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Simplified immunoassay for rapid Dengue serotype diagnosis, revealing insensitivity to non-specific binding interference

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ABSTRACT

Proof of concept of an immunoassay, which is easy to implement, for rapid Dengue virus (DENV) serotype diagnosis, in the early infection stage, is reported. The four-layer assay is immobilized onto a thin gold film and relies on a low cost, disposable polymer biochip for optical surface plasmon resonance sensing and detection. The protocol comprises Neutravidin-Biotin mediated monoclonal antibody (MAB) attachment as the functionalized sensing element. Formation of the MAB-DENV complex results in a pronounced thickness change that is optically recorded in real time, employing a microfluidic set-up. Virus presence is confirmed by atomic force microscopy from the same sample. Serum samples were collected from a patient in acute febrile state. Simultaneous serological analysis by means of the reverse transcription polymerase chain reaction, independently, confirmed presence of DENV2 and DENV3. The protocol proved applicable in presence of strong non-specific binding interference that originates from, and is caused by, various blood, serum and other body fluid constituents. False positive indications for both, negative serum and blood control samples were not observed. The achievable limit of detection was estimated to be 2×10^4 particles/ml. Eventually, the method can be modified towards detection of other viruses by using the same protocol.

1. Introduction

Since no commercial vaccination is yet available, infections with the Dengue virus (DENV) and Zika virus [1] are a persistent threat in many densely populated urban as well as rural areas in tropical and subtropical regions. Dengue fever outbreaks reveal an apparent seasonal oscillating cycle, in relation with dry and wet seasons. The day active mosquitoes *Aedes aegypti* and *Aedes albopictus* act as vectors of the DENV and Zika that belong to the genus *Flavivirus* of the Flaviviridae family. The virus is present in four serotypes, DENV-(1-4), which substantially differ in their antigenic activity [2]. Infections exhibit a broad clinical spectrum that ranges from weak to strong influenza like symptoms, known as Dengue fever (DF) to severe forms, denoted as Dengue hemorrhagic fever (DHF) and, revealing much higher mortality, the Dengue shock syndrome (DSS). Commonly, a first infection features relatively mild influenza type symptoms, along with temporally high fever, rather clearly diagnosed by experienced clinical personal. Second infections frequently require comprehensive clinical treatment.

It is now understood that appearance of DHF or DSS is more likely to occur with a secondary infection, while involving exposure to a different DENV serotype [3]. Simultaneous or alternating appearance of different DENV serotypes during epidemic phases thus may lead to critical clinical conditions, and result in a substantial increase of DHF and DSS cases. High virulence is actually reported for the DENV serotype 2 in the Northeast Region of Brazil. For required immediate and appropriate medical treatment in specialized medical institutions for tropical diseases, as well as epidemic control, it therefore would be beneficial to clearly and rapidly identify appearance and local distribution of DENV serotypes. This requires serological analysis. Considerable DENV titer has been observed in serum, saliva and urine [4]. Moreover, serological DENV diagnosis is time critical: in

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the initial febrile phase, which commonly lasts from 3 to 7 days, the virus concentration is high. After a longer period, due to the body immune response, it drops below the detectable amount, while the concentration of DENV specific antibodies IgG and IgM increases [5].

Present diagnostic methods, such as RT-PCR (reverse transcription polymerase chain reaction) and a recently developed ELISA (enzyme-linked immunosorbent assay) kit for the NS1 (non-structural glycoprotein-1) viral protein require a quite demanding laboratory infrastructure, well trained personal, and are time consuming. Moreover, transport of frozen serum or blood samples over a long distance from remote areas to laboratory centers is almost excluded in most tropical countries with poor infrastructure conditions. Here, an attempt has been made to develop an easy applicable, affordable and disposable assay and associated diagnostic device. It is anticipated for use in small local medical institutions, designed as a so-called PoC (Point of Care) device, eventually being operated by available medical personal.

Surface plasmon resonance (SPR) sensing, in connection with a specially designed polymer biochip, is used in this communication for real time monitoring of virus attachment, directly out of diluted patient serum onto a nanometer thin layer of monoclonal antibodies to DENV2. The functionalized biochip is loaded into a separate optical instrument, denoted as the SPR reader. It houses all components, as a micro-fluidic cell for transporting the diluted serum analyte to the sensing chip surface, as well as optical and electronic components for optically monitoring the SPR phenomenon and indicating DENV presence.

There exists a relatively large body of literature, which describes the optical SPR sensing technique, and which is out of the scope of this communication [6]. Principally, SPR it is a non-labeling analytical sensing method [7]. Unlike ELISA or Immunofluorescence, where specific adsorbents cause color changes or fluorescent emission, an SPR sensor detects specific binding of an antigen to an associated antibody - or vice versa - by the increase of the total layer thickness or mass coverage, respectively, as a result of antigen-antibody complex formation [8]. However, the composition and constituents of body fluids like urine, saliva, blood, plasma, account for pronounced non-specific binding effects that interfere with the specific binding event. Until today, this prevents that SPR and other non-labeled methods are reliably and routinely employed for medical diagnostics [9,10]. Numerous attempts have been reported towards reduction of non-specific binding effects [11–13]. In this work, a practical method is presented, which largely eliminates the abovementioned severe limitations [14].

