IMMUNOGLOBULIN M±CAPTURE BIOTIN-STREPTAVIDIN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES TO DENGUE VIRUSES

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Abstract. A biotin-streptavidin system was adapted to an IgM-capture ELISA for detection of dengue antibodies in human sera. To develop this assay, high titers of antibodies to flavivirus were purified by ion-exchange chromatography (DEAE-cellulose) and labeled with biotin. Heavy chain–specific goat anti-human IgM was first bound to the wells of a polystyrene microtiter plate, followed by binding of IgM in test specimens, and the use of tetravalent dengue antigens (dengue 1–4), biotin-labeled anti-flavivirus IgG, and streptavidin-peroxidase conjugate. The sensitivity and specificity of the IgM-capture biotin-streptavidin ELISA (IgM-BS-ELISA) in acute sera were 83.3% of patients with dengue infection and 95.3% of nondengue-infected cases, respectively. The positive predictive value was 92.4% and the negative predictive value was 89.2%. The efficiency of test was 90.4%. In convalescent sera, the sensitivity and specificity of IgM-BS-ELISA were 100% and 92.6%, respectively. The predictive values of positive and negative results were 90.3% and 100%, respectively. The efficiency of test was 95.6%. The agreement rate of IgM-BS-ELISA and standard hemagglutination inhibition test was good: \( \kappa \) (kappa) values were 0.79 for acute sera and 0.91 for convalescent sera. The correlation between two methods was quite good, with correlation coefficients (r) of 0.76 for acute sera and 0.85 for convalescent sera (\( P < 0.001 \)). The results indicate that the IgM-BS-ELISA is highly sensitive, specific, simple to perform, and rapid.

Dengue fever and dengue hemorrhagic fever (DHF) remain a major public health problem in Thailand and in many other tropical countries.¹ Dengue hemorrhagic fever and dengue shock syndrome have been the leading causes of pediatric morbidity and mortality. Serologic diagnosis of dengue infection by hemagglutination inhibition (HI) test is a conventional method that requires paired sera for meaningful results. Various versions of IgM-capture ELISAs for detection of dengue virus antibodies have been reported.²⁻⁷ However, the sensitivities of these tests are limited to 50–78% in acute sera. Determinations of IgM and IgG are able to distinguish between primary and secondary dengue virus infections.⁸⁻¹⁰ Streptavidin is a protein extracted from the bacterium *Streptomyces avidinii* with a molecular weight of 60,000 and has four binding sites with high affinity (\( K_a = 10^{15} \) M) for biotin, which is a water-soluble vitamin with a molecular weight of 244.¹¹

We report the development of a IgM-capture biotin-streptavidin ELISA (IgM-BS-ELISA) for detection of dengue antibodies in acute and convalescent sera of dengue and nondengue-infected patients.

MATERIALS AND METHODS

**Serum samples.** All sera were collected from patients (202 cases) < 15 years of age who were admitted to the Ratchaburi Hospital and healthy students (48 volunteers) after informed consent was obtained in Ratchaburi Province during March–September 1993. The subjects enrolled in this study were confirmed by a combination of clinical diagnosis and serologic HI test. One hundred two cases were diagnosed as dengue infections and classified as primary or secondary infections based on the results of the test according to standard World Health Organization definitions.¹² Most acute and convalescent serum samples of dengue cases were collected on fourth, fifth, seventh, and ninth days of onset of disease, respectively. Nondengue cases (100 cases) included nine with measles, 10 with other viral infections, 29 with pyrexia of unknown origin, 42 with upper respiratory tract infections, seven with pneumonia, and three with vomiting. Examination of the control samples by the HI test revealed no evidence of recent dengue infection. Blood samples (3–5 ml) were obtained from the patients by venipuncture and the serum was separated and stored at −20°C.

**Antigens.** Dengue and Japanese encephalitis (JE) virus antigens were obtained from the Virus Research Institute, Department of Medical Science, Ministry of Public Health (Nonthaburi, Thailand). They were produced by sucrose acetone extraction of the brains of suckling mice infected with the following prototype virus strains: dengue-1 (DEN-1) Hawaii, DEN-2 Tr 1751, DEN-3 H-87, DEN-4 H-241, and JE JAGAR #01. The lyophilized antigens were stored frozen at −20°C and resuspended in distilled water before use.

**Hemagglutination inhibition test.** The HI test was conducted using the method of Clarke and Casals¹³ adapted for use in a microtiter plate.

**Purification of anti-flavivirus IgG.** The convalescent-phase human antisera with broadly reactive high titers of HI antibodies against dengue and JE viral antigens were collected from DHF patients with a secondary type of immune response. After precipitation by saturated ammonium sulfate solution, anti-flavivirus IgG fractions were separated from hyperimmune anti-flavivirus globulin by anion exchange chromatography. The DEAE-cellulose (Pharmacia, Uppsala, Sweden) was equilibrated in 0.01 M potassium phosphate buffer, pH 8.0. The dialyzed globulin was applied to the column in the same buffer. The IgG, which did not adsorb to DEAE-cellulose under this condition, passed through the column with the starting buffer. Other globulins were eluted with 0.02 M potassium phosphate buffer, pH 8.0, 0.1 M potassium phosphate buffer, pH 8.0, and 0.1 M sodium chloride solution. Each fraction (3 ml) was collected and the optical density (OD) at 280 nm was measured. The fractions with high OD values were pooled, concentrated by lyophilization, and stored at −20°C until labeled with biotin.
Preparation of biotin-linked anti-flavivirus IgG. Anti-flavivirus IgG was covalently conjugated to biotin using the method essentially as described by Nerurkar and others. Briefly, 1 mg of IgG was dissolved in 1 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.2, and clarified by centrifugation at 300 \( \times g \) at 4°C for 10 min. It was then dialyzed against 0.1 M sodium bicarbonate, pH 8.2, at 4°C overnight, clarified by centrifugation again, and mixed with 1 mg/ml of freshly prepared N-hydroxy succinimidobiotin in dimethylsulfoxide. The mixture was rotated gently at room temperature for 4 hr. It was then extensively dialyzed against 0.15 M PBS, pH 7.2, and clarified by centrifugation. The supernatant was mixed with an equal volume of glycerol and stored at -20°C.

