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Research Article

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Evaluation of Diagnostic Potential of Anti-Peptide Antibodies for Dengue Virus Infection

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Abstract : Dengue virus is diagnosed by antibodies, viral antigen, and nucleic acids. Serological techniques detect only at late acute phase that delay the appropriate treatment. Viral detection methods are expensive and required skilled professionals. Therefore, a rapid and accurate dengue diagnosis with cost effective is of paramount importance for effective control of dengue outbreaks. As antibodies of synthetic peptides reported in our previous work showed the potential binding between Dengue virus and antibodies, the present study aims to use anti-peptide antibodies for the purpose of diagnosis. The objective of the present study is to evaluate anti-peptide antibodies generated for three synthetic peptides of envelope glycoprotein for their diagnostic potential against dengue virus. Indirect ELISA was performed for detecting the antigen. A total of 80 serum samples from dengue infected and non-infected patients were evaluated using three anti-peptide antibodies raised against three synthetic peptides of envelope glycoprotein of dengue virus. Anti-peptide antibodies showed high sensitivity of 91%, 82%, and 90% against viral antigen for anti-peptide1 antibody, anti-peptide2 antibody and anti-peptide3 antibody respectively. The specificity of the anti-peptide antibodies was also found to be high in anti-peptide2 antibody and anti-peptide3 antibody. These anti-peptide antibodies detect the viral antigen by serological method. Therefore, the anti-peptide antibody based diagnosis can be a dependable alternative approach for the early diagnosis of the disease. This is the first article reporting anti-peptide antibodies generated against synthetic peptides of envelope glycoprotein of dengue virus for the serological diagnosis of dengue fever.

Keywords: *Dengue virus, Anti-peptide antibodies, Peptides, Diagnosis, Specificity, ELISA, Sensitivity*

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1. INTRODUCTION

Dengue fever (DF) is an arboviral disease that poses severe health burden in tropical and subtropical countries. Annually, it is estimated that 390 million people are infected and 96 million develop clinical disease[1]. The severity of this disease can be judged from simple statistics that over 40 percent (2.5 billion) of the world's population in tropical and subtropical countries continue to live under the threat of contracting dengue infections and 24,000 deaths are reported annually worldwide[2]. It is prevalent in the countries like China, Singapore, Malaysia, America, India, and Pakistan. Dengue virus is a mosquito borne Flavivirus, and positive strand encapsulated RNA virus. The size of genomic RNA is approximately 11 kb. The virus consists of three structural proteins and seven nonstructural proteins[3,4]. The viral genome encodes for 3400 amino acids[5]. *Aedes aegypti* and *Aedes albopictus* are major dengue causing vectors. It stores the infectious agent in different organs, like salivary glands and central nervous system. *Aedes albopictus* is also a minor vector for dengue infection. The two basic methods for establishing a laboratory diagnosis of dengue infection are detection of the virus and viral nucleic acid or detection of anti-dengue antibodies or a combination of these techniques[6].

1.1. Serological methods

Serological methods are indirect methods as it detects the antibody response to dengue viral infection. It is the right choice of diagnosis during the acute phase of infection because of the fact that IgM primary antibody and IgG antibody appear at the end of the first week of illness. Currently, the most widely applied in routine serological techniques include hemagglutination inhibition (HI), Immunoglobulin M (IgM) - capture enzyme linked immunosorbent assay (MAC ELISA), Complement fixation (CF), Neutralization tests (NT), and immunoglobulin G ELISA test. HI test is based on the principle that red blood cells are aggregated by dengue antigens when it is mixed with negative sera. However, the haemoagglutination is inhibited in the positive sera as it has anti-dengue antibodies to interact with dengue antigen[6]. The complement fixation test has more disadvantages, so it is not used for routine dengue diagnosis. Neutralization test identifies the primary serotype. This test is a sensitive and specific method for dengue viral diagnosis. It is a high cost method[7]. MAC-ELISA has good sensitivity and specificity but only used five or more days after the onset of fever. This method is possible for single serum samples that measure the IgMantibodies[8]. IgG ELISA is a method for serological diagnosis. This method identifies the IgG antibodies. The major limitation of these techniques is the high cross-reactivity[7]. Moreover, the dengue antibodies are detected around the fifth day of disease onset, making this technique unfeasible for rapid diagnosis[9].

