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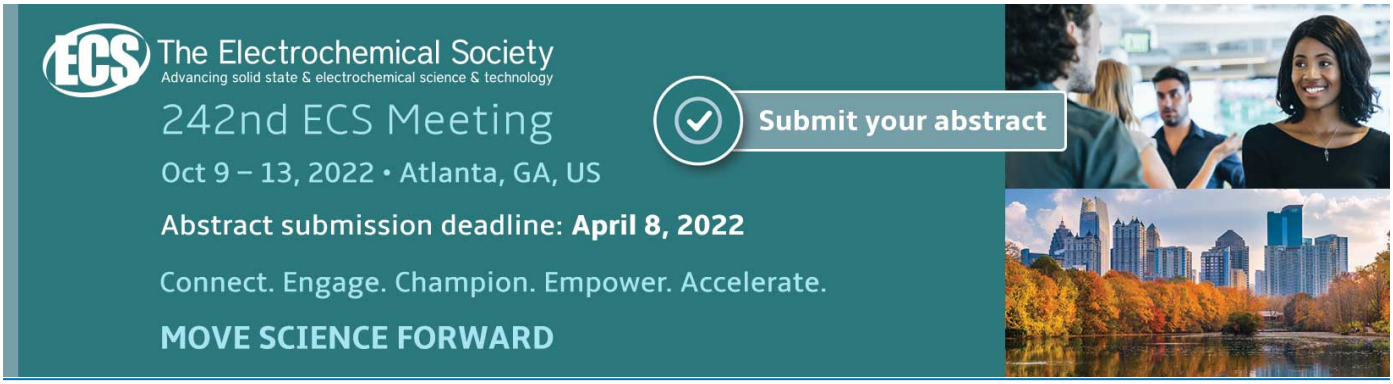

Computational antigenic epitope prediction of clinical Indonesian Dengue virus NS1 protein

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Abstract. The identification of human Non-Structural-1 (NS1) protein epitopes will help us better understand Dengue virus (DENV) immunopathogenesis. In this study, several online and offline bioinformatic prediction tools were exploited to predict and analyze T-cell and B-cell epitopes of DENV NS1 consensus sequences originated from Indonesian clinical isolates. We identified a potential peptide at $NS1_{155--163}$ (VEDYGFGIF) which interact with MHC-I allele HLA-B*40:01 and showed high binding affinity (IC_{50}) scores ranging between 63.8 nM to 183.9 nM for all Indonesian DENV serotypes. Furthermore, we have succeeded identified a region at the C-terminal of Indonesian DENV NS1 protein between 325--344 as part of discontinuous antigenic epitope which conserved for all serotypes. Our analyses showed this region could induce strong and persistent antibody against all DENV serotypes by interacting with MHC-I molecule and also recognized by B-cell receptor. The identification of DENV NS1 T-cell and B-cell epitopes may help in the development of a new vaccine, drug discovery, and diagnostic system to help eradicate dengue infection.
Keywords: dengue; NS1 protein; epitope; computational; vaccine

1. Introduction

Dengue virus (DENV) symptoms range from asymptomatic to severe dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) [1]. The DENV exist in four types, commonly known as serotype 1, 2, 3, and 4 which closely related, but antigenically distinct. The infection by one serotype does not contribute cross-protective immunity against the others due to antigenic differences [2]. DENV has a genome length of 11 kilobases and a single open reading frame that produces a 3,400-amino-acid-long polyprotein precursor. The genome consists of three structural proteins: Capsid, pre-Membran, Envelope, and seven nonstructural proteins: Non-Structural-1 (NS1), NS2A/2B, NS3, NS4A/4B, NS5 [3].

DENV NS1 is a 43-48 kDa glycoprotein that dimerizes in the endoplasmic reticulum lumen of infected cells. The NS1 protein is secreted and circulated in the bloodstream of an infected person [4]. Several studies describe attempts to use the NS1 protein as a target for prevention, treatment, and diagnosis [5-7]. In the context of dengue vaccine development, the NS1 protein epitopes are represented by MHC-I and MHC-II molecules, and which are also promising targets for T-cells immune response

[8]. Furthermore, subunit vaccine based on the NS1 protein have been investigated under non clinical conditions [9].

