



SYNTHETIC PEPTIDES AS AN EMERGING TOOL FOR THE SEROLOGICAL DIAGNOSIS OF DENGUE VIRUS INFECTION

Biological Science

R. Praveena

Department of Zoology, Fatima College, Madurai – 625018, Tamil Nadu, India

J. Asnet Mary*

Assistant Professor, Department of Zoology, Fatima College, Madurai – 625018, Tamil Nadu, India *Corresponding Author

ABSTRACT

Dengue fever (DF) is one of the fastest emerging arboviral diseases and has become a major public health concern across the world. It is caused by dengue virus, belonging to genus flavivirus. It causes an acute, systemic febrile illness. Specific treatments for Dengue viruses are not available so far. It is imperative to diagnose the dengue virus at the early stages of infection to curb mortality. The basic laboratory diagnosis methods detect antigen or antibodies. In this study, peptide antigen was used to diagnose the virus Indirect ELISA. Three synthetic peptides, Peptide-1-LEHGSCVTMAKDKPTL, Peptide-2-DRGWGNGCGLFG and Peptide-3-CGQKKAHNGRLITANP were used for the serological diagnosis. Accuracy, sensitivity and specificity were calculated for their potential to differentiate positive and negative samples. All statistical analyses were performed using MedCalc software. The results showed that Peptide-1, Peptide-2 and Peptide-3 have high sensitivity of 90%, 96.7%, 95% respectively. The specificity of the peptides is also 70%, 75%, 70% for Peptide-1, Peptide-2, Peptide-3 respectively. The results suggested that the peptide based diagnosis is an alternative method for the early diagnosis of Dengue virus.

KEYWORDS

Dengue virus, Peptides, Diagnosis, Specificity, ELISA, Sensitivity

INTRODUCTION

Dengue fever is the most important arthropod-borne viral infection of human. Globally, 2.5 billion people are at risk of infection and ~5000,000 persons are hospitalized with dengue haemorrhagic fever (DHF) annually, in tropical and subtropical countries (WHO, 2008). Dengue virus belongs to the genus flavivirus within the *Flaviviridae* family. The virion comprises a spherical particle, 40-50nm in diameter with a lipopolysaccharide envelope. It is a positive, single stranded RNA virus; contain approximately 10.7kb in length and is translated to a single long polypeptide that is cleaved by proteases resulting in generation of three structural proteins (capsid (C), membrane (M) and envelope (E) glycoprotein and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Perera et al., 2008). The structural proteins include capsid (C), membrane protein (prM/M) and envelope (E). prM and E are released from the polyprotein by signalase cleavage in the ER, but remain anchored on the luminal side of the membrane. The C is also anchored in the ER membrane (on the cytoplasmic side) by a conserved hydrophobic signal sequence at its C-terminal end. This signal sequence is cleaved by the viral NS2B-NS3 protease (Randolph and Stollar, 1990). Envelope protein is an essential component for initiating infection, mediating virus binding to cell receptors and the subsequent fusion step (Crill and Roehrig, 2001). Envelope glycoprotein is involved in the early events of dengue virus infection such as attachment of the virus to the host cell surface and engagement of fusion between viral and host cell membrane. It consists of three functional domains (I, II and III) of which domain III is critical for virus absorption to the receptors expressed on the host cell surface (WHO, 2008).

DENV causes a febrile illness with myriad clinical manifestations which range from common dengue fever to severe, life threatening dengue hemorrhagic fever (DHF). Less than 3% of patients develop the most severe, life threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). The subsequent heterotypic dengue virus infection is the most significant risk factor for the development of DHF/DSS (Burke et al., 1988; Guzman et al. 1990; Halstead et al., 1970). There is no specific therapeutic agents and efficient vaccine available. Therefore, early and accurate diagnosis of the disease is imperative for managing the disease. So far, different techniques are available that detect the dengue virus, viral nucleic acid or antigen or antibodies (WHO, 2008).

The two methods for laboratory diagnosis of dengue infection are detection of the virus or detection of anti-dengue antibodies. Serology is currently the most widely applied in routine diagnosis. Hemagglutination inhibition, Complement fixation, Neutralization tests, Immunoglobulin M capture enzyme linked immunosorbent assay and immunoglobulin G ELISA test are performed in the serological diagnosis of dengue virus. Although these techniques are sensitive and specific, it is not cost effective (Paula and Fonesca,

2004). In virus isolation, it is more specific. However, it is time-consuming and expensive, needing expertise, an inability to differentiate primary and secondary infections, and requiring acute samples. Identification of both serotype and genotype, nucleic acid detection also need acute sample. The tools are very expensive, handling and differentiation of primary and secondary infection are also the major problem of dengue viral diagnosis (WHO, 2008). The major impediment in dengue diagnosis is lack of rapid and cost-effective assays tools.

