CHAPTER VI: EVALUATION OF IDENTIFIED EPITOPES FOR DENGUE VIRUS ANTIBODY DIAGNOSTIC ASSAY

6.1 Introduction

Clinical symptoms are the first stage of diagnosis of Dengue. Clinical presentation of Dengue virus infection can vary from being asymptomatic to symptomatic to severe dengue. In the acute phase, generally, patients are presented with a sudden onset of fever followed by nausea, aches, headaches, and pain[1]. However, these symptoms in the acute phase are not unique to Dengue and are also reported for other febrile diseases. Therefore, a confirmed laboratory diagnosis is very necessary to prevent the severity of the disease. Early and effective treatment can lessen the impact of dengue on society by preventing severe morbidity and mortality.

Various methods of Dengue diagnosis include i) cell culture; ii) viral nucleic acid by NAAT; iii) DENV antigens; and iv) specific DENV antibodies[2]. While virus isolation offers the most precise test, virus culture necessitates a biosafety level 2 laboratory, a lot of time, labor, and expert staff training[3]. Serum samples are stable under these climate circumstances, and serological testing, which was created to analyze antigen/antibody reactions during dengue infection, is often less expensive and easier to execute. The most popular point-of-care diagnostic assay for dengue virus infection is a commercial Rapid Diagnostic Test (RDT) kit. However, when employing DENV NS1 antigen detection, false positives caused by antigenic cross-reactivity between Dengue and Zika infections have been documented[4]. This has become a major concern in rapid diagnosis.

Because of the issue of cross-reactivity, it is very crucial to identify specific targets on Dengue proteins for use in diagnosis. In this regard, the identification of conserved and specific epitopes of DENV proteins could be of great importance. Antigenic determinants, or epitopes, are a class of molecules on the surface of antigenic molecules that have a unique structure and exhibit immunological activity[5, 6]. They are considered to be immunoreactive regions on antigenic molecules. Their ability to induce the production of antibodies or sensitized lymphocytes allows them to be identified by the generated antibodies. Normally epitopes are of only 5–7 amino acid residues, and at most 20 amino acid residues.

The B cell epitope is currently the focus of epitope study because binding to BCRs stimulates B cells to generate antibodies. Prediction has been the subject of ongoing research, and advances have been achieved in computational prediction techniques as well. Early in silico methods used primary sequence-based antigen data for linear epitope

prediction.

The antigen binding site of antibodies is known as complementarity-determining regions, or CDRs. Antibody light and heavy chains each consists of three CDRs[7]. Complementarity-determining regions (CDRs) must be precisely identified in order to understand and regulate antigenic interactions[8]. Identifying antibody residues that interact with B cell epitopes on the antigen is one method to achieve this. Naturally, this necessitates identifying B cell epitopes, which may be accomplished by designating antigen residues that bind to CDRs, necessitating the identification of CDRs[7, 9].

Therefore, we have developed an indirect ELISA-based diagnostic assay using epitope-based antigens. We have selected the two best epitopes to design a recombinant synthetic peptide SP7 and also checked the diagnostic potential of the synthetic peptide. Evaluation of the diagnostic potential of SP7 was performed using 52 Dengue positive, 20 Healthy Control and 15 J.E positive samples.

6.2 Materials and methods

6.2.1 Sample collection

Lab confirmed samples were collected from Gauhati Medical College & Hospital. Abbott Bioline Dengue NS1 Ag Test Kit used to classify patients as dengue or non-dengue. JE positivity was determined using ICMR-NIV J.E IgM Capture ELISA kit .

6.2.2 Construction of a synthetic peptide P7

Two immunodominant peptides of Envelope protein were linked by linker to build a recombinant synthetic peptide (SP7). These two epitopes were connected with GGGGS linker in between and GS linker is added at the N and C terminal of the peptide. SP7 was commercially synthesized (GL Biochem Shanghai Ltd).

6.2.3 Prediction of B cell epitope of SP7 construct

SP7 construct was designed with the best potential epitopes joined by linkers. However, to confirm the presence of epitope region in the construct, B cell epitope predictions were performed. Linear B cell epitopes were predicted by BepiPred2.0 for Dengue virus 2 E and NS1 protein. Prediction scores of more than 0.5 (yellow region) were considered as B cell epitopes. Conformational B cell epitopes were predicted by ElliPro.

