Evaluation of a Commercially Available Immunoglobulin M Capture Enzyme-Linked Immunosorbent Assay Kit for Diagnosing Acute Dengue Infections

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Received 11 March 1999/Returned for modification 15 June 1999/Accepted 2 July 1999

Dengue fever and the more severe dengue hemorrhagic fever are common diseases that occur throughout tropical regions of Southeast Asia and the Americas. The dengue viruses (DEN) that cause these clinical illnesses consist of four serotypes, DEN-1, -2, -3, and -4. Antibody profiles elicited by primary and secondary DEN infections differ (2, 6). Primary infections result in the initial appearance of detectable DEN-specific immunoglobulin M (IgM) antibodies after approximately 3 to 5 days of illness, peaking within approximately 2 weeks of illness. Approximately 3 months after infection, DEN-specific IgM antibodies become undetectable (4). In the acute and early-convalescent phases of illness, DEN-specific IgG antibodies appear at low levels and usually remain below IgM antibody levels for 2 to 4 weeks. During secondary infection, DEN-specific IgM levels remain low to absent whereas IgG increases rapidly to very high levels over 2 weeks and is easily detectable. Given that a large portion of DEN infections occurring in endemic regions are secondary infections, diagnosing these infections by the use of IgM detection assays remains a challenge.

Recently, commercially available kits for the detection of anti-dengue virus (anti-DEN) immunoglobulin M (IgM) antibodies have been developed. These standardized assays have greatly enhanced our ability to effectively diagnose DEN infections. We conducted an evaluation of a test kit manufactured by MRL Diagnostics Inc. that is designed to detect anti-DEN IgM antibodies. Eighty paired samples from DEN-infected individuals were tested by the MRL DEN Fever Virus IgM Capture enzyme-linked immunosorbent assay (ELISA), the PanBio Duo ELISA, the PanBio Rapid Immunochromatographic Test (PRIT), and the IgM-IgG antibody capture (MAC/GAC) ELISA. All infections were confirmed by either PCR-assisted detection of DEN transcripts or by DEN isolation in C6/36 cells. Seventeen paired samples from individuals with no evidence of acute DEN infection were used as negative controls. The PRIT had the best sensitivity (100%), whereas the MAC/GAC ELISA and the PanBio Duo assay had the highest levels of specificity. The MRL ELISA and the PanBio Duo assay were the top performers when taking into consideration both sensitivity and specificity. All assays were able to detect DEN-specific antibodies in samples from patients with either primary or secondary infections, regardless of the infecting DEN serotype.

MATERIALS AND METHODS

Serum samples. One hundred sixty-one acute- and convalescent-phase serum samples from 80 DEN-infected patients were used in this evaluation. The number of days between acute- and convalescent-phase sample collections ranged from 2 to 14, with a median of 5 days. All infections were confirmed by either PCR-assisted detection of DEN transcripts in serum (3) or DEN isolation in C6/36 cells. Serum specimens from cases involving infections with each of the four DEN serotypes were included among the specimens: DEN-1, n = 16; DEN-2, n = 27; DEN-3, n = 32; and DEN-4, n = 5). The patients consisted of 34 males and 30 females, ranging in age from 2 to 50 years. The ages and genders of 16 patients were not available, and the exact time of onset of illness was available for a total of 32 patients.

Seventeen paired serum samples that demonstrated no evidence of recent DEN infection by serology, virus isolation, and PCR were used as negative controls. These pairs of samples were obtained at least 7 days apart. At the time of sample collection, all control subjects had febrile illnesses that were clinically similar to DEN but were subsequently proven to be unrelated to DEN. The days between collection of the acute- and convalescent-phase negative-control samples ranged from 6 to 14, with a median of 8 days. The acute- and convalescent-phase sera for 10 of the negative-control samples were positive for DEN by hemagglutination inhibition (HI) assays, but no increases in titer were noted, supporting the presence of a non-DEN febrile illness. Paired samples from two control subjects had stable DEN HI titers of 1:80, three had titers of 1:40, two had titers of 1:20, and one each had titers of 1:640, 1:320, and 1:160. All serum samples were obtained after informed consent was provided by the patient and were part of earlier studies that were conducted under human use protocols approved by the Committee for the Protection of Human Subjects, US NAMRU 2.

Serological assays for DEN. (i) MRL DEN Fever Virus IgM Capture ELISA. The MRL IgM capture assay (MRL Diagnostics, Cypress, Calif.) is designed to detect human serum antibodies to DEN-1, -2, -3, and -4. Ninety-six-well microtiter plates are coated with anti-human IgM. Test serum, diluted 1:100 in sample dilution buffer, is added to each well, and following a 1-h incubation at room temperature, a DEN antigen cocktail is added. The DEN strains used in preparing the antigen cocktail included TH-Sman (DEN-1), TH-36 (DEN-2), H87 (DEN-3), and H241 (DEN-4). Antigen bound to DEN-specific IgM is detected with a flavirus group-specific monoclonal antibody–horseradish peroxidase conjugate. In this assay, tetramethylbenzidine and hydrogen peroxide are used as substrates. The results of this assay are reported as index values. Index values are calculated by dividing the specimen optical density (OD) value read at 450 nm (corrected for blank readings) by the OD of the cutoffibrator preparation. This serum preparation was formulated to give optimum differentiation between
negative and positive sera. An index value of >1.00 is presumptive evidence for the presence of anti-DEN IgM antibodies, while an index value of <1.00 indicates the absence of DEN-specific IgM antibodies.