1.2. Nucleic acid detection

Another traditional dengue diagnostic method is a direct method wherein a dengue virus and viral antigens are identified. Inoculation of newborn mice, inoculation on mammalian cell cultures, inoculation of adult mosquitoes and inoculation on mosquito cell cultures methods are used for viral isolation. Intracerebral inoculation and mammalian cell culture has more disadvantages. So, it is not recommended for the routine dengue diagnosis. In dengue virus isolation in the most sensitive or least method is mosquito inoculation. Compared to other techniques mosquito cell culture has many advantages. It is a standard, sensitive and process of several samples at the same time[7]. The various methods of molecular diagnosis are useful in the dengue viral diagnosis[3]. RNA-RNA hybridization is a sensitive technique used in the nucleic acid hybridization technique[7]. RT-PCR is mostly used for the plasma or serum samples. The combination of RT-PCR, IgM and IgG ELISA increases the accuracy and sensitivity of the laboratory diagnosis of dengue virus infection[9]. Multiplex real time RT-PCR assays are used to study the pathogenesis of dengue disease[6]. Real time RT-PCR performed using SYBR Green1 assay. It is a useful assay for the diagnostic test in early stages of DH and DHF and before the appearance of IgMantibodies[10]. The NASBA (Nucleic Acid Sequence Based Amplification) assay adapted to dengue virus. So, it is a useful method for studying dengue infections in field studies[6]. A prototype reverse transcription recombinase polymerase amplification (RT- RPA) assay detects the DENV genome without cross reacting with other arbovirus. Tan *et al*., (20018) recently reported single tube pan dengue reverse transcription recombinase polymerase amplification (RT-RPA) method for early detection of DENV[11]. This method is comparable to Real Time RT-PCR[-12]. The major impediment in dengue diagnosis is lack of specific, rapid and cost-effective assays tools. Viral antigen based detection methods are more specific. However, it is time-consuming, expensive; needs skilled technicians and sophisticated equipment, an inability to differentiate primary and secondary infections, and requiring acute samples. In case of a serological method, IgM antibodies can be detected only 4-6 days of infection. IgG specificity is reduced to cross-reactivity among Flaviviruses[13]. Recently, anti-peptide antibodies are used for the viral diagnosis. In our previous work, we have reported the interaction between anti-peptide antibodies that generated for three peptides and Dengue virus using DOT-PLOT and Indirect ELISA[14]. As the result was quite encouraging, we anticipated whether they can bind with the viral antigens in the patient's serum. Hence, the objective of the present study is to evaluate the potential application of three anti-peptide antibodies for diagnosing dengue virus and to examine their sensitivity and specificity by comparing clinical positive samples and negative samples using ELISA. The schematic diagram of using of peptide antibodies as diagnostic agents is illustrated in Figure 1. It describes the method of applying anti-peptide antibodies for diagnosing dengue virus. First, the patients serum sample, as source for viral antigen, is allowed to bind onto the substratum, over that anti-peptides antibodies are added. After that, enzyme conjugated secondary antibody is used to bind which will react with chromogen. The optical density of wells is directly proportionate with the viral load in the serum sample. Therefore, anti-pepide antibodies would pave a way for the early diagnosis of dengue with high sensitivity and accuracy with cost-effective in the near future.