Epitopes are antigen sites that bind specifically to a lymphocyte receptor or antibodies, eliciting either cellular or humoral immune response. The precise position of an epitope is crucial in many biomedical applications such as vaccine development, diagnosis and disease treatment [10, 11]. Ideally, during vaccine development, we should select an epitope that is conserved over the pathogen's stages and covers a large population. In this regard, the epitope must also bind to more than one major histocompatibility complex (MHC) allele in order to improve the vaccine candidate's protective activity. [12-14]. In the last decades, vaccine development exclusively depends on biochemical and immunological experiment which are very expensive, time-consuming, and laborious. With the aid of in silico study, many researchers are now able to predict the epitope of the proteins of interest and in the same time reducing wet-lab experiments [11].

DENV NS1 is essential to the development of new and safe methods for controlling the disease, and may help us to better understand DENV biology [9]. The consensus sequence of DENV NS1 protein from Indonesian isolates was used in this study to predict the best T-cell and B-cell antigenic epitopes. The prediction of DENV NS1 epitopes from Indonesian isolates is a critical step toward developing a new subunit vaccine and a more accurate diagnosis system to protect the Indonesian population from dengue infection.

2. Materials and methods

2.1. Materials retrieval of DENV NS1 protein sequences

All the available nucleotide sequences of NS1 protein from DENV 1 to 4 Indonesian isolates were retrieved from National Center for Biotechnology Information (NCBI). The nucleotide sequences were translated into amino acid and selected via multiple sequence Alignment in ViPR website (http://www.viprbrc.org/brc/msa.spg?method=ShowCleanInputPage &decorator =flavi_dengue) to generate consensus NS1 sequence for each DENV serotype.

2.2. Antigenecity prediction

We used the online server VaxiJen 2.0 to determine the potent antigenic peptide from Indonesian DENV NS1 protein. A prediction accuracy of between 70% and 89% is achieved by using the physicochemical properties of the input protein as input. We set the threshold value analysis at 0.7 and evaluated the best antigenicity scores of every NS1 conserved sequences for each DENV serotype.

2.3. CD8+ and CD4+ T-cell epitope identification

NetCTL 1.2 was used to identified the T-cell epitope of the Indonesian DENV NS1 protein [15] with a 0.95 threshold to provide sensitivity and specificity value of around 90% and 95%, respectively. The best prediction scores were calculated by combining the score of proteasomal processing, TAP transport, and MHC binding and translated the value into sensitivity/specificity [16]. For each DENV serotype, the optimal NS1 epitope location was chosen for future analysis. The Immune Epitope Database (IEDB) website has an MHC-NP prediction tool that can determine the likelihood that a particular peptide was naturally processed and bound to a certain MHC molecule [17]. For the prediction of NS1 peptides binding to specific MHC-I molecules, we used MHC-I Binding Predictions tools from the IEDB and calculated the IC_{50} [18]. Peptides with a length of nine residues and a binding affinity < 200 nM were selected for further investigation. Most antibody responses against the pathogen are dependent on $CD4^+$ T-cell. We used NetMHCII 2.2 server to predict the possible T-cell epitopes and selected the NS1 peptide that specific to MHC class II molecule with binding affinity < 50 nM [19].

2.4. Threading modeling

The consensus Indonesian DENV NS1 protein 3D models were predicted using I-TASSER server after translating the nucleotide sequence into amino acids. The I-TASSER server could be access at

http://zhang.bioinformatics.ku.edu/I-TASSER [20]. To build reliable models, the I-TASSER uses threading, ab initio modeling, and structural refinement [21]. The results from threading modeling were saved in PDB format and used for further analysis to predict the discontinuous B-cell epitope.