6.2.4 Physiochemical properties of SP construct

The physicochemical characteristics of the synthetic peptide, such as its molecular

weight, half-life, sequence length, aliphatic index, instability index, theoretical pI, and grand average of hydropathicity, were investigated using the Protparam server[10]. The predicted instability index of a protein defines its status: proteins with values less than 40 are categorized as stable, whereas those with values more than 40 are categorized as unstable. The volume occupied by protein's aliphatic side chains—is known as its aliphatic index. The Grand Average of Hydropathy was calculated using the total hydropathy found for each sample.

6.2.5 Determination of Complementary Determining Region of Neutralising Antibody

Immunoglobin (Ig) hypervariable areas known as complementarity-determining regions (CDRs) limit the binding of [11]specific antibodies (shown in Figure 6.1). They are part of the B-cell and T-cell receptor-containing variable chains of antibodies. These molecules attach to their distinct antigen. Complementarity-determining region CDR were identified using the online server AbRSA[12]. The variable (V) and constant (C) regions that make up the light and heavy chains each contain three CDR loops, which make up the main part of the antigen-binding site (Figure 6.1).

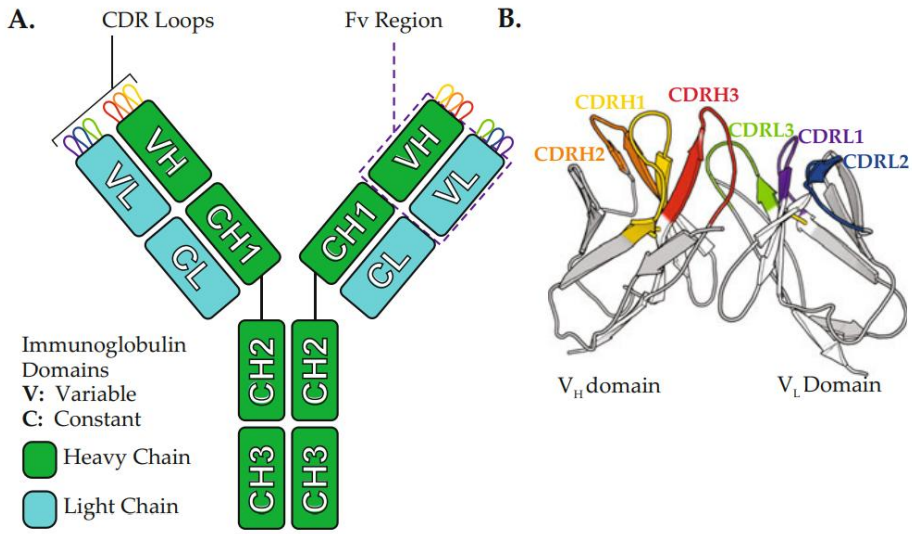

Figure 6.1 A) Structure of an antibody molecule, -B) Complementary Determining Region of Antibody. The image is adapted from a previously published paper[13].

6.2.6 Docking of synthetic peptide with Neutralising antibody (NAb)

3D structure of SP7 was modeled using the AlphaFold2 server[14]. The best model was selected for docking study. Cluspro v.2.0[15] docking software was used to molecularly bind the synthetic peptide P7 with the neutralizing antibody (NAb) 4UTA[16] using default settings. Cluspro provides cluster scores based on rigid docking and pairwise RMSD energy minimization. Based on the lowest energy weight score and members, the final P7-NAb complex was visualized using PyMOL software. Interaction studies of residues for best-docked models were performed using PDBsum[17].

