Discrimination between primary and secondary dengue virus infection by using an immunoglobulin G avidity test

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Abstract

Discrimination between primary and secondary dengue infections is important, as the possibility of DHF is more in secondary infection. Therefore, there is need to develop a test that can distinguish between primary and secondary serological responses. The traditionally-used haemagglutination inhibition (HI) test, which is recommended by the World Health Organization, is complicated to perform. We standardized an enzyme-linked immunosorbent assay kit with some modifications to discriminate between primary and secondary dengue infections. Sera from 72 patients with acute dengue infection were tested. Seventy-one of the 72 patients were correctly classified (18 of 18 patients with primary dengue and 53 of 54 patients with secondary dengue). We conclude that this rapid and simple test is an excellent alternative to the HI test for discriminating between primary and secondary dengue virus infections during the acute phase of dengue.

Keywords: Dengue; Discrimination; Primary and secondary infections; Immunoglobulin G avidity test.

Introduction

Dengue infection (DI) is among the most important arboviral diseases in India in terms of both morbidity and mortality[1]. Dengue virus is a member of the flaviviridae family, with four serologically related but antigenically distinctive serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Acute infection due to dengue virus is generally asymptomatic and may present with classical dengue fever (DF), a mild illness, or its severe form, dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS)[1]. DHF, which is life-threatening, has been postulated to result from immune enhancement after a second (heterotypic) infection by a different serotype. The hypothesis on antibody-dependent enhancement can be used for the establishment of an early diagnostic test to distinguish the primary from the secondary infection and to know the immunological status of the patients infected with dengue virus. Keeping in view the increased possibility of DHF in secondary infections, it is important to discriminate between primary and secondary infections[2,3].

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Traditionally, the haemagglutination inhibition (HI) test has been used to detect and differentiate between primary and secondary dengue virus infections\[4\]. Patients are classified as having secondary dengue virus infections when the HI test titre in their sera is greater than or equal to 1:2560, and are classified as having primary dengue virus infection if the HI test titre is less than 1:2560\[4\]. However, when the interval between the acute- and the convalescent-phase samples is less than 7 days, or the convalescent phase specimens are not available, haemagglutination inhibition test is difficult to interpret\[4,5\]. Moreover, the requirements of serum pre-treatment with acetone or kaolin to remove non-specific inhibitors makes HI test a tedious one. Furthermore, this test cannot give an early diagnosis\[4,5\].

Innis et al.\[6\] first proposed the classification of primary and secondary dengue infections by determining the ratio of dengue virus IgM antibodies to the dengue virus IgG antibodies. The acute-phase sera of patients with primary dengue virus infections show higher IgM/IgG ratios, as compared to the patients with secondary infections who show lower IgM/IgG ratios. The ratio of IgM/IgG higher than 1.78 was considered as a marker of primary infection and less than that was considered as a marker of secondary infection\[6\]. The IgG antibody avidity test is a very useful tool for differentiating between primary and secondary immune responses\[7\]. The avidity assay is based on the fact that the first antibodies synthesized after an antigenic challenge or primary infections have a lower affinity for the antigen than those produced later on. In the secondary infection, the rapid antibody response is characterized by the production of high-avidity antibodies\[2\].

The present study was performed in the Department of Microbiology, Maulana Azad Medical College and Associated Lok Nayak Hospitals, New Delhi, from September 2005 to December 2006. The study group included 150 patients clinically suspected of having dengue infection, attending the outpatient department and admitted in medical wards of Lok Nayak Hospital, New Delhi. The WHO criteria were followed for inclusion or exclusion of a case of dengue infection\[4\]. Acute-phase blood samples were collected within 4–8 days of infection, and a convalescent-phase sample was obtained after 8–15 days of onset of fever. Confirmation of acute dengue was obtained by the detection of IgM antibodies and demonstration of a ≥ 4-fold change in reciprocal IgM antibody titres in paired serum samples. IgG and IgM antibodies were detected by using PanBio IgG and IgM capture ELISA kit.

**Materials and methods**

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Primary and secondary dengue infections were defined by using the following diagnostic criteria. Primary dengue virus infection was characterized by the presence of IgM antibodies during the acute-phase and seroconversion (appearance of the IgG antibodies along with IgM antibodies) in the convalescent phase\(^2\), or the acute phase serum sample was positive for both IgM and IgG and the ratio of IgM/IgG was greater than 1.78\(^6\). A secondary dengue infection was characterized by the presence of both IgM and IgG antibodies in the acute-phase serum sample and also the IgM/IgG ratio was lesser then 1.78\(^3\).

Following the above criteria, 18 patients were classified as primary dengue infection cases and 54 patients as secondary infection cases. Samples whose absorbances were above the limit of the ELISA reader were retested using higher dilution, i.e. 1:1000. The IgG avidity test was standardized by using dengue indirect IgG ELISA kit (PanBio), the procedure of which was modified by introducing an urea incubation step. The test was performed in the same manner as reported in a previous study on standardization of dengue IgG avidity test\(^2\), excepting that the commercial kit used by us was PanBio dengue IgG indirect ELISA kit. Serum samples were diluted 1:100. The samples were then dispensed in duplicate into dengue antigen-coated wells. The samples were then incubated for half-an-hour at room temperature. After the incubation period first differential washing with PBS was done. After first washing half of the wells were washed with phosphate-buffered saline (pH 7.2) which contained urea, and the other half were rinsed with phosphate-buffered saline without urea. After five washing cycles, the test was performed as per the manufacturer’s instructions. The avidity index (AI), expressed as a percentage, was calculated as the ratio of the optical density with urea to the optical density without urea multiplied by 100.