2.5. B-cell continuous and discontinues epitope prediction

The NS1 B-cell continuous epitopes were predicted using BCPred method from BCPREDS web server, which can be accessed at http://ailab.cs.iastate.edu/bcpreds/ [22]. This approach utilizes a subsequence kernel-based SVM classifier that was trained on a dataset of linear B-cell epitopes that had been homology-reduced. BCPred outperforms AAP (AUC 0.7), EPMRL (AUC 0.728), and SVMTriP (AUC 0.702) with an AUC of 0.758 [23]. We used the ElliPro online tool to determine the B-cell discontinuous epitopes of the Indonesian DENV NS1 protein. The 3D structures of NS1 proteins in PDB format from threading modeling of I-TASSER server were used for the analysis. ElliPro outperforms structure-based prediction tools such as DiscoTope and CEP with AUC value of 0.732 [23]. To enhance the prediction's validity, we adjusted the cutoff value to 0.7 and the maximum distance to 6 Å.

3. Results

3.1. Sequence analysis of Indonesian DENV NS1 protein

In this study, a total of 151 sequences of Indonesian DENV NS1 protein from 1975 to 2015 were retrieved from NCBI. The consensus sequences of Indonesian DENV NS1 protein were generated by using Unipro UGENE v1.1.8.0 software. A conserved sequence was determined on a peptide with five or more amino acids which is homolog in the same position along with the NS1 protein sequences for all Indonesia DENV serotypes. We found 16 regions of conserved sequences with a range between 5--13 amino acids as shown in figure 1.

Figure 1. Position of predicted CD8⁺ and CD4⁺ epitope on consensus NS1 amino acid sequences. The conserved sequence analysis was done by using BioEdit ver 7.2.5. The fully conserved regions amongst DENV are highlighted in yellow box. The rectangles in two color represent the anticipated T-cell epitope regions: red for CD8⁺ and green for CD4⁺.

3.2. CD8+ and CD4+ T-cell epitope prediction

Based on the NetCTL server, we found 7 positions out of 16 conserved regions for all Indonesian DENV serotypes that showed the best score for Cytotoxic T Lymphocytes (CTL) epitopes. We determined the total score of the epitopes by analyzing the binding affinity to MHC-I alleles, proteosomal cleavage and TAP transport. To mimic the natural process of immunogenicity and antigenicity of an epitope, all epitopes were evaluated using MHC-NP and pMHC-I tools. By combining these analyses, we found the most potent peptide eliciting the immune response is $NS1_{155--163}$ (table 1).

Table 1. Potential CD8+ T-cell epitopes and interacting MHC class I alleles, as well as overall processing score, MHC-NP, pMHC-I immunogenecity, and antigenicity score.

Position	Epitopes	Serotype	MHC-NP score	MHC class I interaction with an affinity of IC50 < 200 nM	pMHC-I immunogenecity score	Antigenicity
11--19	RELKCGSGI	DENV 1	HLA-B*53:01; 0.0397	HLA-B*40:02; 13.6 (0.12)	-0.36102	antigen (1.091)
	KELKCGSGI	DENV 2	HLA-B*44:03; 0.0575	HLA-B*40:02; 21.3 (-0.09)	-0.36102	antigen (1.091)
		DENV 3		HLA-B*40:01; 79.4 (-0.66)		
		DENV 4		HLA-B*49:01; 132.7 (-0.88)		
26--34	HTWTEQYKF	DENV 1	HLA-B*57:01;0.4415	HLA-B*58:01; 28.7 (1.09)	-0.0297	non-antigen (0.175)
		DENV 2		HLA-A*32:01; 46.7 (0.88)		
		DENV 3		HLA-B*57:01; 73.5 (0.69)		
		DENV 4		HLA-A*23:01; 199.1 (0.25)		
155--163	VEDYGFGIF	DENV 1	HLA-B*53:01; 0.6366	HLA-B*40:01; 96 (0.32)	0.25304	antigen (1.619)
	VEDYGFGVF	DENV 2	HLA-B*53:01; 0.6283	HLA-B*40:01; 63.8 (0.60)	0.1994	antigen (1.586)
		DENV 3		HLA-B*18:01; 88.7 (0.46)		
	VEDYGFGMF	DENV 4	HLA-B*53:01; 0.7131	HLA-B*40:01; 183.9 (0.04)	0.07268	antigen (1.638)
192--200	KAVHADMGY	DENV 1	HLA-B*44:03; 0.6080	HLA-B*15:25; 36.1(1.09)	-0.02347	antigen (1.259)
		DENV 4		HLA-B*15:01; 110.2(0.61)		
	RAVHADMGY	DENV 2	HLA-B*44:03; 0.4650	HLA-B*15:25; 24.7 (1.27)	-0.02347	antigen (1.259)
		DENV 3		HLA-C*03:02; 60.4 (0.88)		

