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Immunospot assay based on fluorescent nanoparticles for Dengue fever detection

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ABSTRACT

Dengue fever is one of the most neglected tropical diseases and of highest international public health importance, with 50 million cases worldwide every year. Early detection can decrease mortality rates from more than 20% to less than 1% and the relevant early diagnosis analyte is the viral non-structural glycoprotein, NS1. Currently, enzyme linked immunosorbent assay (ELISA) is the method of choice to detect NS1. However, this is a time consuming method, requiring 3–5 h, and it is the bottleneck for routine of clinical analysis laboratory in epidemic periods, when hundreds of samples should be tested. Here we describe an easy method combining principles of fluorophore linked immunosorbent assay (FLISA) and enzyme linked immunospotting (ELISPOT). For detection, we used mouse anti-NS1 IgG labeled with fluorescent nanoparticles. The presented procedure needs only 4 μL of serum samples and requires 45–60 min. The detection limit, 5.2 ng/mL, is comparable to ELISA tests. The comparison of 83 samples with a commercial ELISA revealed a sensitivity of 81% and specificity of 88%. The use of fluorescent nanoparticles provides a higher sensitivity than an assay using usual fluorescent dye molecules, besides avoiding bleaching effects. Based on the results, the proposed method provides fast, specific and sensitive results, and proves to be a suitable method for Dengue NS1 detection in impoverished regions or epidemic areas.

1. Introduction

Neglected tropical diseases (NTD) are a multiple of diverse diseases strongly associated with poverty, flourishing in impoverished environments and thriving best in tropical areas. Due to these characteristics, they have low visibility in the rest of the world and limited access to support services. Among the NTD, Dengue fever (DF) has emerged as a rapidly spreading vector-borne disease affecting mainly poor, urban populations and it is also the leading cause of hospital admissions in several countries (WHO, 2010). DF results from infection with a virus transmitted mainly by *Aedes aegypti*, a species of mosquito with a global distribution. The incidence of DF has dramatically grown over the world in recent decades. Over 2.5 billion people are now endangered from Dengue. The World Health Organization (WHO, 2012) currently estimates 100 million cases of Dengue fever, 500,000 cases of Dengue hemorrhagic fever (DHF) and resulting in 2.5% of annual mortality. The disease is now endemic in more than 100 countries in Africa, America, Eastern Mediterranean, South-east Asia and Western Pacific. The threat of a possible outbreak of DF now exists in Europe and local transmission of DF was reported for the first time in 2010. For DHF, medical care can save lives, decreasing mortality rates from more than 20% to less than 1% (Allwinn, 2011).

To provide medical care, DF diagnosis should be fast, specific, sensitive and with low cost. Nowadays, there are different tools used for diagnosis by isolating the virus, detecting viral antigen or RNA in serum or tissues (Levi et al., 2007), and detecting specific antibodies or proteins in the serum (Guzman and Kouri, 1996). Serologic tests have been routinely used for diagnosis of DF due to their simplicity and rapidity in comparison to the other methods. Enzyme linked immunosorbent assay, ELISA, has become the most widely used serologic test for DF diagnosis in the past few years due to its simplicity and little sophisticated equipment (Gubler, 1998). These assays are predominantly based on the detection of immunoglobulin, which consists of IgM and IgG antibodies produced against the virus at day 5 or 6 of illness (Vaughn et al., 1997). Due to the necessity of diagnosing DF in

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early stages, a highly conserved viral non-structural glycoprotein (NS1) of the Dengue virus has been used as a high potential analyte target for early Dengue detection (Datta and Wattal, 2010; Linares et al., 2012). Dengue NS1 antigen has been detected in the serum of DF infected patients as early as one day post onset of symptoms. During acute phase, high NS1 level exists as membrane-associated and secretory forms by the virus; both forms are demonstrated to be immunogenic (Lima et al., 2010).

NS1 detection using ELISA is based on the principle of a solid phase sandwich enzyme-linked immunosorbent assay with colorimetric detection. A monoclonal antibody is adsorbed on the solid phase and works as capture antibody for binding the antigen. Another monoclonal antibody conjugated to an enzyme is used to detect the antigen through a colorimetric enzymatic reaction (Najioullah et al., 2011). Although Dengue NS1 ELISA offers a sensitive approach with detection in the low ng/ml range, the technique also shows some disadvantages. ELISA tests require a large number of incubation and washing steps. These make the procedure difficult to automate for screening large sample number, and significantly prolong the time up to 5 h to get the results (Alcon et al., 2002; Young et al., 2000). In addition, in many cases, efficient antibody adsorption on microplates requires overnight incubation time. For routine tests in clinical analysis laboratories during epidemic periods, the test duration is relatively long, as hundreds of samples should be tested at the same day (Liu et al., 2003; Velappan et al., 2008).