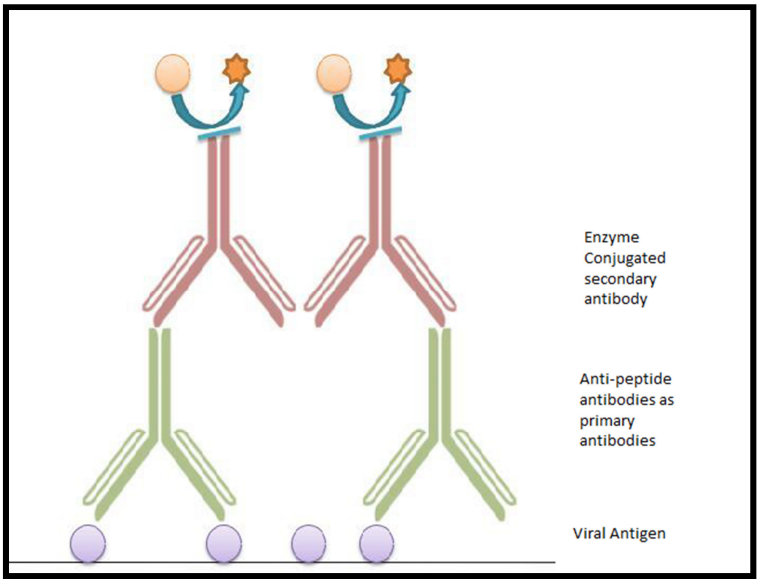

Fig 1. Schematic outline on the application of anti-peptide antibodies as diagnostic agents

2. MATERIALS AND METHODS

2.1. Sample collection from patients and healthy volunteers

The preserved serum dengue positive serum samples were obtained from Meenakshi Mission Hospital and Research Centre (MMHRC), Madurai, India, after they were tested positive with IgM, IgG and NS1 ELISA diagnostic kits. The study was approved by the ethical committee of MMHRC, Madurai, India (MMHRC/IEC/No. 12/2018). The samples consisted of dengue infected 32 male and 28 female patients, during the period of November 2018 to January 2019. The patient age group ranged from 3 to 75 years. Serum samples from 20 healthy volunteer blood donors from Madurai area in the age group of 17 to 55 years were used as a negative control after examining their health for recent viral infections, blood borne infectious diseases and fever. All serum samples were stored at -20°C prior to use.

2.2. Selection of peptides and generation of anti-peptide antibodies

Three synthetic peptides such as LEHGSCVTTMAKDKPTL of domain I, DRGWGNGCGLFG of domain II and CGQGKAHNGRLITANP of domain III of envelope glycoprotein of Dengue virus were used for the generation of anti-peptide antibodies. These peptides were chosen based on the *in silico* identification of immunodominat peptides in four serotypes of Dengue virus. The peptides were chemically synthesized by GenScript, USA. And then a standard immunization protocol was followed by immunizing rabbits with KLH conjugated synthetic peptides[14]. The specificity and interactions between peptides and anti-peptide antibodies were evaluated by DOT-BLOT and ELISA which was described by our previous report. A standard immunization protocol was followed by immunizing rabbits with KLH conjugated synthetic peptides[14].

2.3. Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed by following the protocol described previously[15]. One hundred microlitre of positive serum samples were added to the microtitre plates. 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, anti-peptide antibody (1:100 dilutions) 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. Horseradish Peroxidase conjugated Goat anti-rabbit secondary antibody was added 1:3000 dilution. The wells were then incubated for 45min at 37°C. After another wash with PBS-T, 100µl of the OPD-H_2O_2 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 H_2SO_4. The absorbance of colour in each well was read at 495 nm. The protocol was followed for optimization with the varying concentration of anti-peptide antibodies and different dilutions of positive samples in 1:10, 1:100, 1:1000, and 1:10000. Based on the standardization, the test samples and negative samples were also analyzed by ELISA at 70 µg 70 µg and 60 µg concentration of anti-peptide antibody-1, anti-peptide antibody-2, and anti-peptide antibody-3 respectively and the primary antibody was added in 1:1000 dilution.

2.4. Accuracy, Sensitivity, and Specificity Testing

2.4.1. Accuracy

The accuracy of a test is its ability to differentiate the patient and healthy cases correctly. To estimate the accuracy of a test, the proportion of true positive and true negative are evaluated in all cases. Mathematically, this can be stated as: Accuracy =TP+TN/TP+TN+FP+FN[16]**;** True positive (TP) = the number of cases correctly identified as patient; False positive (FP) = the number of cases incorrectly identified as patient; True negative (TN) = the number of cases correctly identified as healthy; False negative (FN) = the number of cases incorrectly identified as healthy.