Position	Epitopes	Serotype	MHC-NP score	MHC class I interaction with an affinity of IC50 < 200 nM	pMHC-I immunogenecity score	Antigenicity
229--237	HTLWSNGVL	DENV 1	HLA-B*53:01; 0.1345	HLA-C*03:03; 98 (-0.04)	0.10482	non-antigen (-0.467)
		DENV 2		HLA-C*03:04; 98 (-0.04)		
		DENV 3		HLA-C*03:02; 184.5 (-0.31)		
		DENV 4				
295--303	GPSLRTTTV	DENV 1	HLA-B*07:02; 0.2595	HLA-B*07:02; 40.2 (-0.46)	0.07752	antigen (1.165)
		DENV 3				
	GPSLRTTTA	DENV 2	HLA-B*07:02; 0.1088	HLA-B*07:02; 75.1 (-1.02)	0.07752	antigen (0.9538)
		DENV 4				
325--333	GEDGCWYGM	DENV 1	HLA-B*53:01; 0.3205	HLA-B*40:01; 61.3 (-0.72)	0.21399	non-antigen (0.100)
		DENV 2		HLA-B*40:02; 141.4 (-1.08)		
		DENV 3				
		DENV 4				

The MHC class II binding prediction tool NetMHCII 2.2 server was utilized and predicted 229 epitopes for all Indonesian DENV NS1 protein. Predictions were obtained for 14 HLA-DR alleles, six HLA-DQ and six HLA-DP. After analysis of all possible epitopes, we found only one peptide as a potential $CD4^+$ T-cell epitopes at position $NS1_{330--338}$ and fitted to all serotypes of Indonesian DENV NS1 protein, interacted mostly with HLA-DRB1*01:01 allele (figure 1). The peptide of all DENV serotypes at position $NS1_{330--338}$ showed a strong binding affinity (IC_{50}< 50 nM) to HLA-DRB1*01:01 ranging from 6.60--9.10 nM (table 2) with a high antigenicity score (1.757--2.004) for all dengue serotype.

Comparing this to other studies, we selected the potential peptide that showed higher affinity for MHC-I (IC_{50}<200 nM) and MHC-II (IC_{50}<10 nM), respectively, to increase the validity of our prediction analysis. The immunogenicity of a predicted T-cell epitope was assessed using its IC_{50} value, with a lower IC_{50} score indicating effective suppression when tested in vitro [24, 25].

3.3. Threading modeling of DENV NS1 protein

The 3D structure of the DENV NS1 protein was predicted using the I-TASSER online server and the validity of the models shown as C-score, TM-score, and RSMD. Higher C-scores indicate better models. The standard for a good model is indicated by a C-score ranging from -5 to 2 [20]. The best predicted Indonesian DENV NS1 protein structure with the maximum confidence score (C-Score) were 1.71, 1.38, 0.76, and 1.25 for DENV 1, DENV 2, DENV 3, and DENV 4, respectively (figure 2).