Mary et al., 2018 demonstrated that the three peptides derived from domain I, domain II and domain III of Envelope glycoprotein of Dengue virus blocked the entry of Dengue virus in Vero and LLC-MK2 cell lines. As synthetic peptides are efficient in binding onto the envelope glycoprotein, it was hypothesized that peptides are able to detect the dengue viral particles. Peptides are polar substances, safe and easy to produce. Hence, it can be evaluated for their ability to detect the disease. Therefore, the present study aimed to evaluate the potential role of three peptides for diagnosing dengue virus and to examine their sensitivity and accuracy in detection of clinically confirmed samples and negative samples using ELISA. The schematic representation of applications of synthetic peptides as diagnostic agent is outlined in Figure 1.

MATERIALS AND METHODS

Sample collection from patients and healthy volunteers

Dengue positive Serum samples collected from Meenakshi Mission Hospital and Research Centre (MMHRC) were used for the study. The samples were collected from dengue infected 32 male and 28 female patients, during the period of November 2018 to January 2019. The patient age group ranges from 3 to 75 years. The dengue infections were confirmed by IgM, IgG and NS1 ELISA. All four dengue serotypes are represented in the patient groups. Serum samples were collected from 20 healthy individuals for negative control. All serum samples were stored at -20°C prior to use.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed by following the protocol described previously by Amin et al., (2013). One hundred microlitres peptides were added to the microtitre wells. It was incubated overnight at 4°C. After the incubation, it was washed thrice. It was then blocked with 1% BSA for one hour. After washing with PBS, positive serum sample (primary antibody) of dilution 1:100 was added to the well and the plates were incubated at 37°C for 1 hour. After incubation the wells were washed thrice with PBS-T. Horse Raddish Peroxidase conjugated Goat anti-human secondary antibody was added 1:3000 dilution. The wells were then incubated for 45 min at 37°C. After another wash with PBS-T, 100µl of the OPD-H₂O₂ substrate solution was added to the wells and incubated at room temperature for approximately 10 min. The reaction was stopped with 100µl of 2.5N H₂SO₄. The absorbance of colour in each well was read at 495 nm.

Sensitivity and Specificity Testing

Sensitivity: The sensitivity of a test is its ability to determine the patient cases correctly. To estimate it, the proportion of true positive was calculated in patient cases. Mathematically, this can be stated as: $\text{Sensitivity} = \text{TP} / (\text{TP} + \text{FN})$

Specificity: The specificity of a test is its ability to determine the healthy cases correctly. To estimate it, the proportion of true negative was calculated in healthy cases. Mathematically, this can be stated as: $\text{Specificity} = \text{TN} / (\text{TN} + \text{FP})$

Statistical analysis

All statistical analyses were performed using MedCalc Software, Ostend, Belgium (version 10.1.2). The selection of cut-off point was performed with a Receiver Operating Characteristic Curve (ROC) analysis and P-value of <0.05 was considered statistically significant. All the experiments were performed in triplicates.

RESULTS AND DISCUSSION

Dengue fever is a major public health concern in tropical and subtropical countries. Efficient and rapid diagnosis is crucial for effective implication of disease management strategies. Multitude approaches attempted to identify individual viruses, includes serological assays and molecular methods. Detection assays based on peptides have become increasingly substantial and indispensable for its advantages over conventional methods (Mohanraj *et al.*, 2017). Though peptides have been available for decades, their adoption in diagnostics has been limited, due to diminish specificity. Recent reports suggested that protein based rather than peptide based is more specific (Navalkar, 2015). The three peptides used in this study were derived from the E protein, corresponding to amino acids 25 - 42 (LEHGSCVTTMAKDKPTL), 98 - 109 (DRGWGNGCGLFG) and 339 - 354 (GQGKAHNGRLITANP) and denoted as Pep-1, Pep-2 and Pep-3 respectively. The peptides were commercially synthesized at GenScript (USA) by Solid phase peptide synthesis. The identity of the peptides was confirmed by LC-MS and its purity was over 96% determined by HPLC analysis.

