessed from (http://tools.iedb.org/conservancy/). This program can compute the degree of epitope conservation at a given identity level within a given protein sequence set. In order to analyze the conserve epitope, there were several steps involved. Firstly, the FASTA format of the predicted linear epitope sequences were entered into the webpage, followed by all 48 protein sequences collected. Then, the calculation was set into '100' as its sequence identity threshold, 'Epitope linear sequence conservancy' as its analysis type and ticked 'Remove duplicate protein sequence' box. The minimum conservation percentages were recorded [1].

2.5 Predicted Epitope Alignment

The online server for this task can be accessed through this link: https://www.genome.jp/tools-bin/clustalw. ClustalW can run multiple alignment of more than two protein sequences at one time. This type of sequence alignment can give a quick but useful overview of the sequence data. A set of sequences derived from different but related organisms can be linked together using multiple sequence alignment. Multiple sequence alignment will be used to align the protein sequences to reveal the conserved motif or residues in the output. To perform the multiple sequence alignment, the FASTA format of all 48 NS1 protein sequences was needed as the input. After keying in the protein FASTA format, the "Align/Assemble" button found on the toolbar was clicked, before "Multiple Alignment" was chosen. The result was kept in a word file.

2.6 Visualization of Sequence Conversion Using WebLogo 3.0

According to reference [24], WebLogo software can be used to generate sequence logos and then represent patterns in a multiple sequence alignment dataset. Sequence logos can reveal important features of an alignment by describing the precise description of sequence similarity. Each logo will be made up of letter stacks, one for each position in the sequence. Each stack's overall height is the height in the stack that corresponds to the relative frequency of the corresponding amino or nucleic acid at that position. WebLogo has been updated to include new features and functions, making it a highly configurable sequence logo generator. The WebLogo website can be found at (http://weblogo.threeplusone.com/). To visualize the conserved epitope or residue in the protein sequences, the multiple sequence alignment was copied from the word file onto the WebLogo 3.0 webpage. The generated result was saved in a PNG file.

3 Results and Discussion

3.1 Predicted Epitopes

The three utilized prediction tools identified 24 linear BCEs located on the NS1 protein. The epitopes have size ranging in 6 to 25 amino acids as any epitopes with amino acid lengths less than 5 and greater than 25 were not considered as epitopes and were eliminated [14]. These epitopes have high probability scores in each prediction tool. Table 2 shows the list of predicted epitopes using the three prediction tools namely the BepiPred, SVMTrip and BCPred. The BepiPred 2.0 predicted seven of the 24 epitopes, the SVMTrip predicted nine, and the BCPred predicted eight. Essentially, the same epitope positions were identified across the four serotypes, with just several amino acids different in length.

The majority of epitopes predicted by these three tools were found to contain overlapping residues, at least in partial. For example, overlapping epitopes predicted by both BCPred and SVMTrip are: (EP5 and EP6); (EP9 and EP10); (EP12, and EP13) and (EP14 and EP15). EP8 by BepiPred 2.0 has also been discovered to coincide with EP9 by SVMTrip in partial, meanwhile EP18 predicted by BCPred has overlaps with EP19 predicted by BepiPred 2.0. Overall, the epitopes that overlap by all three prediction tools are (EP15,16,17), and (EP22,23,24). The results reveal that the three prediction methods agree well, with nearly the same areas indicated as epitopes by more than one prediction tool [1].

Table 2. The predicted linear B-cell epitopes of DENV NS1 protein using three distinct prediction tools