Table 2. $CD4^+$ T-cell epitopes interacting to MHC class II alleles with binding affinity < 50 nM

Position	Epitopes	Serotype	Alleles	Binding Affinity (nM)	Antigenicity
330—338	WYGMEIRPV	DENV 1	HLA-DRB1*01:01	8.20	antigen (2.004)
	WYGMEIRPL	DENV 2	HLA-DRB1*01:01	6.60	antigen (1.757)
	WYGMEIRPL	DENV 4	HLA-DRB1*01:01	6.90	antigen (1.757)
	WYGMEIRPI	DENV 3	HLA-DRB1*01:01	9.10	antigen (1.799)

The average TM-score (RMSD) of the best model for NS1 protein were 0.95+0.05 (3.1+2.2 Å), 0.91+0.06 (3.7+2.5 Å), 0.82+0.09 (4.9+3.2 Å), 0.89+0.07 (4.0+2.7 Å) for DENV 1, DENV 2, DENV 3, and DENV 4, respectively. The TM-score with a value near to 1 indicating a better structural match [26]. The result indicating that predicted models of consensus NS1 protein for all serotype showed high similarity with the structural protein references.

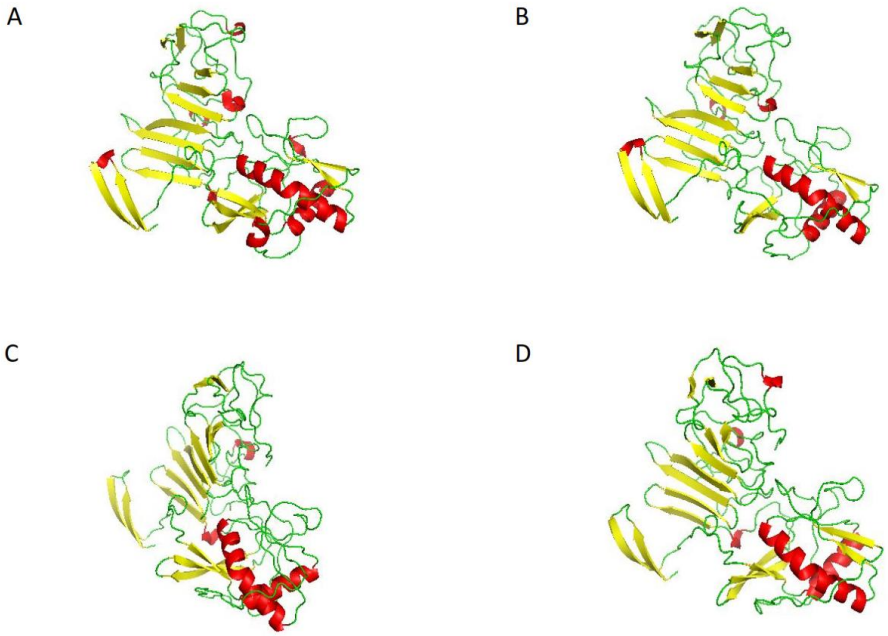

Figure 2. Predicted 3D NS1 Protein Models Built by I-TASSER. (A) DENV 1, (B) DENV 2, (C) DENV 3, and (D) DENV 4.

3.4. B-cell continuous and discontinous epitope prediction

The B-cell continuous epitopes of Indonesian DENV NS1 protein were predicted by using BCPREDS web server. We selected the epitopes with a length of 20-mer, a cut-off value of more than 0.9 and a classifier specificity set at 75%. To increase the validity of the prediction, we employed the VaxiJen2.0 for every predicted peptide to determine the antigenicity of the epitopes. Interestingly, we found only $NS1_{325--344}$, which is conserved for all DENV serotypes with the best score for both predictor servers (table 3).

The prediction of B-cell discontinuous epitopes of Indonesian DENV NS1 protein were carried out by using ElliPro online tool. This prediction tools elaborate the protein antigenicity, solvent accessibility and flexibility on the basis of protein-antibody interactions [27]. Previous study showed that ElliPro was outperformed compare to other epitope structural-based prediction tools with an AUC value of 0.732 [28]. Analysis of discontinuous peptide of Indonesian DENV NS1 protein was set by using the NS1 protein 3D model built by I-TASSER (figure 2).