2. Materials and methods

Blood samples were collected by venipuncture from a mid aged patient at the Manaus clinic of the Tropical Medicine Foundation of Amazonas (FMTAM), suspected of acute Dengue infection, with 2 to 5 days of symptoms. Several samples were centrifuged and then frozen and stored at −70 °C. A first serum fraction was used [15] for pre-analysis by PCR and virus isolation. Extraction of viral RNA was performed with the QIAmp viral RNA Mini Kit (QIAGEN), following manufacturer's instructions. For reverse transcription (RT), AccessQuick Kit™ RT-PCR System (Promega) was used. After RT, each cDNA was amplified by the procedure developed by Lanciotti et al. [16]. The PCR primers used are universal for Dengue (DENV-1 and DENV2). Then, using serotype-specific primers, during the nested PCR reaction, the Dengue serotype was clearly identified as DENV2. Details of this bio-molecular analysis are out of the scope of the present work.

Furthermore, for the serological diagnosis, the detection of viral protein NS1 was performed with the Platelia Dengue NS1 AG test, according to the manufacturer's instructions. The Platelia Dengue NS1 Ag assay, developed by Bio-Rad in partnership with the Pasteur Institute in France, is an ELISA test and enables the detection of NS1 Dengue virus antigen, as soon as the first clinical signs appear. The ability to detect the presence of the NS1 antigen offers earlier detection of the infection, compared to the conventional serological methods currently used.

Dengue virus isolation of the used infected serum samples was performed by two of the authors at Laboratório Entomologia Aplicada and Fundação de Medicina Tropical both from Manaus, Amazonas by using the method of culture in continuous cell lines, being selected mosquito cells C6/36 (*Aedes albopictus* clone), based on the work by Igarashi [17]. The technique used for identification of cultures showing, or not, CPE (Cytopathic effect), relied on the immune-fluorescence of the Dengue serotypes. Highly specific monoclonal antibodies (MAB_D) against each of the four Dengue virus serotypes were provided by Oswaldo Cruz Foundation (FIOCRUZ), again identifying DENV2. Required fluorescein-conjugated secondary antibodies were obtained commercially.

Two remaining original serum samples (0.5 ml, freeze stored at −80 °C over several weeks) have been 10:1 diluted with PBS solution for the SPR immune sensing analysis, as outlined here. The immunoassay under consideration comprises a four layer arrangement, following a recently suggested route, utilizing Neutravidin (NA)-Biotin mediated antibody attachment. A sketch is illustrated in Fig. 3, where the protocol reads as follows: first, a self assembled mono-molecular layer of sulfonated Biotin (Sulfo-NHS-S-S-Biotin, SB) is attached onto the gold film surface. The SB-layer binds via its disulfide groups covalently onto the gold metal surface below. Its outer Biotin molecules connect to the next, layer above, which is a mono-layer of attached NA. This protein is a tetramer, with four identical domains for Biotin binding, therewith forming a molecular bridge between the biotinylated DENV specific MAB (MAB_D) above, and the SB layer below. The diluted serum is then admitted and flowing over the functionalized surface. DENV antigen, if present in the solution, will attach onto the outermost immobilized layer of MAB_D's.

The surface functionalization scheme, applied in this work, was taken from Ref. [18]. Briefly, a clean surface of a 50 nm thin gold layer is an essential pre-condition for efficient activation of a disposable optical SPR chip. Its physical appearance, along with a sketch of the SPR function are illustrated in Fig. 2, and described in detail in Refs. [19,20]. Possible organic and inorganic surface contaminations on the gold surface were removed by storage over a period of 3 h in hot 30% hydrogen-peroxide solution. The surface afterwards is rinsed with de-ionized water, nitrogen dried and again rinsed with 100% ethanol. Subsequently, 1 mM SB is dissolved in ethanol (p. A grade) and the optical chip kept for 1 h in the solution, in order to deposit a mono-molecular SB-binding layer for NA attachment. SB and NA were purchased from Pierce Inc., while biotinylated MAB's against DENV2 were obtained from Genway Biotech, Inc, both from the USA. Subsequently, the chips were rinsed with ethanol, and stored in a PBS (Phosphate Buffered Saline) solution at 4 ° C over an extended period. A clear indication and test for self assembled mono-molecular formation was the presence of a strong hydrophobic, i.e. water repelling surface.

Afterwards, the SB coated optical SPR chip, is loaded into the SPR reader instrument, as illustrated in Fig. 4. This optical instrument operates in the so-called angular interrogation mode, utilizing the angular position of the surface plasmon resonance. It houses a diode laser light source, lenses for optical beam guidance and propagation, an image detector, along with required electronic components. The SPR resonance manifests itself in Fig. 5 as a black line in the electronically recorded and digitized image, where radiation is absorbed, and it exhibits approximately a parabolic intensity profile. The SPR response can be determined using Fresnel's theory of reflection of light at each interface of the four layer system shown in Fig. 1. The intensity reflection coefficient for a four layers system can be written