6.2.7 Standardisation of indirect ELISA assay and determining seropositivity

A checkerboard ELISA method was used to determine the optimum concentration of the antigen (P7) and antibody for standardisation of the assay. Anti-DENV antibody affinity of the synthetic peptide was checked with DENV1(NR-4746), DENV2(NR-2556) and DENV3(NR-15526) Envelope monoclonal antibodies. We have obtained these monoclonal antibodies as a gift from BEI Resources, NIAID, NIH. The optimum concentration of antibody(1:200) and peptide (1.25 ng/µl) was selected. We have used Nunc Maxisorp 96-well polystyrene plates. After resuspension of the peptides in their respective solvent, 50µl of peptides was coated at 1.25ng/µl concentration in coating buffer (Carbonate-bicarbonate pH 9.6) in duplicates and the plates were kept overnight at 4°C. Unbound peptides were washed off using wash buffer (PBS pH 7.4 with 0.05% Tween 20 -PBST) and 100µl of Blocking buffer (5% BSA in PBS pH 7.4) were added to each well and incubated at room temperature for 3 hours. The plates were washed three times with wash buffer followed by the addition of 50µl diluted serum in each well (serum dilution ratio 1:200 in PBS) and incubated for 1 hour at 37°C. After washing the plates three times HRP tagged Anti-human IgG antibody (ab97225) was added in each well and incubated for 1 hour at 37°C. the unbound antibodies were washed off three times using a wash buffer. 50µl of TMB substrate(Sigma-T0440) was added in each well and incubated for 20 minutes at room temperature protected from light. The peroxidase reaction was stopped by adding 50µl of 1 N H2SO4 in each well. Absorbance was checked at 450nm.

6.2.8 Evaluation of the diagnostic potential of synthetic peptide

The diagnostic potential of SP7 synthetic peptide was evaluated with 52 DENV-positive and 35 DENV negative samples (20 Healthy and 15 J.E positive) collected from GMCH. A cut-off value of 0.567 (cut-off=mean+ 3SD) was determined based on the seroreactivity of 20 healthy human sera.

6.2.9 Determining Sensitivity and specificity

Sensitivity is a test's ability to identify an individual with the disease as positive whereas specificity is a test's capacity to identify as negative a person who does not have a disease. A highly sensitive test indicates few false negative results, and thus fewer cases of disease are missed. A high specificity indicates that the test can correctly differentiate between disease and non-disease, minimizing the number of false positives. Sensitivity and specificity were calculated using the following formula-

$$\text{Sensitivity} = \frac{Number\ of\ true\ positives}{Total\ number\ of\ individuals\ with\ the\ disease}$$

$$\text{Specificity} = \frac{Number\ of\ true\ negatives}{Total\ number\ of\ individuals\ without\ the\ disease}$$

6.3 Results

6.3.1Visualization of SP7 construct

Visualization of the SP7 construct was performed using PyMOL software. The structure of the construct reveals the helical nature of the two epitopes (Epitope 1 and Epitope 2) joined by a linker GGGGS shown as a coil structure (shown in Figure 6.2). Terminal linkers GS are also shown as coil structure.

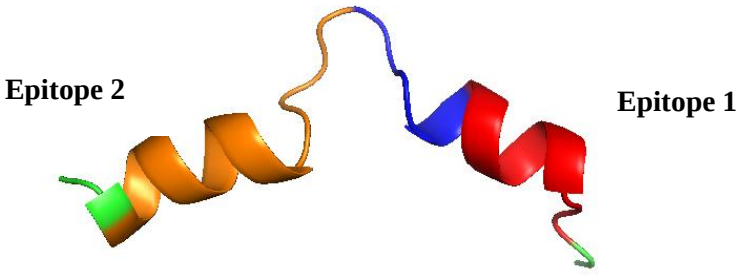

Figure 6.2: 3D model of the synthetic peptide SP7 ,**Green-GS linker Red-Epitope1 Orange- Epitope2 Blue-GGGGS linker**

6.3.2 B cell epitopes of SP7 construct

Linear and conformational B cell epitope regions (Figure 6.3 and Figure 6.4 respectively) were identified in the designed SP7 construct which proves the epitopic characteristics of the synthetic peptide .