We selected the promising epitopes with score above 0.7, maximum distance at 6 Å (Angstrom) and fit to the conserve region for all DENV serotypes. We found one region at N-terminal and another at C-terminal of NS1 protein were matched with our criteria for the best discontinuous epitope prediction (figure 3).

Table 3. Predicted continous antigenic epitopes of Indonesian DENV NS1 protein by using BCPrep method

Serotype	Position	Epitope	BCPrep score	Antigenicity
DENV 1				
	106	PQPMEHKYSWKSWGKAKIIG	1.000	non-antigen (0.550)
	130	NTTFIIDGPNTPECPDDQRA	0.997	non-antigen (0.027)
	325	GEDGCWYGMEIRPVKEKEEN	0.993	antigen (0.916)
DENV 2				
	105	RPQPTELKYSWKTWGKAKML	1.000	antigen (0.786)
	135	IDGPETAECPNTNRAWNSLE	0.988	non-antigen (0.340)
	325	GEDGCWYGMEIRPLKEKEEN	0.992	antigen (0.844)
DENV 3				
	104	LTPQPMELKYSWKTWGKAKI	1.000	antigen (0.891)
	128	TQNSSFIIDGPNTPECPNAS	0.995	non-antigen (0.173)
	325	GEDGCWYGMEIRPINEKEEN	0.981	antigen (1.039)
DENV 4				
	104	LTPPVNDLKYSWKTWGKAKI	1.000	antigen (0.708)
	132	TFLIDGPDTSECPNERRAWN	0.985	non-antigen (0.128)
	324	LGEDGCWYGMEIRPLSEKEE	0.943	antigen (0.872)