2.4.2. Sensitivity

The sensitivity of a test is its ability to determine the patient's cases correctly[16]. To estimate it, the proportion of true positive was calculated in patient cases. Mathematically, this can be stated as:

Sensitivity = TP / TP + FN

2.4.3. Specificity

The specificity of a test is its ability to determine the healthy cases correctly. To estimate it, the proportion of true negatives was calculated in healthy cases[16]. Mathematically, this can be stated as:

Specificity = TN / TN+FP

3. STATISTICAL ANALYSIS

All statistical analyses were performed using MedCalc software (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.

4. RESULTS

Optimizing the concentration of Anti-Peptide Antibodies

Anti-peptide antibodies were generated and characterized for the three peptides. The sequences of three peptides, molecular weight and positions in envelope glycoprotein of Dengue virus are listed in Table 1. Anti-peptide antibodies are denoted as APA-1, APA-2 and APA-3. The concentration of anti-peptide antibodies for serological diagnosis was standardized from three different concentrations such as low concentration (1µl to 10µl), average concentration (20µl to 100µl with the interval of 10) and then high concentration (150µl and 200µl). Optimization of the concentration of anti-peptide antibodies was performed for detecting positive samples in different dilutions of primary antibodies. Primary antibody is the sera from positive and negative control groups.

Table 1. Synthetic peptides used for generating anti-peptide antibodies

Peptide no	Amino acid sequence	Position	Length	Molecular Weight (Da)	Structural domain	Anti-peptide antibodies
Pep 1	LEHGSCVTTMAKDKPTL	25 - 42	17	1831.13	Domain I	APA1
Pep 2	DRG WGN GCG LFG	98 - 109	12	1238.34	Domain II	APA2
Pep 3	CGQGKAHNGRLITANP	340 - 354	15+1	1636.84	Domain III	APA3

4.1. Diagnosis of positive samples by anti-peptide antibodies

All the clinical samples obtained from MMHRC were positively confirmed by commercial IgG, IgM and NS1 diagnostic kits. The samples were tested with three peptide antibodies for evaluating their potential use as a diagnostic agent. Of the 60 samples tested, anti-peptide antibodies-1 diagnosed 56 samples, anti-peptide antibodies-2 and anti-peptide antibodies-3 diagnosed 55 samples respectively (Supplementary Table 1). Statistical summary of anti-peptide antibodies diagnosed positive samples are tabulated in Table 2. Arithmetic mean is 0.3491 and the standard deviation is 0.12 for APA-1. APA-2 has the arithmetic mean value of 0.337 and the standard deviation is 0.1361. Anti-peptide antibody-3 has the mean value of 0.3091,standard deviation is 0.1108 with p value of <0.0001.

Table 2. Statistical summary of anti-peptide antibodies diagnosed the positive cases

Antibodies	Sample size	Arithmetic mean	Median	Standard deviation	Standard error of the mean	Significance level P
APA1	60	0.3491	0.377	0.1228	0.01373	<0.0001
APA2	60	0.337	0.3685	0.1361	0.01522	<0.0001
APA3	60	0.3091	0.33	0.1108	0.01231	<0.0001

4.2. Sensitivity and specificity of anti-peptide antibodies

A total of 60 positive samples and 20 negative samples tested, and the sensitivity of APA-1 was 91% whereas the specificity is 65% (Figure 2A). It diagnosed the diseased 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 distribution of data of healthy subjects and dengue patients are given in Figure 2B. Sensitivity of APA-2 was 82% whereas the specificity is 85% (Figure 3A). The results revealed that the sensitivity is less compared to APA-1, but 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 APA-3 was 90% whereas the specificity is 81% (Figure 4A). It diagnosed the diseased cases accurately without losing its specificity. It was found to be as high as 82%. 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.