Sensitivity and specificity of anti-peptide antibodies

Sensitivity of Peptide-1 was 90% whereas the specificity is 70% (Figure 2A). It diagnosed the positive cases accurately but the specificity was found to be low. It revealed that it predicted the healthy cases also as positives. The OD readings were similar in patients and in healthy donors. The distributions of data of healthy subjects and dengue patients are given in Figure 2B. Sensitivity of Pep-2 was 97% whereas the specificity is 75% (Figure 3A). The results revealed that the sensitivity is higher than Peptide-1. However, it was less likely to diagnose the healthy cases as positives. The distributions of data of healthy subjects and dengue patients are given in Figure 3B. Sensitivity of Peptide-3 was 95% whereas the specificity is 70% (Figure 4A). It diagnosed the diseased cases accurately but the specificity was found to be 70%. It is apparent that the antibody has shown differential binding with positive samples and negative samples. The distributions of data of healthy subjects and dengue patients are given in Figure 4B. The overall sensitivity and specificity of three peptides are tabulated in Table 2. Pep-2 has high sensitivity and specificity. It is a fusion loop peptide of domain II envelope glycoprotein of dengue virus.

The use of peptides as antigens in serological diagnosis has major advantages as it is safe and easy to produce in a reproducible manner. The choice and design of the peptide are of major importance for a successful peptide ELISA (Langedijk *et al.*, 1996). Most peptides that have been used in serology represent continuous epitopes because it is difficult to detect antibodies against complex discontinuous epitopes using small linear peptides. In addition, it is difficult to mimic the antigenic surface of large globular proteins accurately with a small linear peptide.

CONCLUSION

Conventional methods of diagnosis of Dengue virus by serological methods are not rapid. Virus isolation and nucleic acid detection would also need sophisticated infrastructure facility and skilled personal. Therefore, synthetic peptides become an alternative emerging tool for the diagnosis of Dengue virus. As it is specific and safe, it can be used as a diagnostic agent using Indirect-ELISA. It would give new insights on the application of peptides for the detection of viruses. Therefore, the peptides will be an alternative and an effective method for diagnosing Dengue virus.

Acknowledgements

The work was supported by UGC-Minor Research Project (F.MRP-6770/16 (SERO/UGC), South Eastern Regional Office, Hyderabad. Authors would like to thank Dr.R. Paramasivan, Scientist E, CRME (ICMR), Madurai for the comments and suggestions.

DECLARATION

The study was approved by ethical committee of Meenakshi Mission Hospital and Research Centre, Madurai

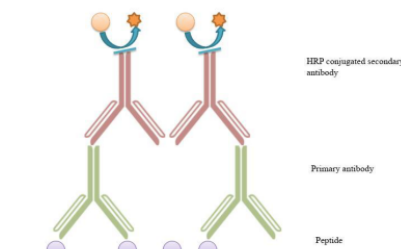


Figure 1 Schematic representation of application of peptides as diagnostic agents.

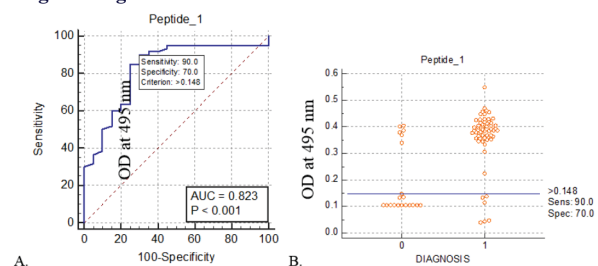


Figure 2. A. Sensitivity and specificity of Peptide-1. **B.** Comparison of OD values in positive and healthy subjects .

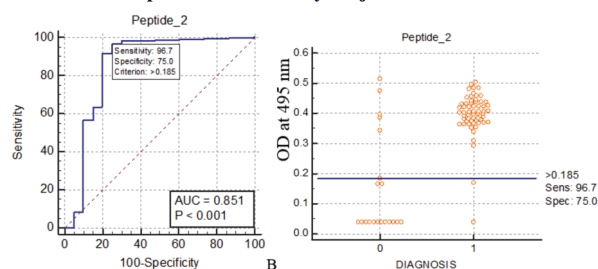


Figure 3. A. Sensitivity and specificity of Peptide-2. **B.** Comparison of OD values in positive and healthy subjects .