Epitope ID	Predicted epitope sequence	Size	Prediction tools/ score	Conservation %				
				All 4D	D1	D2	D3	D4
EP1	^{7}NWKGRE^{12}	6	BepiPred 2.0	50	100	50	83.33	50
EP2	^{26}HTWTEQYKFQADSPK ^{40}	15	BepiPred 2.0	80	100	80	100	80
EP3	^{48}KAWEEGVCGIRSATRLENIM^{67}	20	SVMTrip/0.346	65	100	80	65	70
EP4	^{83}DMKFTVVVGDVAGILAQGKK^{102}	20	SVMTrip/0.229	40	85	40	60	45
EP5	^{106}PQPMEHKYSWKSWGKAKIIG^{125}	20	BCPred/1	60	95	70	75	60
EP6	^{115}WKSWGKAKIIGADVQNTTFI^{134}	20	SVMTrip/0.342	50	90	50	60	55
EP7	^{130}NTTFIIDGPNTPECPDDQRA^{149}	20	BCPred/0.997	60	90	65	75	60
EP8	^{140}TPECPDDQRAW^{150}	11	BepiPred 2.0	63.64	90.91	63.64	72.73	63.64
EP9	^{146}DQRAWNIWEVEDYGFGIFTT^{165}	20	SVMTrip/0.297	70	95	75	80	70
EP10	^{151}NIWEVEDYGFGIFTTNIWLK^{170}	20	BCPred/0.89	75	95	85	90	75
EP11	173DSYTQVC179	7	BepiPred 2.0	28.57	85.71	28.57	42.86	42.86
EP12	^{182}RLMSAAIKDSKAVHADMGYW^{201}	20	SVMTrip/0.749	85	100	85	85	90
EP13	^{194}VHADMGYWIESEKNETWKLA^{213}	20	BCPred/0.872	65	100	75	80	65
EP14	^{208}ETWKLARASFIEVKTCIWPK^{227}	20	SVMTrip/0.223	60	95	70	70	60
EP15	^{217}FIEVKTCIWPKSHTLWSNGV^{236}	20	BCPred/0.752	85	95	90	90	85
EP16	^{228}SHTLWSNGVLESEMII^{243}	16	BepiPred 2.0	81.25	93.75	100	93.75	81.25
EP17	^{230}TLWSNGVLESEMIIPKIYGG^{249}	20	SVMTrip/0.243	75	95	85	80	75
EP18	^{242}IIPKIYGGPISQHNYRPGYF^{261}	20	BCPred/0.965	65	95	75	75	65
EP19	^{248}GGPISQHNYRPGYFTQTAGPWHLGK^{272}	25	BepiPred 2.0	80	100	84	88	80

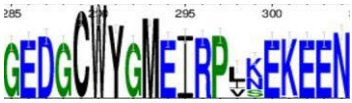
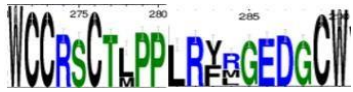
EP20	[262]TQTAGPWHLGKLELDFDLCE[281]	20	SVMTrip/0.599	75	95	80	90	75
EP21	[289]EHCGNRGPSLRTTTVTGKII[308]	20	BCPred/0.961	65	100	75	80	65
EP22	[325]GEDGCWYGMEIRPVKEKEEN[344]	20	BCPred/0.993	85	100	95	85	90
EP23	[311]WCCRSCTLPPLRYRGEDGCW[330]	20	SVMTrip/1.000	85	95	100	95	85
EP24	[338]VKEKEENLVKS[348]	11	BepiPred 2.0	63.64	90.91	81.82	63.64	63.64

Notes: The list of epitope sequences correlate to the D1 NS1 protein (ARO84722.1). The epitope conservation analysis score ranged from a minimal to a maximal percentage. The percentages stated are the minimum conservations. DENV serotypes: D1 (DENV1), D2 (DENV2), D3 (DENV3), and D4 (DENV4).

3.2 Conservation Analysis of the Predicted Epitopes

The conservation analysis of each predicted epitope within each of the four serotypes, as well as between the four serotypes, is shown in Table 2 above. Only seven predicted epitopes were found to be substantially conserved (over 80%) within a particular serotype using all prediction tools. Meanwhile the minimal percentage of epitope conservation between the four serotypes ranged from 28.57% to 85%. Except for EP4 and EP11, all other epitopes exhibited greater than 50% conservation across the four serotypes. Eleven epitopes were more than 70% conserved amongst the four serotypes, with four of them, EP12, EP15, EP22, and EP23, being 85% conserved. Table 3 below shows the most conserved epitopes sequences that were visualized by WebLogo 3.0 tool. The predicted epitopes were displayed as stacked sequences.

Table 2. The visualization of the most conserved epitopes sequences by WebLogo 3.0.

Epitope ID	Epitope sequence	Visualization
EP12	^{182}RLMSAAIKDSKAVHADMGYW^{201}	
EP15	^{217}FIEVKTCIWPKSHTLWSNGV^{236}	
EP22	^{325}GEDGCWYGMEIRPVKEKEEN^{344}	
EP23	^{311}WCCRSCTLPPLRYRGEDGCW^{330}	

Notes: The higher the letter stacks, the higher the frequency of the corresponding amino acid at that position.