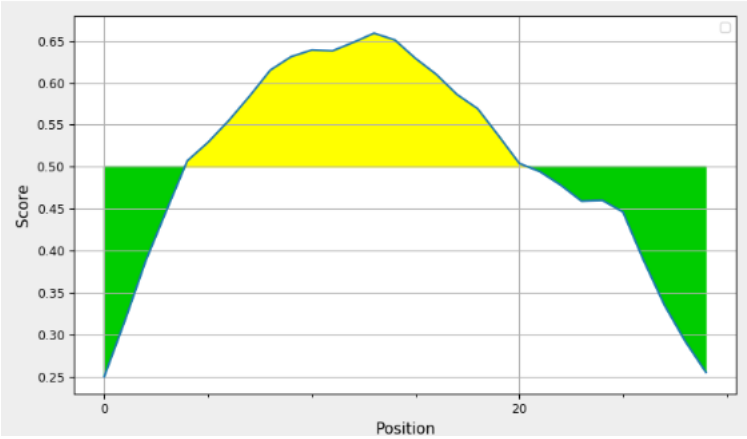

Figure 6.3: Linear B cell Epitope region of SP7 as predicted by BepiPred 2.0

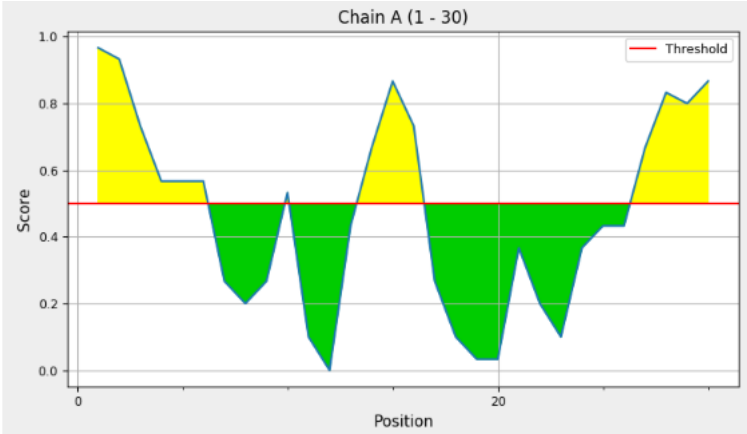

Figure 6.4: Conformational B cell Epitope region of SP7 as predicted by ElliPro.

6.3.3 Physiochemical properties of SP7 construct

Physiochemical analysis of the SP7 construct reveals an instability index score of 33.31 which indicates the construct is stable (Table 6.1). The peptide appears to be somewhat acidic based on the analysis's 6.18 pI (isoelectric point) value and has an antigenicity score of 0.6941. The protein is thermostable, as indicated by the estimated aliphatic index of 61.67; a higher aliphatic index value corresponds to greater thermostability. The

computed grand average of hydropathicity (GRAVY) was -0.383; a negative number suggests that the protein is hydrophilic and will interact with molecules of water. Overall, the physicochemical characteristics of the peptide construct were found to be significant, according to ProtParam
analysis.

Properties	
Antigenicity	**0.6941**
Instability index:	**33.31**
Aliphatic index	**61.67**
Grand average of hydropathicity (GRAVY)	**-0.383**
Molecular weight	**3010.27 Da**
Theoretical pI	**6.18**

Table 6.1: Physiochemical properties of SP7 construct

6.3.4 Determination of CDR region

CDR regions of both heavy and light chain of Neutralising Antibody (NAb) was determined using AbRSA server. CDR region is shown below in the figure and the residues are highlighted in colour.

>4UTA_2|CHAINS HEAVY CHAIN

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  1 EVQLVESGGGLVQPGGSLRLSCSASGFTFSTYSMHWVRQAPGKGLEYVSAITGEGDSAFY   60
 61 ADSVKGRFTISRDNSKNTLYFEMNSLRPEDTAVYYCVGGYSNFYYYYTMDVWGQGTTVTV  120
121 SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ  180
181 SSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPLEDDD  240
241 DKAGWSHPQFEKGGGSGGGSGGGSWSHPQFEK
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>4UTA_3|CHAINS LIGHT CHAIN

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  1 RSEIVLTQSPATLSLSPGERATLSCRASQSISTFLAWYQHKPGQAPRLLIYDASTRATGV   60
 61 PARFSGSRSGTDFTLTISTLEPEDFAVYYCQQRYNWPPYTFGQGTKVEIKRTVAAPSVFI  120
121 FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS  180
181 TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
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- CDRs are highlighted in colors (CDR1, CDR2, CDR3).
- The gray letters indicate the non-variable-domain region.
- The underlined black letters indicate variable domain of heavy chain while the other black letters indicate variable domain of light chain.