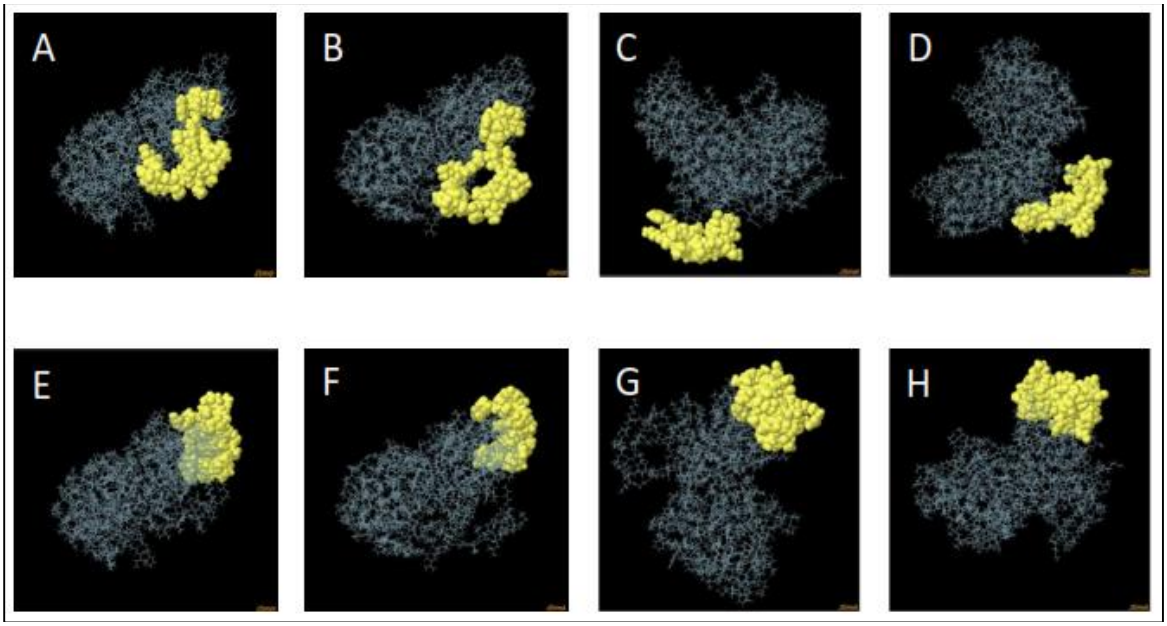

Figure 3. The 3-D representation of discontinuous epitopes of all Indonesian DENV NS1 Protein. Upper panel for discontinuous epitopes at N-terminal; (A) DENV 1, (B) DENV 2, (C) DENV 3, (D) DENV 4. The lower panel for discontinuous epitopes at the C-terminal; (E) DENV 1, (F) DENV 2, (G) DENV 3, (H) DENV 4.

The highest Protrusion Index (PI) scores of discontinuous epitope at N-terminal of NS1 protein were 0.825, 0.807, 0.933 and 0.836 for DENV 1, DENV 2, DENV 3, and DENV 4 respectively. A residue with a higher PI score correspond to larger solvent accessibility. The PI scores at the C-terminal were slightly different compared with the scores at N-terminal (table 4).

Table 4. Predicted discontinous antigenic epitopes of Indonesian DENV NS1 protein

Position	Serotype	Residues	Number of Residues	PI Score
N-terminal	DENV 1	A:D1, A:S2, A:G3, A:C4, A:V5, A:I6, A:N7, A:W8, A:K9, A:G10, A:R11, A:E12, A:L13, A:K14, A:C15, A:G16, A:S17, A:G18, A:I19, A:F20, A:K189, A:D190, A:S191, A:K206, A:N207, A:E208, A:T209, A:W210	28	0.825
	DENV 2	A:D1, A:S2, A:G3, A:C4, A:V5, A:V6, A:S7, A:W8, A:K9, A:N10, A:K11, A:E12, A:L13, A:K14, A:C15, A:G16, A:S17, A:G18, A:I19, A:F20, A:I21, A:K189, A:D190, A:N191, A:L206, A:N207, A:D208, A:T209, A:W210	29	0.807
	DENV 3	A:D1, A:M2, A:G3, A:C4, A:V5, A:I6, A:N7, A:W8, A:G10, A:K11, A:E12, A:L13, A:K14, A:C15, A:G16, A:S17, A:G18, A:I19, A:F20	19	0.933

Position	Serotype	Residues	Number of Residues	PI Score
	DENV 4	A:D1, A:M2, A:G3, A:C4, A:V5, A:V6, A:S7, A:N9, A:G10, A:K11, A:E12, A:L13, A:K14, A:C15, A:G16, A:S17, A:G18, A:I19, A:K189, A:D190, A:Q191, A:K206, A:N207, A:T209, A:W210	25	0.836
C-terminal	DENV 1	A:L279, A:C280, A:G282, A:T283, A:T284, A:V285, A:V286, A:V287, A:D288, A:E289, A:H290, A:C291, A:G292, A:N293, A:R294, A:L298, A:R299, A:T300, A:T301, A:T302, A:V303, A:T304, A:G305, A:K306, A:I307, A:I308, A:H309, A:E310, A:W311, A:C312, A:C313, A:R314, **A:R336, A:P337, A:V338, A:K339, A:E340, A:K341, A:E342, A:E343, A:N344**, A:L345, A:V346	43	0.779
	DENV 2	A:F279, A:C280, A:G282, A:T283, A:T284, A:V285, A:V286, A:V287, A:T288, A:E289, A:D290, A:C291, A:G292, A:N293, A:R294, A:R299, A:T300, A:T301, A:T302, A:A303, A:S304, A:G305, A:K306, A:L307, A:I308, A:T309, A:E310, A:C312, A:R314, **A:P337, A:L338, A:K339**	32	0.788
	DENV 3	A:T302, A:V303, A:S304, A:G305, A:K306, A:L307, A:I308, A:H309, A:E310, A:C312, A:R314, **A:P337, A:I338, A:N339, A:E340, A:K341, A:E342, A:E343, A:N344**, A:M345, A:V346, A:K347	39	0.809
	DENV 4	A:G259, A:E279, A:C280, A:P281, A:G282, A:T283, A:T284, A:V285, A:T286, A:I287, A:Q288, A:E289, A:D290, A:C291, A:D292, A:H293, A:R294, A:L298, A:R299, A:T300, A:T301, A:T302, A:A303, A:S304, A:G305, A:K306, A:L307, A:V308, A:T309, A:Q310, A:W311, A:C312, A:C313, A:R314, A:S315, A:C316, **A:E334, A:I335, A:R336, A:P337, A:L338, A:S339, A:E340, A:K341, A:E342, A:E343, A:N344,** A:M345, A:V346	49	0.755