(ii) PanBio DEN DUO IgM and IgG capture ELISA. The PanBio DEN DUO assay (PanBio Pty. Ltd., Windsor, Australia) is a combination kit in a microwell plate format that is designed to detect both IgM and IgG antibodies to DEN in separate reactions by the use of a capture method. Details of the procedure for this test have been previously described (4). Whether a sample is positive or negative is determined by the IgG or IgM cutoff ratio. These ratios are determined by dividing the sample OD value read at 450 nm by the cutoff value, which is determined by using the IgG and IgM cutoff Calibrator serum that is supplied with the kit. A result of ≤1 indicates a positive sample. IgG ratios of >3.0 are indicative of a secondary antibody response, whereas IgG ratios of between 1 and 3 suggest a primary antibody response.

(iii) PanBio Rapid Immunochromatographic Test (PRIT). The PRIT is a chromatographic assay (PanBio Pty. Ltd.,) that is capable of detecting both IgM and IgG antibodies and can be completed in approximately 5 min. A description of this assay and the results of an evaluation of its sensitivity and specificity have been previously published (7). A positive result is indicated by the presence of a visible IgM and/or IgG band. A primary DEN infection is said to be present if only an IgM band is visible. If both an IgM and an IgG band are visible, the infection is considered to be secondary. If only an IgG band is visible, a DEN infection is considered positive.

(iv) IgM antibody capture (MAC)-IgG antibody capture (GAC) ELISAs. The MAC and GAC (MAC/GAC) assays are based on a microwell plate capture format and are used to detect both IgM and IgG antibodies against DEN. A detailed description of these assays was published previously (2). A positive result is obtained when the unit value for a sample is ≥0.80. This value is calculated by using the formula 100 × (A492 of test sample − A492 of NS)/A492 of PS − A492 of NS), where PS and NS represent weakly positive and negative control standards, respectively. A primary infection is indicated when the IgM-to-IgG index value ratio is ≥1.78, and a secondary infection is indicated when the ratio is <1.78. In IgM-negative cases, a secondary infection is said to exist when there is a twofold increase in the anti-DEN IgG value with an absolute IgG index of ≥100 (7).

(v) HI assays. HI was performed on each acute- and convalescent-phase sample pair to confirm the presence of DEN infection and as a gold standard for determining whether the DEN infection was secondary or primary. The method for conducting HI assays was described previously by Clarke and Casals (1). The HI assay detects both IgM and IgG antibodies against DEN. Serocconversion from a negative to a positive test and a fourfold rise in HI titer are considered indicative of infection. The HI assay is also capable of distinguishing between primary and secondary infections. A secondary infection is suggested when early-convalescent-phase HI titers of ≥1:2,560 are obtained. Samples with titers of <1:2,560 are considered to be associated with primary infections.

RESULTS

The sensitivity and specificity of the MRL ELISA were evaluated and compared to those of the above-described assays, using well-characterized sera from DEN-infected patients. Of the acute- and convalescent-phase-paired samples from 80 patients, the MRL assay was able to identify 77 as positive for DEN, (96%) while the PanBio Duo ELISA and the MAC/GAC ELISA identified 74 (92%) and 62 (78%), respectively, showing positive results in 100% of the samples from patients with either primary or secondary infections, we sought to evaluate the accuracy of these tests at actually predicting whether the infection was primary or secondary. Of the samples positive by each assay, the PanBio Duo ELISA correctly predicted 63% (20 of 32) of the primary infections and 82% (28 of 34) of the secondary infections. The PRIT predicted 76% (20 of 26) of the primary infections and 76% (32 of 42) of the secondary infections, while the MAC/GAC ELISA predicted 83% (24 of 29) of the primary infections and 100% (39 of 39) of the secondary infections. Table 1 shows the ability of each test to detect anti-DEN IgM antibodies based on the DEN serotype causing the infection and on whether the infection was secondary or primary. For primary and secondary infections, the PRIT performed the best, showing positive results in 100% of the samples from patients infected with any of the four DEN serotypes. The MRL ELISA did well, showing positive results for all of the samples from...
patients with DEN-1, -3, or -4 primary infections and for all but one of the samples from patients with primary DEN-2 infections. For secondary infections, the PanBio Duo assay was positive for all samples. The MRL ELISA also performed well, showing positive results for all but one DEN-2 and one DEN-3 secondary-infection sample.

