Diagnosis of Dengue Virus Infection by the Visual and Simple AuBioDOT Immunoglobulin M Capture System

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Received 9 October 2002/Returned for modification 3 December 2002/Accepted 12 August 2003

The Dengue IgM Capture ELISA (MAC-ELISA) is the immunoenzymatic system recommended by the Pan American Health Organization and the World Health Organization for the serological diagnosis of dengue virus infection due to its high sensitivity, ease of performance, and use of a single acute-phase serum sample. However, tests with this enzyme-linked immunosorbent assay (ELISA) system are time-consuming and require equipment for washing, incubation, and reading of the results. AuBioDOT is a multistep visual diagnostic immunoassay that uses technology based on the immunoglobulin M (IgM) capture ELISA principle. This system uses white polyethylene opaque plates as the solid phase, colloidal gold as the marker, and silver ion amplification. It does not require special equipment, it is totally manually operated, and it can be performed in less than 1 h. The sensitivity and specificity of AuBioDOT for the detection of anti-dengue virus IgM antibodies were studied with a panel of 336 serum samples (150 serum samples from patients with suspected or serologically confirmed dengue virus infection, 186 serum samples from healthy blood donors and patients without dengue virus infection). The results were compared with those obtained by the MAC-ELISA. A sensitivity of 97.7% and a specificity of 97.1% were obtained. The concordance of the two tests was 97.3%, with a kappa index of 0.94. The application of AuBioDOT for the detection of anti-dengue virus IgM antibodies is recommended as an alternative method for the diagnosis of dengue virus infection, both for clinical diagnosis and for seroepidemiological surveillance. The system is useful under field conditions and in laboratories and requires little equipment.

Dengue fever and dengue hemorrhagic fever continue to be major public health problems in the tropical world. The dengue virus is transmitted to humans by the bite of a domestic mosquito, Aedes aegypti, the principal vector, although some other species such as Aedes albopictus are of importance (34).

A definitive diagnosis of dengue virus infection can be made only in the laboratory and depends on isolation of the virus, detection of viral antigen or RNA in serum or tissues, or detection of specific antibodies in the patient’s serum (8, 10, 33).

In the last two decades, the enzyme-linked immunosorbent assay (ELISA) has become the most widely used serological test for the diagnosis of dengue virus infection, providing high degrees of sensitivity and specificity (2, 18, 20, 21). At present, this system is an invaluable tool for the surveillance of dengue fever, dengue hemorrhagic fever, and dengue shock syndrome.

The Dengue IgM Capture ELISA kit (MAC-ELISA) for detection of anti-dengue virus immunoglobulin M (IgM) antibody is based on the capture of dengue virus-specific IgM antibodies in serum or whole blood dried on filter paper strips. This system has been developed by the Arbovirus Laboratory at the “Pedro Kourí” Institute (IPK) and has been evaluated and applied, with good results (10, 29, 31, 32; J. L. Pelegrino, S. Vázquez, M. G. Guzmán, A. Valdivia, and G. Rogés, Tenth Annu. Clin. Virol. Symp. Annu. Meet. Pan Am. Group Rapid Diagnosis, 1994). Some laboratories in Central and South America have used the MAC-ELISA as the reference test for the diagnosis of dengue virus infection (10).

The MAC-ELISA is a good tool for the diagnosis of dengue virus infection; however, it is time-consuming and requires an ELISA reader. In recent years, different rapid immunoassays for the detection of anti-dengue virus IgM antibodies have been developed (4, 26, 27, 35, 36).

This paper reports on the development of a rapid IgM capture assay for the detection of anti-dengue virus antibodies that uses the basic principles of the AuBioDOT immunoassay technology (22). The AuBioDOT IgM capture (AuBioDOT) test for the detection of anti-dengue virus antibodies is a multistep immunoassay that uses manual operation, visual reading, and colloidal gold-labeled conjugated monoclonal antibodies. In this study, the AuBioDOT assay was compared with the MAC-ELISA.