CDR of Heavy chain

CDR1-26GFTFSTY32

CDR2-52TGEGDS57

CDR3-99GYSNFYYYYTMDV111

CDR of Light chain

CDR1-26RASQSISTFLA36

CDR2-52DASTRAT58

CDR3-91QQRYNWPPYT100

Figure 6.5- Identifying CDRs of Antibody using AbRSA server

6.3.5 Docking of SP7 with Neutralizing Antibody

Docking analysis shows that the SP7-NAb complex binding is stable. Interaction studies by pdbsum reveals that SP7 is specifically binding to the CDR regions of the neutralizing antibody (Figure 6.6)

Figure 6.6: Docked complex of SP7 (Cyan) and NAb(Green)

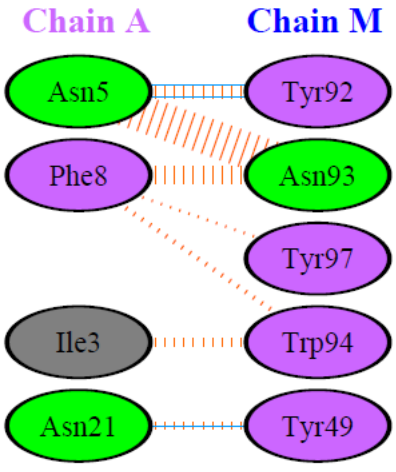

Figure 6.7a: SP7 interaction with **light** chain

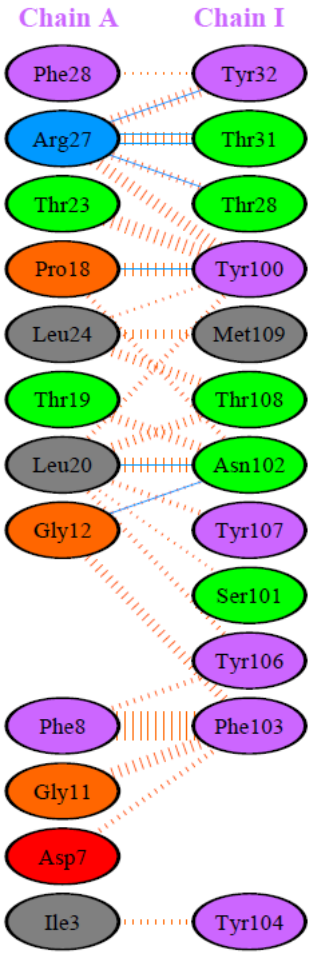

Figure 6.7b:SP7 interaction with **heav**y chain

6.3.6 Interacting residues of CDRs and Nab

The number of interacting residues between CDR regions and the neutralizing antibody shows that SP7 was able to specifically bind to all CDRs of the antibody. In the heavy chain number of interacting residues are 3 and 9 for CDR1 and CDR3 respectively and non with CDR2. Interestingly in the light chain 4 number of interacting residues can be seen with CDR2 and SP7.

Chain	CDR1	CDR2	CDR3
Heavy (I)	**3**	**0**	**9**
Light (M)	**0**	**4**	**0**

Table 6.2: Number of interacting residues with CDRs of Nab

6.3.7 Evaluation of the diagnostic potential of SP7

Based on the cut-off value for the synthetic peptide SP7, we examined the seroreactivity of dengue-positive, dengue-negative and J.E positive samples. In-house designed synthetic peptide SP7 successfully detected 48 dengue positive out of 52 positive samples and 32 dengue negative out of 35 negative samples as shown in Figure 4. None of the 15 J.E positive samples were detected as dengue positive by SP7, thereby eliminating the possibility of cross Flavivirus reactivity. The sensitivity and specificity of the developed assay were found to be 92.3% and 91.42% respectively (Table 6.3).