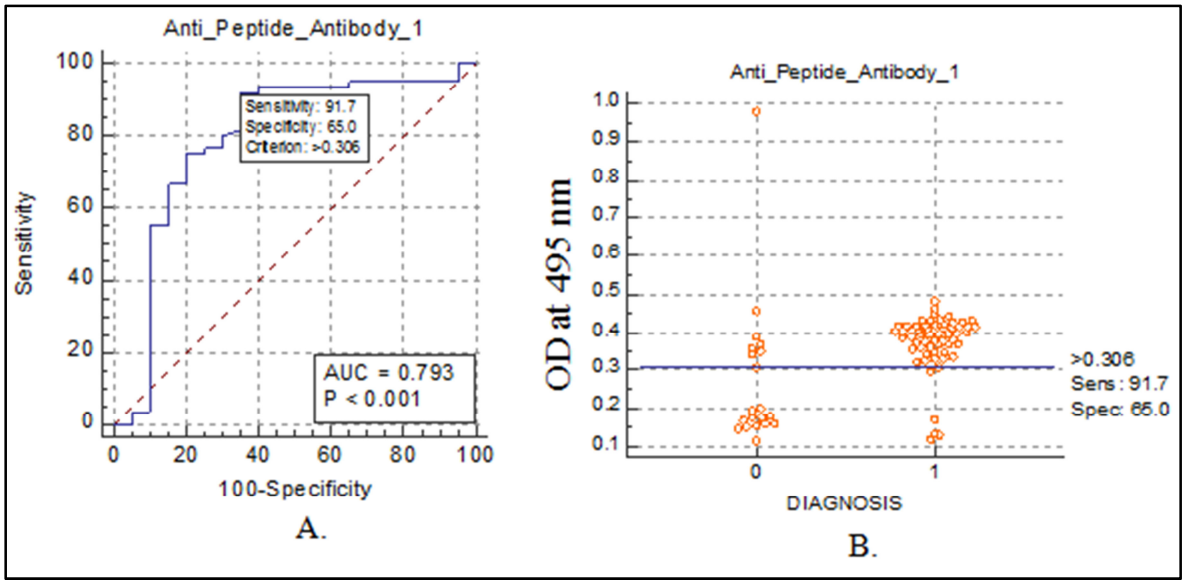

Fig 2. **Diagnostic potential of APA – 1.** *A. Sensitivity and specificity of APA-1. B. OD values of positive samples (>0.306) and healthy subjects (<0.306) using scatter plot*