MATERIALS AND METHODS

Serum samples. Different serum panels were studied. Panel 1 consisted of sera from 110 patients with a clinical diagnosis of dengue virus infection during the 1997 dengue epidemic in Santiago de Cuba, Cuba (17). Panel 2 consisted of sera from 10 patients clinically diagnosed with dengue virus infection and serologically confirmed to have dengue virus infection during the 1995 dengue epidemic in Panama (5, 25). Panel 3 consisted of sera from five patients clinically diagnosed with dengue virus infection and serologically confirmed to have dengue virus infection during the 1988 dengue epidemic in Ecuador (1). Panel 4 consisted of sera from 20 patients clinically diagnosed with dengue virus infection and serologically confirmed to have dengue virus infection during the 1994 dengue epidemic in Nicaragua (5, 11). Panel 5 consisted of sera from five patients from Mexico clinically diagnosed with dengue virus infection and serologically confirmed to have dengue virus infection. Panel 6 consisted of sera from 126...
healthy Cuban blood donors collected in 1994 and 1995. Panel 7 consisted of sera from 20 patients with serologically confirmed hepatitis A (Hepatitis Virus Laboratory, IPK). Panel 8 consisted of sera from 10 patients with serologically confirmed influenza A virus infection (Influenza Laboratory, IPK). Panel 9 consisted of sera from 30 patients confirmed to have leptospirosis (Leptospira Laboratory, IPK).

**Virus and antigens.** The dengue virus antigens used in both the MAC-ELISA and the AuBioDOT test were obtained from the brains of dengue virus-infected mice by the sucrose-aceton method (3). The following strains were used: dengue virus serotype 1 (strain Hawaii), dengue virus serotype 2 (strain New Guinea C), dengue virus serotype 3 (strain H-87), and dengue virus serotype 4 (strain H-241).

**Colloidal gold-conjugated anti-dengue virus complex monoclonal antibody.** Monoclonal antibody H3/6 (14, 24) was conjugated to colloidal gold by procedures previously described by Hirsbeers and Ross (15) and Goodman et al. (7). Colloidal gold particles 20 nm were prepared by the protocol described by Oliver (23). Briefly, monoclonal antibody H3/6 in borax (final concentration, 10 µg/ml; pH 9.2) was passively conjugated to 20-nm gold particles (pH 9). After 10 min of incubation at room temperature (RT), the conjugate was blocked for 5 min with 0.1 M phosphate buffer (pH 8.1%) bovine serum albumin (BSA) fraction V (no. 44155HV; BDH). After centrifugation at 12,000 g for 45 min at 4°C, the supernatant was decanted and the final conjugated pellet was resuspended in 0.1 M phosphate buffer (pH 8.1%) BSA-0.05% sodium azide (no. 6688; Merck).

The final conjugate was stored at 4°C at a concentration of 10 optical density (OD) units measured at 520 nm.

**AuBioDOT assay.** White opaque polystyrene strip plates (Greiner, Frickenhausen, Germany) were sensitized at 37°C for 3 h with 0.5 µg of goat IgG anti-human IgM (µ chain specific) antibody (no. I 0759; Sigma) in phosphate-buffered saline (PBS; pH 7.2 to 7.4) (coating buffer). Twenty microliters of undiluted positive and negative control serum samples and test serum samples were added to the wells of the plate, and the plates were incubated for 15 min at RT. After the plates were washed, 50 µl of a pool of antigen containing 64 hemagglutination units of the four dengue virus serotypes was added, and the plates were incubated for 15 min at RT. After the plates were washed, 20 µl of colloidal gold-conjugated anti-dengue virus complex monoclonal antibody (H3/6) was added to each well. The conjugate was diluted at 1 OD unit measured at 540 nm in PBS (pH 8.0)–1% BSA. The plate was incubated for 15 min at RT. After the plate was washed, the addition of 20 µl of silver enhancer solution (code RP492; Amersham) allowed amplification of the reaction within 10 min. An insoluble color reaction (from clear gray to a dark color) takes place in situ. The color intensity is proportional to the concentration of anti-dengue virus IgM antibodies in the serum sample. The reading was carried out by simple visual inspection. All washing steps were first carried out with distilled water (to eliminate excess reagents), followed by three washes with PBS-0.05% Tween 20.

The plates were coated, dried, sealed, and stored at 4°C. The stability assay was performed on plates kept at 4 and 37°C for 1 week.

