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Design and synthesis of multiple antigenic peptides and their application for dengue diagnosis

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ABSTRACT

Major difficulty in development of dengue diagnostics is availability of suitable antigens. To overcome this, we made an attempt to develop a peptide based diagnosis which offers significant advantage over other methods. With the help of in silico methods, two epitopes were selected from envelope protein and three from NS1 protein of dengue virus. These were synthesized in combination as three multiple antigenic peptides (MAPs). We have tested 157 dengue positive sera confirmed for NS1 antigen. MAP1 showed 96.81% sera positive for IgM and 68.15% positive for IgG. MAP2 detected 94.90% IgM and 59.23% IgG positive sera. MAP3 also detected 96.17% IgM and 59.87% IgG positive sera. To the best of our knowledge this is the first study describing the use of synthetic multiple antigenic peptides for the diagnosis of dengue infection. This study describes MAPs as a promising tool for the use in serodiagnosis of dengue.

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1. Introduction

Dengue is a vector borne disease caused by dengue virus (DENV) of the *Flaviviridae* family occurring throughout tropical and subtropical countries. Dengue virus is further classified into four serotypes (DENV1-4) each serotypes is capable of causing dengue. Recently a fifth serotype DENV-5 is also reported [1]. Dengue is considered the most important flaviviral disease in terms of mortality and morbidity. It is a potential public threat causing 50 to 100 million possible cases every year and around 3 billion people are at risk of infection [2]. Dengue infection exist in wide range of disease spectrum from mild infection to dengue fever (DF) to more fatal dengue hemorrhagic shock (DHS) and dengue shock syndrome (DSS).

Dengue virus share antigenic similarities with other flaviviruses that co-circulate in an endemic area that makes detection of anti-dengue antibodies difficult using commercially available kits as these kits use mixtures of inactivated virus preparations or recombinant envelope proteins [3]. The four serotypes of dengue virus are antigenically related but genetically different. Positive stranded RNA of dengue virus encodes for three structural proteins viz. capsid (C), pre membrane (prM), envelope (E) and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The protein ectodomain of the envelope glycoprotein consists of three structural domains (domain I, II and III) which help in viral attachment to host cells. Receptor binding region is located in EDIII domain of envelope protein [4]. This domain contains multiple type and subtype specific neutralizing epitopes [4–6]. EDIII is considered as an important candidate that can be considered for dengue diagnosis. Niu et al. [7], have developed a tetravalent protein by connecting the receptor-binding EDIII of the four serotypes using baculovirus expression system. This rEDIII protein resulted in 100% specificity and 93% sensitivity with predefined dengue sera. rEDIII produced in *P. pastoris* showed 86.96% sensitivity and 99% specificity for IgM and 86.97% sensitivity and 65.25% specificity for IgG with dengue positive sera [8].

Circulating NS1 in the serum of acute DF and DHF patients is one of attractive target for diagnosis. NS1 is a highly conserved glycoprotein, released from infected cells as soluble polymers as early as day 1 post-infection [9]. It remains in circulation for several days and is detectable in the serum of patients with primary and

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secondary dengue infections and elicits specific antibody response. However the level of NS1 antigen is comparatively less during secondary infection due to *anti*-NS1 antibodies. Recently Saito et al. [10] established the applicability of NS1 antigen detection by ELISA in urine samples of acute dengue infection. Sankar et al. [11], have described the use of *anti*-NS1 IgM antibody detection for acute dengue infection. High level of IgG in response to NS1 is observed exclusively during secondary infection [12].

In the absence of vaccine, the severity and transmission of dengue can be managed by early and accurate diagnosis of infection. The gold standard of dengue diagnosis is based on anti- envelope antibodies and NS1 antigen detection in serum. The serology assays include ELISA, indirect immunofluorescence, hemagluttination inhibition assay [13], microneutralization and biosensor based assays [14]. A number of commercial kits (Panbio Dengue early ELISA kit, Dengue NS1 Ag ELISA kit, Dengue NS1 Ag strip kit, SD BIOLINE dengue duo strip kit) have been developed and subjected to field evaluation. The sensitivity and specificity of these assays vary significantly depending on the dengue serotype and lineage. Conventional detection of dengue specific IgG and IgM antibodies strictly hampers dengue diagnosis in areas endemic for more than one flavivirus.

Considering the importance of E and NS1 proteins in the pathology of dengue virus infection, we selected these two proteins to identify epitopes for detecting anti IgM and IgG antibodies. DNAstar approach showed potential epitope regions in domain I, III of E protein and NS1 protein. Most of the epitopes possess secondary structure and hydrophilic in nature. The method described in this study offers significant advantage over other methods viz. ease of production, quality control and flexibility in inclusion of peptides from different regions of the viral proteins for antibody detection. We synthesized these epitopes as linear peptides and in combination as MAP and evaluated their efficacy by detecting anti IgM and IgG antibodies in NS1 positive sera.