3.3 Comparison of the Predicted Epitopes with Previous Studies

All the predicted epitopes were compared with the previously published epitopes as referred to in research study by reference [1]. Numerous biochemical experiments have demonstrated that the epitopes against DENV1 NS1 protein can generate humoral immunogenic responses. Table 4 below illustrates the predicted epitopes that coincide between the three prediction tools and previously reported antigenic areas in prior studies. Several of the predicted epitopes were found to be overlapping with or partially comprising regions that were previously discovered capable of being recognized by mouse antibodies or natural mAbs produced against the NS1 protein of different DENV

serotypes. Therefore, it is plausible to conclude that at least parts of the predicted epitopes are immunogenic in nature [1].

Table 3. The conserved epitopes overlapped within different tools and by previous studies.

BepiPred 2.0	SVMTrip	BCPred	Previous studies
	EP6 (115-134)	EP5 (106-125)	90-109[(i,iii)], 111-130[(i)], 112-123[(ii)]
EP8 (140-150)	EP9 (146-165)	EP10 (151-170)	133-152[(i)]
	EP12 (182-201)	EP13 (194-213)	154-161[(iv)]
	EP14 (208-227)	**EP15 (217-236)**	187-206[(i,iii)]
EP16 (228-243)	EP17 (230-249)		193-204[(ii)]
EP19 (248-272)		EP18 (242-261)	230-239[(v)]
EP24 (338-348)	**EP23 (311-350)**	EP21 (289-308)	266-274[(ii)]
		EP22 (325-344)	294-302[(ii)]

i: Saxena et al. [25]; ii: Chen et al. [26]; iii: Jiang et al. [27]; iv: Masrinoul et al. [28]; v: Pushpakumara et al. [1]

Based on the result, EP12 (^{182}RLMSAAIKDSKAVHADMGYW^{201}) has overlapped with the epitope identified in the study by in reference [25,27] which is located on position 187-206 amino acids. In addition, EP15 (^{217}FIEVKTCIWPKSHTLWSNGV^{236}) has partially overlaps with the epitope studies by reference [1] which encompass an amino acid area spanning 230-239 amino acids. Therefore, as the immunodominance of overlapping epitopes were proven by various biochemical tests, EP12 (^{182}RLMSAAIKDSKAVHADMGYW^{201}) and EP15 (^{217}FIEVKTCIWPKSHTLWSNGV^{236}) can be the promising therapeutic candidates against four DENV serotypes.

Meanwhile, according to the studies from reference [1,26], although EP22 (325-344 amino acids) and EP23 (311-330 amino acids) also has high conservancy level (85%), these epitopes should be ruled out as vaccine candidates for all DENV serotypes as they were located in the carboxy terminal region of the DENV NS1 protein. Reference [1] discovered that the carboxy terminal region of the DENV NS1 protein (271-352 amino acids) appears to be more sequence conserved with the other two flavivirus groups: Japanese Encephalitic Virus (JEV) and West Nile Virus (WNV) (data not shown), showing that the epitopes on this region cannot be used to construct vaccine specific to DENV. Additionally, it has been reported that antibodies against the epitopes in these regions can cross-react with vascular epithelium, which may contribute to vascular leakage associated with severe dengue infection [1,26].

4 Conclusion

The strategy of using bioinformatics tools in the epitope prediction is basically a promising approach as it is more cost-effective and timesaving. The linear BCEs predicted for the DENV NS1 protein by three prediction tools, BepiPred 2.0, SVMTrip, and BCPred, are consistent with each other as well as with the immunodominant epitopes found in previous studies. The predicted epitopes, EP12 and EP15, were

specific for the Dengue group and showed more than 85% conservancy among the four DENV serotypes, indicating its potential to serve as the vaccine targets against four serotypes of DENV. The conserved epitope sequences (EP12, EP15, EP22 and EP23) have also been successfully visualized using WebLogo 3.0 which provide detailed description of the sequence similarity within a multiple sequence alignment.

These predicted epitopes need to be validated further in the laboratory before being applied in other applications. Additionally, simply predicting the linear BCEs is insufficient to gain a DENV vaccine candidate, as it is unlikely to generate the immune response required for effective immunization by itself. Therefore, a more holistic approach is required whereby other additional properties such as the existence of T-cell epitopes, conformational BCEs, and antigenicity of anticipated peptides are being investigated.

Besides, this study can also be improved by increasing the number of samples by covering samples from another region other than Southeast Asia such as Brazil and other countries in which Dengue is endemic. Covering DENV samples from a wide geographical area could provide insight into how a future study could be constructed to determine the dominance of different serotypes in each region over time, hence can be indicators of future DENV serotype outbreaks in different regions.

Acknowledgement. The author would like to acknowledge the support of the Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia for providing the facilities and financial support for this research.

Conflict of Interest Statement. The author agrees that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declares absence of conflicting interests with the funders.

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