Immunoglobulin M-capture biotin-streptavidin ELISA. The IgM-BS-ELISA, which is modified from that described by Innis and others, was used to determine dengue SA. The IgM-BS-ELISA had a sensitivity of 83.3% (85 of 102 cases) and a specificity of 95.3% (141 of 148 cases). The predictive values of positive and negative results were 92.4% and 89.2%, respectively. The efficacy of test was 90.4%, as shown in Table 1. Using convalescent sera, the sensitivity was 100% and the specificity was 92.6% (137 of 148 cases). The predictive values of positive and negative results were 90.3% and 100%, respectively. The efficiency of the test was 95.6% (Table 2). By kappa analysis, there was a good agreement between the IgM-BS-ELISA and HI test in acute sera with \( \kappa = 0.79 \) and very good agreement in convalescent sera with \( \kappa = 0.91 \). The relationship between OD values of the IgM-BS-ELISA and anti-log of HI titers was determined by calculating the correlation coefficients (r), which were 0.76 for acute sera (Figure 1) and 0.85 for convalescent sera (Figure 2) \( (P < 0.001) \).

DISCUSSION

The IgM-BS-ELISA had a sensitivity of 83.3% in detecting anti-dengue IgM in the acute sera of dengue-infected cases. All convalescent sera were positive by this assay, giving a 100% correlation with the HI result. The incorporation of the biotin-streptavidin system into the ELISA seems to increase the sensitivity. This may be due to several advantages inherent in the system. Biotin is a small molecule that can easily be covalently coupled to antibody with high specificity. It does not affect the antigen-binding capacity of immunoglobulin. Moreover, one molecule of streptavidin can efficiently bind four molecules of biotin and results in an amplification of the antigen-antibody reaction. Of the dengue infections serologically confirmed by the HI test, 100 (98%) of 102 cases were secondary infections whereas only two (2%) of 102 cases were primary infections. This study revealed that the IgM was still detected not only in primary dengue infections, but also in secondary dengue infections. In acute sera, most of the negative samples (14 of 17 cases, 82.3%) were obtained before the fifth day after onset of the disease. The negative results could be due to the specimens having been collected before the ap-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Diagnosis of dengue infection by IgM-biotin-streptavidin (BS)-ELISA compared with the hemagglutination inhibition (HI) test in acute sera</th>
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</thead>
<tbody>
<tr>
<td>IgM-BS-ELISA</td>
<td>Dengue cases</td>
</tr>
<tr>
<td>Positive</td>
<td>85</td>
</tr>
<tr>
<td>Negative</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
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<th>Table 2</th>
<th>Diagnosis of dengue infection by IgM-biotin-streptavidin (BS)-ELISA compared with the hemagglutination inhibition (HI) test in convalescent sera</th>
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</thead>
<tbody>
<tr>
<td>IgM-BS-ELISA</td>
<td>Dengue cases</td>
</tr>
<tr>
<td>Positive</td>
<td>102</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
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The appearance of IgM or the masking effect of high levels of dengue IgG in secondary infections. The appearance of dengue IgM in sera was reported on the fourth day and the highest IgM was reported on the seventh day after onset of the disease. Usually, the level of dengue IgM antibodies in sera may persist for approximately 30–90 days or may extend to 252 days. It was demonstrated that all negative patients were positive in convalescent sera. This was also observed in 23.7% of the dengue-positive cases with no detectable IgM in acute specimens that seroconverted to IgM positive convalescent serum phase, indicating that IgM might not always be present in the acute serum specimens of dengue patients.

The IgM-BS-ELISA showed a specificity of 95.3% in acute sera and 92.6% in convalescent sera of non-dengue-infected cases. The sensitivity and specificity of the IgM-BS-ELISA and the HI test were comparable. The IgM-BS-ELISA is more specific than the HI test because it determines IgM antibodies instead of total immunoglobulins, which show high cross-reactivity among flaviviruses. However, some cross-reactions with JE antigen were observed in this study with the weaker reactions than the homologous system. The IgM-BS-ELISA has several advantages over the conventional HI test since it does not require extraction and absorption steps of sera and can detect early IgM in single acute serum. It is rapid; the total test time is about 9 hr, but that of the HI test is approximately two weeks. In addition, the IgM-BS-ELISA gives the color result that can be read with the naked eye as a qualitative method or measured by spectrophotometer as a quantitative method, which enhances its usefulness. Although the IgM-BS-ELISA is highly sensitive and specific, its performance requires 20% normal human serum that is negative for dengue antibodies. In endemic areas, it is difficult to obtain a large amount of such dengue-negative sera. Since the IgM-BS-ELISA is simple to perform, it can be implemented for a large-scale study. This method can be used to confirm recent dengue infections when the result of an HI test is inconclusive and the sera submitted for dengue diagnosis are single specimens. Thus, the IgM-BS-ELISA should be useful in the rapid diagnosis.
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Figure 2. Correlation between dengue IgM-biotin-streptavidin-ELISA optical density units and hemagglutination inhibition (HI) titers (mean of anti-log) using convalescent sera, $r = 0.85$, $P < 0.001$. of dengue infections and appropriate for developing countries.

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