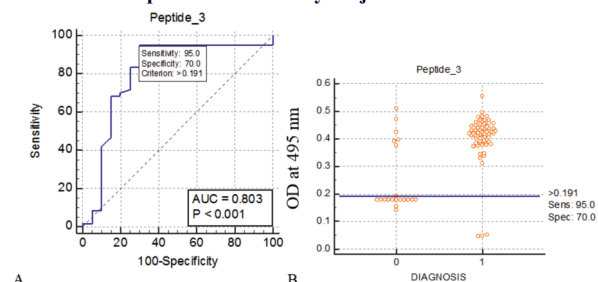


Figure 4. A. Sensitivity and specificity of Peptide-3. **B.** Comparison of OD values in positive and healthy subjects .

Table 1. Synthetic peptides used in the present study

Peptide no	Amino acid sequence	Position	Length	Structural domain
Pep 1	LEHGSCVTTMAKDKPTL	25 - 42	17	Domain I
Pep 2	DRGWGNGCGLFG	98 - 109	12	Domain II
Pep 3	CGQKAHNGRLITANP	340 - 354	16	Domain III

Table 2. Sensitivity and specificity of peptides in diagnosing the clinically confirmed positive samples

Peptides	Sample size	Positive group a	Negative group b	Sensitivity (%)	Specificity (%)	Standard error
----------	-------------	------------------	------------------	-----------------	-----------------	----------------

Peptide_1	80	60 (75.00%)	20 (25.00%)	90	70	0.0556
Peptide_2	80	60 (75.00%)	20 (25.00%)	96.67	75	0.0686
Peptide_3	80	60 (75.00%)	20 (25.00%)	95	70	0.0698

REFERENCES

1. (50-52). Burke, D., A. Nisalak, D. Johnson, and R. Scott. A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg.* (1988) 38:172–180
2. Amin, N., Pupo, M., Aguilar, A., Camacho, F., Alvarez, M., Cabllero, Y., Diaz, D., Garcia, A., Reyes, O., Guzman, M.G., Stott, D., & Acosta, A. (2013) Immunogenicity of NS4b Dengue 3 virus Mimotope presented to the Immune system as Multiple Antigen Peptide System. *Virology*, p.
3. Asnet Mary John, Jittmitrathap A, Chattanadee S, Alwin Prem Anand A, Shenbagarathai R, Leaungwutiwong P. In vitro analysis of synthetic peptides in blocking the entry of dengue virus.
4. Crill, W.D. and Roehrig, J.T. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. *J. Virol.* (2001) 75, 7769–7773.
5. Guzman, M., G. Kouri, J. Bravo, M. Soler, S. Vazquez, and L. Morier. Dengue hemorrhagic fever in Cuba. *Am. J. Trop. Med. Hyg.* (1990). 42:179–184.
6. Halstead, S., S. Nimmannitya, and S. Cohen. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J. Biol. Med.* (1970) 42:311–328.
7. Langedijk, J. P., W. G. Middel, W. M. Schaaper, R. H. Meloen, J. A. Kramps, A. H. Brandenburg, and J. T. van Oirschot. 1996. Type-specific serologic diagnosis of respiratory syncytial virus infection, based on a synthetic peptide of the attachment protein G. *J. Immunol. Methods* 193:157–166.
8. Mohanraj, U, Chander, S., Chavan, YG (2017) Peptide based viral detection systems for effective diagnosis of common viral infections in India, *Current Protein and Peptide Science*, 18(9).
9. Navalkar KA, Johnston SA, Stafford P. (2015) Peptide based diagnostics: are random-sequence peptides more useful than tiling proteome sequences? *J. Immunol Methods*, 417:10-21.
10. Paula, S D. & Fonseca, BAL (2004). Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. *The Brazilian Journal of Infectious Diseases : An Official Publication of the Brazilian Society of Infectious Diseases*, 8(6), 390–398.
11. Perera R., Kuhn RJ. Structural proteomics of Dengue virus. *Curr Opin Microbiol.* (2008) 11(4): 369–377.
12. Randolph, V. B., and Stollar, V. Low pH-induced cell fusion in flavivirus-infected *Aedes albopictus* cell cultures. *J. Gen. Virol.* (1990). 71, 1845±1850.
13. WHO: Report of the Scientific Working Group on dengue. Document WHO/TDR/SWG/08 Geneva, Switzerland, World Health Organization; 2008