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A custom-designed recombinant multiepitope protein as a dengue diagnostic reagent

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Abstract

Currently, dengue fever is the most important re-emerging mosquito-borne viral disease, with the major proportion of the target population residing in the developing countries of the world. In endemic areas, potentially fatal secondary dengue infections, characterized by high anti-dengue IgG antibody titers, are most common. Most currently available commercial dengue diagnostic kits rely on the use of whole virus antigens and are consequently associated with false positives due to serologic cross-reactivity, high cost of antigen production, and biohazard risk. This has prompted the need to develop an alternate antigen to replace the whole virus antigen in diagnostic tests. We have designed and expressed a novel recombinant protein antigen by assembling key immunodominant linear IgG-specific dengue virus epitopes, chosen on the basis of pepscan analysis, phage display, and computer predictions. The recombinant dengue multiepitope protein was expressed to high levels in *Escherichia coli*, purified in a single step, yielding >25 mg pure protein per liter culture. We developed an in-house enzyme-linked immunosorbent assay (ELISA) to detect anti-dengue antibodies in a panel of 20 patient sera using the purified recombinant dengue multiepitope protein as the capture antigen. The ELISA results were in excellent agreement with those obtained using a commercially available diagnostic test, Dengue Duo rapid strip test from PanBio, Australia. The high epitope density, careful choice of epitopes, and the use of *E. coli* system for expression, coupled to simple purification, jointly have the potential to lead to the development of an inexpensive diagnostic test with a high degree of sensitivity and specificity.

Keywords: Dengue virus; Multiepitope protein; Dengue diagnostic test; Ni–NTA chromatography

Dengue fever (DF) and its more severe manifestations, namely, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are caused by infection with the mosquito-borne dengue viruses, which are members of the family *Flaviviridae* [1]. There are four closely related, antigenically distinct, serotypes (1–4) of dengue viruses, each of which can cause disease. In recent decades, there has been a dramatic increase in the incidence dengue infections, with about 100 million cases of DF occurring each year [2]. Globally, about 2.5 billion people are estimated to be at risk from dengue. The lack of a licensed dengue vaccine, in conjunction with predicted climatic changes and population growth is projected to place 5–6 billion people at risk of dengue transmission in the coming decades [3].

Dengue infections may be clinically inapparent or may result in non-specific febrile illness, DF or DHF [4,5]. Severe plasma leakage can lead to fatal DSS and

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mortality rates for untreated patients can be as high as 40–50% [6]. Early diagnosis, followed by supportive care, and symptomatic treatment through fluid replacement are the keys to survival in cases of severe dengue infection [4,7]. Definitive diagnosis of dengue infection depends on the identification of the virus, virus-encoded antigens, viral genomic RNA or the virus-induced antibodies [2,8]. Virus identification through its isolation can take several days and may not always be successful due to very small amounts of viable virus in the clinical samples. Viral antigens can be detected by immunohistochemistry or immunofluorescence. However, the complexity of these assays and their high cost preclude their routine use. Viral RNA can be detected with a high degree of sensitivity, using coupled reverse transcription and polymerase chain reaction (RTPCR). Besides being expensive and requiring sophisticated equipment, the RTPCR assay is subject to wide variability, as demonstrated in a recent study conducted by the European Network for Diagnostics of Imported Viral Disease [9]. Further, a shortcoming common to all these methods is the narrow time window (~5 days), available for successful detection, which coincides with the febrile period during which patients are viremic [10,11]. Often, dengue-infected patients do not seek immediate medical care as the initial manifestations are usually asymptomatic or mild fever. This precludes diagnostic tests based on the identification of the virus or its RNA genome because of the short duration of viremia. Thus, in a majority of cases the only feasible diagnostic test would have to be based on the identification of anti-dengue antibodies.

Numerous dengue diagnostic kits, in a multiplicity of formats, have become available recently [12]. Most of these kits rely on the use of whole virus antigens (produced in tissue culture or suckling mice brain) for the detection of anti-dengue antibodies in patient sera, and are consequently associated with an inherent biohazard risk. One kit, which has replaced the whole virus antigen with insect cell-expressed dengue envelope protein, eliminates this risk [13]. However, all these kits are expensive due to the high costs associated with antigen production, making them unaffordable for use in the economically weaker countries where dengue is mostly prevalent. Apart from this, a major shortcoming of the commercial kits is that they do not differentiate between infections due to dengue and other flaviviruses (such as Japanese encephalitis and yellow fever viruses). Additionally, sera from patients with typhoid, malaria, and leptospirosis also tend to score positive using these kits.

There is currently a need for developing cost-effective, safe, and simple diagnostics that combine sensitivity and specificity. In dengue endemic areas, secondary infections (infection of individuals seropositive for dengue due to a prior exposure) are most common and often associated with DHF and DSS [10,14]. During secondary infection with dengue virus, high levels of IgG serum antibodies appear within 3–5 days after onset of illness, peak by about 2 weeks and then decline gradually during the following several months [4]. As these infections are characterized by significant serum IgG titers, we focused on developing a recombinant antigen designed to detect the IgG class of anti-dengue antibodies. To this end, we adopted a novel approach that entails the creation of a multiepitope protein consisting of several key IgG-specific, immunodominant epitopes, encoded by the major structural envelope (E) protein and the non-structural (NS) proteins 1 and NS3 [1]. The E protein is the major structural component [15] and the most immunogenic of all the dengue viral proteins, eliciting the first and longest-lasting antibodies [16,17]. Immunodominant epitopes on the E protein are well-documented [18–20]. Amongst the non-structural proteins, NS1 [21–24] and NS3 [16,22] are reported to elicit significant antibody responses, particularly in secondary infections [25], with the former being more immunogenic than the latter. Epitopes on these proteins have been mapped using synthetic peptides spanning entire proteins [18,21] or defined regions based on computer predictions [22,23], phage displayed peptides [24,26] and recombinant fragments [19,20] on the basis of reactivity towards patient sera [18,22] or monoclonal antibodies [19–21,24,26].

This multiepitope protein, r-DME-G (recombinant dengue multiepitope protein, specific to IgG), was expressed to high levels in *E. coli* and purified efficiently in a single step by affinity chromatography. Using an in-house enzyme-linked immunosorbent assay (ELISA), we demonstrate that this recombinant synthetic protein is able to accurately identify patient sera that contain anti-dengue virus antibodies. In this paper, we describe the design of r-DME-G protein, its expression, purification, and a preliminary evaluation of its utility to serve as a diagnostic reagent in the detection of dengue infections.

Materials and methods

Materials

Escherichia coli host strain DH5α for routine recombinant plasmid manipulations was purchased from Invitrogen, USA. *E. coli* host strain SG13009, harboring the *lacI* repressor encoding plasmid pREP4 (kan^r), for recombinant protein expression was from Qiagen, Germany. The plasmid pQE60 (amp^r), Ni–NTA superflow resin, and anti-His monoclonal antibody (catalog 34660) were also from Qiagen. All secondary antibody–enzyme conjugates [anti-mouse IgG-alkaline phosphatase (AP), anti-mouse IgG-horseradish peroxidase (HRPO) and anti-human IgG-HRPO], and the AP substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) were from Calbiochem, USA. The HRPO substrate 3,3′,5,5′-tetramethylbenzidine (TMB)