The test was performed several times using different concentrations of urea, 6M urea for 10 min, 7M urea for 10 min, and 8M urea for 5 min. Since variable results have been obtained by several investigators when ELISA was used to test avidity using different commercial plates, different sources of antigen and different urea concentrations\(^9\), we tested different formats such as concentration of urea and time for urea incubation step to standardize the procedure and followed the same method as de Souza et al.\(^2\).

**Statistical analysis**

We used SPSS version 12 statistical software for the statistical analysis. Mann Whitney’s test was used to check whether the avidity level was significantly different between primary and secondary dengue infections. A receiver operating characteristic (ROC) curve analysis was employed using Analyze-it software, to evaluate the accuracy of the test.

**Results**

The study used various incubation schedules of 6M for 10 min, 7M for 10 min and 8M for 5 min. The mean avidity index for primary infection was 38.24, 22 and 15.11 for urea at 6M for 10 min, 7M for 10 min and 8M for 5 min respectively, while the mean avidity indices for secondary infection were 78.94, 72 and 43.7 for urea at 6M for 10 min, 7M for 10 min and for urea at 8M for 5 min, respectively (Figure). The use of 6M urea for 10 min could differentiate only 13 out of 18 primary dengue infections and 44 out of 54 secondary dengue infections. Whereas 8M urea for 5 min could differentiate 15 out of 18 primary dengue infections and 49 out of 54 secondary dengue infections. The use of 7M urea for 10 min differentiated best between the primary and
The secondary infections. This washing schedule could correctly classify all 18 primary and 53 secondary dengue infections, and was chosen to evaluate IgG AI. Avidity indices ranged from 14–32 for primary infections and 27–106 for secondary infections with 7M for 10 min. The mean AI for primary infection was 22 ± 5.4 and for secondary infection was 72 ± 12.2. The mean AI of the 18 primary dengue infections was significantly lower than the 54 secondary dengue infections (P < 0.001) (Table). The cut-off point of ≥27.4% IgG AI was chosen for the

**Figure:** IgG antibody avidity indices in sera from patients with primary and secondary dengue infections with washing schedules of 7M urea for 10 min and 8M urea for 5 min and 6M for 10 min

<table>
<thead>
<tr>
<th>Avidity Index (AI) range</th>
<th>Data for patients with</th>
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<tr>
<td></td>
<td>Primary infection</td>
<td>Secondary infection</td>
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<td></td>
<td>No. (%) of patients</td>
<td>No. (%) of patients</td>
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<tr>
<td>≥27.4</td>
<td>0 (0.0)</td>
<td>53 (98.14%)</td>
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<tr>
<td>&lt;27.4</td>
<td>18 (100.0)</td>
<td>22 (5.4)</td>
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<td>Total</td>
<td>18 (100.0)</td>
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**Table:** Performance of IgG avidity test
Immunoglobulin G avidity test to discriminate between primary and secondary dengue virus infections

Classification of primary and secondary infections. At this cutoff point, the IgG avidity test provided correct classifications of 71 of 72 patients [18 of 18 patients with primary dengue (100%) and 53 of 54 patients with secondary dengue (98.14%)].

The avidity test showed 98.67% sensitivity, 100% specificity.

Discussion

Dengue hemorrhagic fever and dengue shock syndrome have been observed to occur frequently with secondary dengue infection. Therefore, it is important to discriminate between primary and secondary infections and to assess the immunological status of patients to know the progression of the disease[4]. The haemagglutination inhibition test is conventionally used as a standard test to differentiate between primary and secondary dengue virus infections. The main disadvantages of the HI test are the requirement of paired samples, serum pre-treatment with acetone or kaolin and goose red blood cells[5,10]. Keeping in mind the disadvantages of HI, alternative assays are needed for differentiating between primary and secondary dengue infections. The utility of the assay in diagnosing a primary infection has been reported for a variety of parasites and viruses like leishmania[11], respiratory syncytial virus (RSV)[12], and rubella[13]. Recently, a few studies have standardized the avidity test and discriminated between primary and secondary dengue infection. To the best of our knowledge, no study is reported from India which has used avidity test for differentiating between primary and secondary dengue infections. The studies carried out by de Souza et al.[2] have shown for the first time that, by using a commercial kit for the diagnosis of dengue, it was possible to discriminate between a case of primary dengue infection and a case of secondary dengue infection by detecting avid IgG antibodies. Our results, obtained by using a commercial IgG indirect ELISA kit, confirmed the results obtained by de Souza et al.[2] We observed a mean AI of 22% during a primary infection and 72% during a secondary infection. The sensitivity and specificity of the test were 98.67% and 100%, respectively, with a single sample. These findings were in tune with the previous studies, which showed that the avidity test was an excellent alternative to HI assay for differentiating between primary and secondary dengue infections. Thus, the avidity test standardized by this study is a simple test which can differentiate between primary and secondary dengue infections.

References