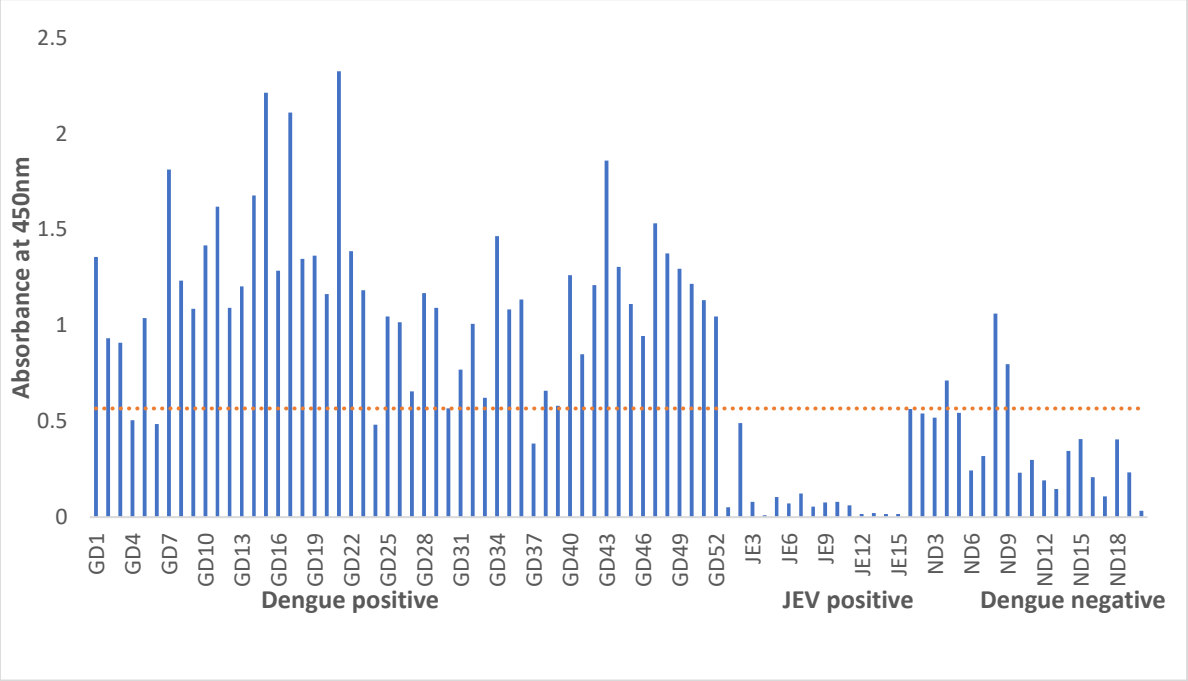

Figure 6.8: Graph showing seroreactivity of SP peptide with dengue positive (GD) J.E positive (JE) and healthy sera (ND). Cut-off is indicated by the horizontal dotted line in the graph.GD=Dengue positive, JE= J.E positive and ND= non-dengue healthy samples**.**

	Dengue positive	Dengue negative
In house test +	48	3
In house test -	4	32
Total	52	35
Sensitivity	48/52*100	92.3
Specificity	32/35*100	91.42

Table 6.3: Sensitivity and specificity of the recombinant peptide SP7

6.4 Discussion

Although serological testing is a common practice to examine patients infected with dengue, the co-circulation of other Flaviviruses, which share many antigen similarities, affects specificity. Therefore, advances are needed to increase the precision of serological testing, such as the identification of novel antigens as tools for identifying antibodies specific to dengue.

Advances in B cell epitope identification using bioinformatic approaches have made epitope mapping easier. The use of predicted B cell epitopes to analyze anti-sera and design vaccines has been well documented by many researchers. In our study, we have used Peptide array and bioinformatic approaches to identify immunodominant regions and epitopes respectively of the viral proteins. The concordance of both approaches proved the authenticity of the study. Overlapping and conserved epitopes were selected to design synthetic peptides for diagnostic study. We have designed the recombinant synthetic peptide using two best epitopes and joined by linker GGGGS[18]. The designed synthetic peptide could correctly identify Dengue-positive samples by ELISA and also can differentiate clearly between serum samples from Dengue and *Japanese encephalitis* virus infection. The diagnostic potential of in-house designed synthetic peptide when evaluated showed a sensitivity of 92.3% and 91.42% specificity.

A similar study was reported by Periera et al (2023) where they have developed IgG ELISA based on a multi-epitope NS1 antigen for antibody detection and achieved 77.42% sensitivity and 88.57% specificity with no cross reactivity with ZIKV-infected subjects [19].

We have used a limited number of freshly collected samples for validation and it needs to be validated in a larger number of samples. This study supports that epitope-based antigens are promising candidates for the use in serodiagnosis of dengue. However, the synthetic peptide was designed using only two potential peptides, the validation of other potential peptides remains and inability to experimentally validate for cross-reactivity/specificity with other flaviviruses (except JEV positive samples) for lack of clinical samples remains a limitation in our study.

6.5 References

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