2. Material and method

2.1. Study subjects

This study was approved by the human ethics committee, AIIMS, New Delhi. A total of 157 samples (between 2 and 10 days after onset of fever) were selected from Department of Medicine, Department of Microbiology, AIIMS, New Delhi and some were gifted from Himalayan Institute of Medical sciences, Dehradun. Ethical approval of this study was obtained from the Institute Ethics Committee, All India Institute of Medical Sciences (AIIMS), New Delhi, India (IEC A423/29–2013). These samples were dengue positive on the basis of clinical findings and NS1 antigen detection by commercial card test kit by Bhat Bio-scan (Bhat Bio-tech India, P, Ltd.)

2.2. Selection and synthesis of peptides

Whole protein sequences of Envelope and NS1 protein from all the strains of dengue virus were screened for hydrophilicity, secondary structures, antigenicity index, amphipathicity, using Bcepred (http://www.imtech.res.in/raghava/bcepred/)/DNAstar software for B cell and T cell prediction. Presence of these epitopes on protein surface was confirmed by PYMOL. 3D structure of protein was developed from SWISSMODEL. Protein sequences were adapted from DENV-2 (Accession no AEV66304.1; E protein and CAA78918.1; NS1), DENV3 (CAA78918.1; NS1), DENV 4 (AEV66304.1, E protein).

2.3. MAP synthesis, purification and characterization

For synthesis of MAP, main aim is to include different epitopes from E and NS1 protein in a single unit. The epitopes showing good prediction of antigenicity and conserved in most serotypes of dengue virus were selected and assembled as MAP. Multiple antigenic peptides (MAPs) were synthesized using Fmoc chemistry on Gly HMP-Tantagel resin as described by Pau et al., [15]. At the N terminal end of each branch, t-Boc protected amino acid was used to prevent further chain elongation. Fmoc-Lys(ivDde)-OH (novabiochem) was incorporated at the beginning of the synthesis for each branch to serve as the branching point. Kaisers Test was performed after addition of every amino acid to check the presence of any free amino acid. Palmitate was attached at the amino terminus. The MAP was purified by gel filtration chromatography, lyophilized and stored at room temperature.

2.4. ELISA

MAPs (400ng/100 μl diluted in 0.05 M carbonate bicarbonate buffer pH 9.6) were coated in the wells of microtitre plates for 4 h at 37 °C. After coating, the wells were washed three times with PBS/0.05% Tween 20 (PBS-T) and blocked with 200 μl blocking solution (5% skimmed milk powder in PBS-T) overnight at 4 °C. After washings, Chikungunya (CHIKV), DENV and negative sera (1: 100 dilutions) was added in duplicate wells and plates were incubated for two hour at 37 °C. Antigen-antibody complex was detected using goat anti human IgM/IgG antibodies conjugated with HRP (1:2000). The plates were washed as above and color was developed with substrate solution containing TMB. The reaction was stopped by adding 100 μl of 1 N sulphuric acid and optical density (OD) was measured at 492 nm. CHIKV sera were used to check the MAPs reactivity towards non-dengue virus. The graphs were prepared by GraphPad Prism software version 5.0.

3. Results

On the basis of antigenic index, surface probability and hydrophilicity obtained from Bcepred/DNAstar software, we have selected six peptides from envelope and three from NS1 protein. These peptides were combined as MAPs of various combinations (Table 1). Surface localization of these selected peptides was necessary to confirm their location on the protein. Surface localization of these sequences was done by PYMOL software program (Fig. 1). From NS1 protein, peptide sequences DNVHTRTEQYKFQPESPSKLASA (NS1.1), IMQVGKRSLRPQPTELRYSWK (NS1.2) and WLRLREKQDAFCDSKLMSAAIKDNRAVHA (NS1.3) are present on the surface of the peptide because they are hydrophilic in nature. From the peptides selected from envelope protein, peptide sequence RGARRMAIL (EDIII.1) was partially buried inside because of the presence of few hydrophobic amino acids. The peptide sequences DFGSVGGVL (EDIII.2) is present on the surface of the protein because of its hydrophilic nature.

3.1. Cutoff value calculation

A cutoff value was determined for MAPs to evaluate their diagnostic efficiency with patient sera. A total of 157 DENV positive, 10 CHIKV positive and 15 healthy human sera were screened using MAPs. Based on the seroreactivity of the 15 healthy human sera tested for IgM, the cut off values for different MAPs were in the range of 0.34–0.37. Based on the cut-off value for these MAPs, we examined the seroreactivity of dengue positive and CHIKV positive

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