Analogous to ELISA principles, another technique has been used for DF detection, which are based on enzyme-linked immunospot (ELISPOT). The term immunospot has been used to refer to a method, where antibodies are immobilized on membrane, compounds secreted by cells are captured and an enzyme conjugated antibody provides the colorimetric detection (Bathoorn et al., 2011; Franci et al., 1986). The use of paper, such as nitrocellulose membranes, has provided an useful and simple base for fast and low cost tests (Liu et al., 2011; Martinez et al., 2010). Cardosa et al. (1988, 1995) reported the development of a dot enzyme immunoassay for the detection of Dengue antibodies. This formed the basis of a commercial Dengue blot kit in which the viral antigens are bound to nitrocellulose membranes instead of microplates. The antibodies are visualized using protein A, which is labeled with horseradish peroxidase (Lam et al., 1996). Although this assay allows field application with diagnosis in peripheral health settings due to the use of membrane as solid support, the tests also involve several time consuming steps, which turns to the same practical disadvantages observed for ELISA.

While efforts have been described to overcome ELISA limitations (Chunglok et al., 2011; Pião et al., 2009; Piletsky et al., 2001; Wan et al., 2012), fluorophore linked immunosorbent assays (FLISAs) have been proven to be a powerful alternative that uses fluorescence rather than enzymatic activity for detection and thus overcome some of the disadvantages of ELISA (Cummins et al., 2006; Liu et al., 2003; Velappan et al., 2008). Compared to sandwich ELISA, this assay has several advantages: rapidity (the time is reduced from 5 to 1.5 h), simplicity and lower costs due to the use of fewer reagents. FLISA still shows similar specificity and sensitivity as the sandwich ELISA (Liu et al., 2003). New approaches have also been described to improve FLISA performance. Miller et al. (2006) used fluorescent dyes conjugated to antibody coated polystyrene beads to detect the analyte on the focus plane by confocal laser scanning microscopy. High sensitive assays have been also described using quantum dot-based fluorescence-linked immunosorbent assays, enhancing the fluorescence signal provided by high intensity of a particle in comparison to a dye (Zhu et al., 2011; Peng et al., 2009).

In this work, we present an immunospot test for Dengue detection, combining FLISA advantages with principles of ELISPOT as an alternative method to replace sandwich ELISA in impoverished regions or epidemic areas. The advantage of combining both techniques is the development of a sensitive and fast sensor. Spot tests based on fluorescence detection have already shown useful combinations of high sensitivity and low complexity (Linares et al., 2007). In the proposed test, a low volume of serum sample is applied onto a nitrocellulose membrane and mouse anti-NS1 IgG labeled with fluorescent nanoparticles are used for Dengue detection. The test takes less time and the fluorescent signal can be measured with a fluorometer or alternatively with an UV-lamp. The possibility of applying the sample directly to a high surface area nitrocellulose membrane allows the use of low volume of samples and avoids the necessity of two antibodies for capture and detection. The use of fluorescent nanoparticles provides lower detection limit in comparison to conventional FLISA, additionally avoiding bleaching effects. As a general view, the immunospot test allows diagnosing DF in less than one hour with high specificity and sensitivity, less complexity and using conventional apparatus in clinical analysis laboratories.

2. Materials and methods

2.1. Chemicals and materials

Bovine serum albumin (BSA), glycine, *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), anti-mouse IgG–FITC antibody, dialysis membranes (MWCO 100 kDa and 130 kDa), Tween 20, sodium carbonate and bicarbonate, potassium phosphate mono- and dibasic were purchased from Sigma-Aldrich (Munich, Germany). FluoSpheres carboxylate-modified microspheres, 5% (w/w), [yellow–green (505/515 nm), orange (540/560 nm) and red (580/605 nm)], 0.04 μm, were obtained from Invitrogen (Carlsbad, United States). Nitrocellulose AC100 membrane and absorbent pad were purchased from Whatman (Maidstone, United Kingdom). ELISA microplate, F96 MicroWell™ plates, was obtained from NUNC (Roskilde, Denmark).

Dengue Virus NS1 glycoprotein mouse monoclonal antibody was obtained from Abcam (Cambridge, United Kingdom). Purification procedure is described in Supplementary Information. NS1 Ag ELISA kits were purchased from Standard Diagnostics (Hagal-Dong, Korea) and Biorad Laboratories (Marnes-La-Coquette, France).

2.2. Serum samples

A total of 83 serum samples were obtained by the São Paulo Institute of Tropical Medicine, University of São Paulo. The tests were approved by the ethics committee from the university. The samples were analyzed for NS1 using the commercial kit Platelia™NS1 (Biorad Laboratories, Marnes-La-Coquette, France). Dengue diagnostic is described in the supplementary information.

2.3. Synthesis of polystyrene nanoparticle–albumin conjugates

An aliquot of 1 mg of albumin at 3 mg/mL was dissolved in phosphate buffer 0.01 mol/L, pH 7.4. Then, 500 μL of a 2% aqueous suspension of carboxylate-modified sphere was added and incubated at RT for 30 min. Subsequently, 0.5 mg of EDC was added and mixed by vortexing and the pH was adjusted to 6.5 ± 0.2 with diluted NaOH. The dispersion was incubated on a shaker for 3 h at RT. To remove unbound proteins, dialysis was performed through a membrane with MWCO of 100 kDa against phosphate buffer 0.01 mol/L, pH 7.4, during 24 h and 5-times change of the external solution.