**DISCUSSION**

The increasing prevalence and incidence of dengue fever and dengue hemorrhagic fever in tropical regions of the world has resulted in the need for improved and standardized serological assays to aid in the diagnosis of these diseases. Several ELISAs have become commercially available, including the PanBio Duo ELISA, the PRIT, and the MRL IgM ELISA. The PanBio assays have been evaluated and shown to have good sensitivity and specificity (5, 7), whereas the MRL assay has had limited evaluation. In this study, we used well-characterized sera from DEN-infected patients to compare the sensitivity and specificity of this assay to those of the others.

Based on the results of this study, the MRL ELISA and the PanBio Duo Assay were the best performers when both sensitivity and specificity are taken into consideration. The PRIT gave results comparable to those of the MRL assay with regard to sensitivity, but the specificity was lower (81%). The MRL and PRIT assays also performed well at detecting anti-DEN antibodies in samples from patients with either primary or secondary DEN infections, although the PanBio Duo assay did as well as the other two assays at detecting antibody during secondary infections.

None of the assays performed particularly well at detecting DEN IgM antibodies in acute-phase samples collected on the day of admission or during an initial clinic visit. The MRL assay and the PRIT were, however, able to detect anti-DEN-specific IgM antibodies in 38 and 56% of the acute-phase samples, respectively. These results are comparable to those achieved with the DEN dipstick ELISA, with which IgM was detected in 40% of acute-phase samples (8).

In earlier published studies, the MAC/GAC ELISA demonstrated a sensitivity of 78% in detecting DEN-specific IgM antibodies in acute-phase samples (2). The reason why this assay performed less well in our studies is not clear but may be related to differences in the types of sera used in the two studies. The DEN patient serum samples in our study were all virus isolation positive, which was not the case in the earlier study. Given that DEN isolation from MAC ELISA IgM-negative samples is more common than from MAC ELISA IgM-positive samples (2), one would expect the number of samples positive by this assay to be small. With the convalescent-phase samples, the detection of IgM antibodies improved for all of the assays tested. It is noted that interpretation of the PanBio Duo assay and MAC/GAC ELISA results relied on an increase in IgG index values in 27 and 10 cases, respectively, to indicate the presence of DEN infection. Therefore, the MRL assay was the most sensitive at detecting DEN-specific IgM antibodies in the virus isolation-positive samples used in our evaluation.

Antibodies generated as a result of infection by any of the four DEN serotypes could be detected by all assays used in this study, regardless of whether the infection was primary or secondary. The PanBio Duo ELISA performed less well than the others at detecting antibodies to DEN-2 infection, and the performance of the MAC/GAC ELISA was comparatively poorer with regard to detection of DEN-3 infections. The reasons for this are unknown. Whether the failure to detect antibodies in these samples is related to lower antibody titers or to genetic differences in strains of the virus causing the infection cannot be determined with the data generated by this study. Because of the small number of DEN-4 infections, very little can be said regarding the comparative abilities of these assays to detect DEN-4-specific antibodies.

The reasons why the sensitivity and specificity varied among the assays evaluated in this study are unknown. These variations may be related to the different methods of preparing the DEN antigens for the assays. Because the details of antigen preparation for the commercial assays are unknown, no conclusions can be made regarding the influence of the procedures on the performance of the tests.

DEN possess epitopes that are shared by other flaviviruses, such as Japanese encephalitis (JE) virus, and as a result, antibodies generated against cross-reactive epitopes during infection may give rise to false-positive tests. This cross-reactivity occurs more commonly with IgG antibodies and less often with IgM antibodies. Because of the present lack of well-characterized sera representative of other closely related flavivirus infections, we were unable to evaluate the extent to which cross-reactivity occurs in the MRL IgM ELISA. With the PanBio Duo ELISA, PRIT, and MAC/GAC ELISA, false-positive reactions with anti-JE antibodies have been demonstrated. Efforts are now under way to obtain well-characterized sera from patients with other flavivirus infections to evaluate the level of cross-reactivity seen with the MRL assay.

**ACKNOWLEDGMENTS**

This research was supported by the Naval Research and Development Command for Work Unit 6.2/S/ESX/2416. We thank Terrisita Porter and Robiyati for assistance in the preparation of the manuscript.

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**TABLE 1. Test results based on DEN virus serotype and type of infection**

| Antibody test         | No. of samples in which DEN antibodies were detected* |
|-----------------------|-------------------------------------------------------|
|                       | DEN-1 (P = n = 10) | S (n = 6) | DEN-2 (P = n = 17) | S (n = 10) | DEN-3 (P = n = 8) | S (n = 24) | DEN-4 (P = n = 1) | S (n = 4) |
| MRL ELISA             | 10 | 6    | 16 | 9     | 8      | 23       | 1      | 4      |
| PRIT                  | 10 | 6    | 17 | 10    | 8      | 24       | 1      | 4      |
| PanBio Duo ELISA      | 10 | 6    | 13 | 10    | 7      | 24       | 0      | 4      |
| MAC/GAC ELISA         | 8  | 6    | 14 | 7     | 7      | 16       | 0      | 4      |

* P, primary infections; S, secondary infections.
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