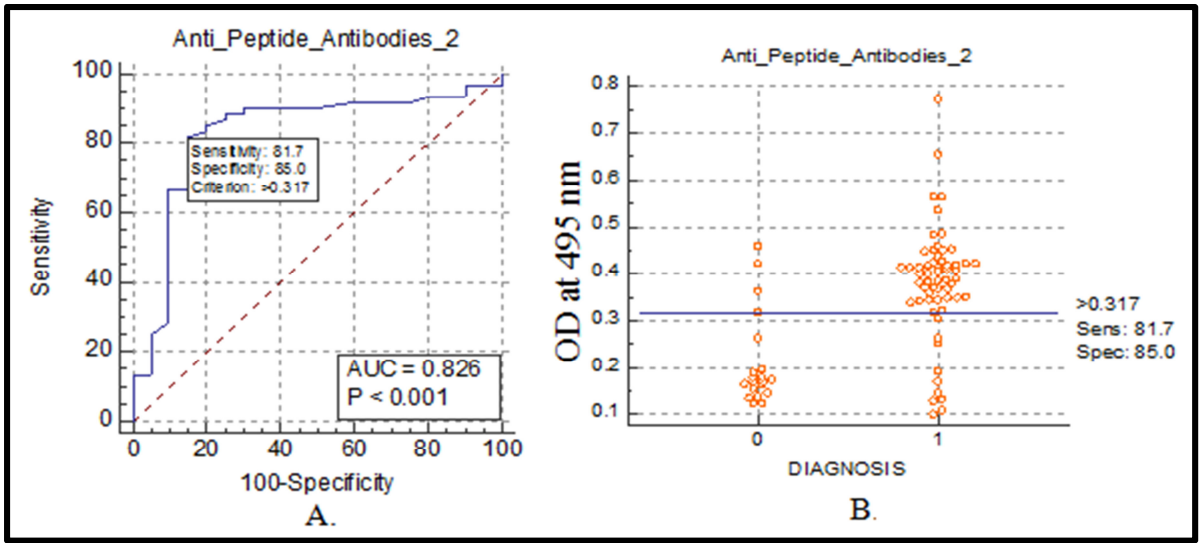

Fig 3. **Diagnostic potential of APA – 2.** *A. Sensitivity and specificity of APA-2 B. OD values of positive samples (>0.317) and healthy subjects (<0.317) using scatter plot.*

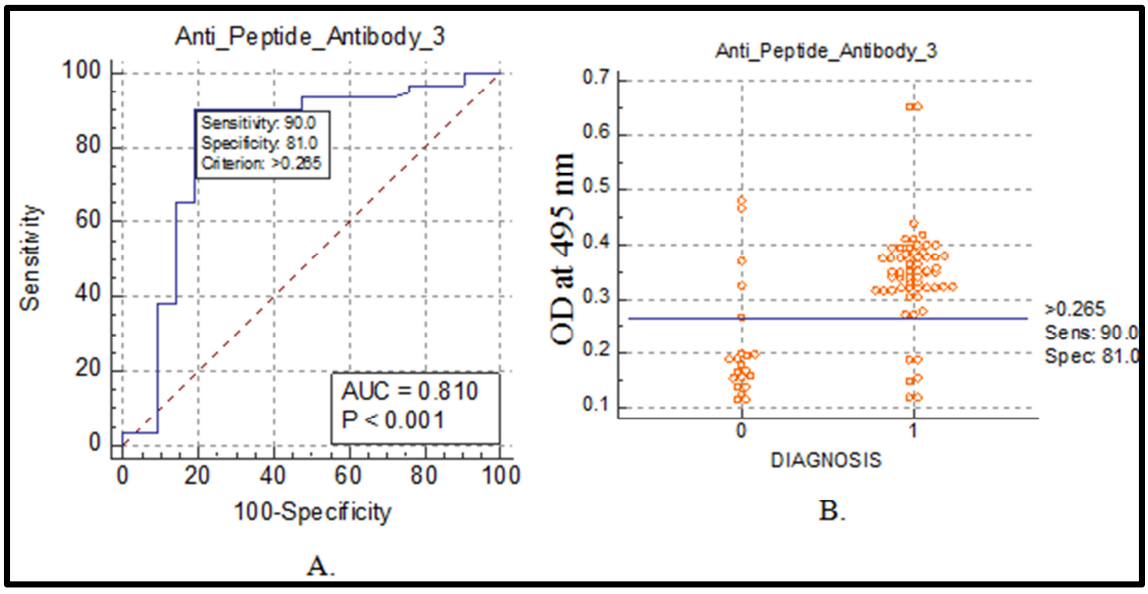

Fig 4. **Diagnostic potential of APA – 3.** *A. Sensitivity and specificity of APA-3 B. OD values of positive samples (>0.265) and healthy subjects (<0.265) using scatter plot.*