4. Discussion

In 1968, the first dengue cases in Indonesia have been reported from the capital city Jakarta and also Surabaya. Increasing number of cases of dengue infections since then have been reported and spreading almost all regions of the country [29]. However, to the best of our knowledge, there are no data about molecular profiling of all Indonesian DENV serotypes especially from specific genes such as NS1 protein. This kind of information could be used for vaccine design, diagnostic or pathogenesis study. In this study, 16 sequences were found conserved with a range between 5-13 amino acids.

Analysis by using MHC-NP showed the peptides $NS1_{155--163}$ interacted mostly with MHC-I allele HLA-B*53:01 with the highest score were 0.6366 for DENV 1, 0.6283 for DENV2-3, and 0.7131 for DENV 4, respectively. Immunogenicity prediction by pMHC-I analysis and antigenicity prediction by VaxiJen2.0 also showed a consistent result for peptide $NS1_{155--163}$ with the highest score of the epitopes compared with the other peptides. These results are in accordance with a previous in vitro study that reported a region of NS1 protein between 141 to 168 as a common epitope region for all DENV serotypes [30]. Several studies have demonstrated that CD8+ T-cells have a protective role in severe DENV murine infection models by reducing the viral burden and at the same time reduce the risk of antibody-dependent enhancement (ADE) [31-34].

Interestingly, the predicted NS1 discontinuous epitope at C-terminal showed the same region with B-cell continuous epitope NS1325--344. The homolog amino acids between continuous and discontinuous B-cell epitope at C-terminal are highlighted in bold (table 4). Previous studies also reported that several region at C-terminal of NS1 protein, 338--352 [35], 331--339 [36], and 273--346 [37], have been identified as potential epitope of DENV. Another discontinuous B-cell epitopes which located at N-terminal of NS1 protein was also predicted in this study. Both discontinuous epitope at C-terminal and N-terminal were highly conserved to all DENV serotypes. However the discontinuous epitope at C-terminal to be more likely promising as new target for DENV vaccine development or detection system since at this region residing more conserved epitopes that could recognized by CD8+ T-cells and CD4+ T-cells.

The combination of databases from verified vaccine experiments and in silico tools method to predict potential epitopes that induce host immune response have a complementary role for design a new generation vaccine [38]. Various online in silico vaccine design tools have been developed recently and many of them have been improved which make feasible to skip over many in vitro screening step, use directly genome sequence, predicting T-cell and B-cell immune epitopes which are promising as new vaccine targets [38, 39]. Epitopes which representing antigenic determinants of a pathogen are attractive target in development of prophylactic and curative vaccines and could be use also to increase the detection limit of dengue diagnosis system [27].

5. Conclusion

Targeting the correct T-cell and B-cell epitopes is very important in order to increase the efficacy of vaccine and elevate the sensitivity and specificity of diagnostic test. In the present study, we elaborated on several different prediction tools to predict the best epitopes of CD8+ T-cells, CD4+T-cells, continuous and discontinuous B-cell of Indonesian DENV NS1 protein. The predicted epitopes in this study are key possibilities for developing a novel dengue vaccine candidate or as a target inducer for producing monoclonal antibodies utilized in the dengue detection system. However, further in vitro and in vivo investigations are required to confirm the current in-silico analysis's findings.

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