5. DISCUSSION

Antibody detection of dengue virus is a drawback due to delayed development of antibodies and the requirement for immediate diagnosis. Detection of viral antigen is an alternative approach instead of detecting the antibodies and viral nucleic acids. In this study, an antigen based detection assay was developed by using the anti-peptide antibodies. The antigen detection was performed based on indirect ELISA. The study reported that the antigen detection assay had a dependable sensitivity (91%, 82%, and 90% for APA-1, APA-2, and APA-3). The specificity of APA-2 (85%) is higher than the two other anti-peptide antibodies (65% and 81% for APA-1 and APA-3). It reveals that APA-2 can be further assessed for its complete potential in detection of viral antigen. This shows that the anti-peptide antibody has the ability to detect the presence of dengue virus in samples. Serum from negative samples may lead to false positive results, which has to be resolved to assure the accuracy of the detection assay. It is the first report interrogated anti-peptide antibodies against dengue viral antigen. However, this has been performed in other virus that proves the effectiveness of anti-peptide antibody. For instance, anti-peptide antibody has the ability to detect the presence of herpes simplex encephalitis virus[17]. Anti-peptide antibodies were used for diagnosis of central nervous system infections against viral antigen by ELISA[18]. Antibodies against dengue NS1 and prM proteins were found to increase the sensitivity of the dengue diagnosis until 15 days[19]. Moreover, detection of antibodies against both proteins was able to differentiate dengue from Japanese encephalitis infection. Fully mapped linear epitope on M protein of JEV was studied and identified one linear B-cell epitope[20]. The epitope was JEV specific and highly conserved among JEV strains. This data could provide an important basis for the potential application of epitope based virus specific diagnosis. Seven monoclonal antibodies recognizing NS1 of DENV were generated by hybridoma technology. These antibodies can be divided into two groups: serotype-specific (DB6-1, DB12-3, and DB38-1) and nonspecific (consisting of antibodies DB16-1, DB20-6, DB29-1, and DB41-2)[21]. This diagnostic platform displayed better specificity and sensitivity than two examined commercial NS1 diagnostic platforms. An anti-NS1 polyclonal antibody raised from a correctly refolded protein expressed by *E.coli* was able to recognize the NS2 found in human serum[22]. A western blot analysis confirmed that the purified anti-rNS1 polyclonal antibody was only able to recognize a single and specific band corresponding to the NS1 protein in infected patients. Polyclonal peptide antisera were used for the detection of YF-C and NS1 proteins and used antisera to investigate NS1 protein expression during YFV infection in mammalian cells and YFV target proteins were detected by all antisera in western blot and immunofluorescence assays[23]. rNS1 antigen was used to detect human anti-NS1 and IgM and IgG Ab. The anti-NS1 Ab response was found in 15 of 17 patients with primary dengue infection and all 16 patients with secondary dengue infection[24]. These results indicated that using the full length rNS1 whose antigenicity is restored as ELISA antigen, a high anti-NS1 antibody prevalence could be detected in patients with either primary or secondary dengue infection. The localization of the 14.7 KD NS2 in respiratory syncytial virus infected cells was detected on NS2 using anti-peptide antibodies elicited against predicted sequence of the NS2 protein[25]. This clearly demonstrated the specificity of the anti-peptide antibodies. An anti-peptide antibody could be used as a safe and specific tool for the diagnosis of infectious bursal disease in chickens. The purified anti-peptide antibodies were screened against native IBDV antigen and it reacted specifically with IBDV[26]. Anti-peptide antibodies and their performance were studied in the detection of surviving in human tumor tissues[27]. The ability of the antibodies to this peptide to detect surviving tumor tissue lysates was demonstrated by immunoblotting. Peptides also elicit immune response in the form of antibodies similar to proteins. Therefore, these anti-peptide antibodies can be used as a diagnostic tool.

6. CONCLUSION

Anti-peptide antibodies have been proven to be an effective tool in diagnosing viruses. Using anti-peptide antibody, antigen could be detected earlier which helps in improved diagnosis and early administration of the disease. In this study, it was found that the anti-peptide antibody has the ability to detect the presence of virus in samples. ELISA is a relatively simple technique and provides a low cost and quantitative analysis with high efficiency. This is the first-of-its-kind report on exploiting anti-peptides antibodies for the serological diagnosis of Dengue virus. The specificity and sensitivity of the anti-peptide antibodies are highly reliable and can be further improved to produce the necessary accuracy in detection of viral antigen. The specificity of anti-peptide antibodies-2 is higher than other two anti-peptide antibodies which showed promising results for diagnosis. The evaluation of combined actionof anti-peptide antibodies in different combinations can also be performed further to increase the sensitivity and specificity in detection of viral antigen.

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9. AUTHORS CONTRIBUTION STATEMENT

J. Asnet Mary conceived the project, designed the study, statistically analyzed data and wrote the paper. J. Jaya Supa Sooriya performed the work and analyzed data.

10. CONFLICT OF INTEREST

Conflict of interest declared none.

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