**MAC-ELISA.** The MAC-ELISA was used as the "gold standard" assay (29, 31, 32; Pelegroino et al., Tenth Ann. Clin. Virol. Symp. Ann. Meet. Pan Am. Group Rapid Diagnosis, 1994). Briefly, Maxisorb plates (no. 469949; Nunc) were coated with goat IgG anti-human IgM (µ chain specific) antibody (no. I 0759; Sigma) and blocked with BSA. Fifty µl of a 1/20 dilution of both the positive and the negative control serum samples and the test serum samples in PBS-0.5% BSA was added. After the plates were washed, 50 µl of a pool of antigens for the four dengue virus serotypes was added to each well at 16 hemagglutination units. After overnight incubation, 50 µl of a dilution of human anti-dengue virus IgG conjugate was added, o-Phenylenediamine (no. L536843 048; Merck) and hydrogen peroxide (no. K2699310 936; Merck) in phosphate-buffered citrate (pH 5) were used as the substrate. The reaction was stopped, and the plates were read at 492 nm. The cutoff for the assay was two times the mean OD for the negative control serum sample.

**EIM for virus antibody detection.** The ELISA inhibition method (EIM) was performed as described by Vázquez and Fernández (28) and Fernández and Vázquez (6), with some modifications. Each well of polystyrene plates (no. 3591; Costar) was adsorbed overnight at 4°C with 100 µl of human anti-dengue virus IgG at a concentration of 10 µg/ml in coating buffer (pH 9.6). On the following day, the plates were blocked with 1% BSA and incubated for 1 h at 37°C. Dengue virus serotype 2 antigen (100 µl) was obtained by the sucrose-aceton method, diluted 1/40 in PBS-0.05% Tween 20, and added to each well. The plates were incubated for 1 h and then washed. Volumes of 100 µl of the respective dilutions of serum 1/20 to 1/10,240 were added. After the plates were washed, 100 µl of human anti-dengue virus IgG conjugate diluted 1/3,000 in PBS-0.05% Tween 20–2% fetal bovine serum was added. Substrate containing o-phenylenediamine was added, and the reaction was stopped after 30 min at room temperature. The test was read at 492 nm. The percent inhibition was calculated for each dilution by the following equation: [1−(OD of serum sample/OD of negative control)]×100.

The titer in each serum sample was the highest dilution with ≥50% inhibition. Those samples with antibody titers of 5,120 (equivalent to 1,280 by the hemagglutination inhibition assay) or higher were considered to be probably infected with dengue virus (30). Titers of 10,240 (equivalent to 2,560 by the hemagglutination inhibition assay) or higher defined a secondary infection (30).

**RESULTS**

**Results by MAC-ELISA and AuBioDOT assay with 150 serum samples from patients with clinically diagnosed and serologically confirmed dengue.** Table 1 shows the results obtained by both tests with the samples from panels 1 to 5. Of the 150 serum samples tested, 130 (87%) and 127 (85%) were positive by the MAC-ELISA and AuBioDOT assay, respectively. Three serum samples were positive by the MAC-ELISA and negative by the AuBioDOT assay. Two of the serum samples showed IgG titers of 10,240 or higher by EIM. The third one showed a titer of 5,120 by EIM.

Twenty serum samples from panel 1 were negative by both tests.

**Results by MAC-ELISA and AuBioDOT assay with 186 serum samples from panels 6 to 9.** The sera from panels 6 to 9 were negative by the MAC-ELISA (Table 2). When the samples were tested by the AubioDOT assay, 180 serum samples were positive by the AuBioDOT assay.

**TABLE 1. Results for 150 serum samples from patients with clinically diagnosed and serologically confirmed dengue infections studied by MAC-ELISA and the AuBioDOT assay**

| Panel no. | Serum sample source | No. of samples positive/total no. tested (%) |
|-----------|---------------------|---------------------------------------------|
| 1         | Santiago de Cuba    | 90/110 (82)                                  |
| 2         | Panama              | 10/10 (100)                                  |
| 3         | Ecuador             | 5/5 (100)                                    |
| 4         | Nicaragua           | 20/20 (100)                                  |
| 5         | Mexico              | 5/5 (100)                                    |
| Total     |                     | 130/150 (87)                                 |

**TABLE 2. Results for 186 serum samples from non-dengue virus-infected patients and healthy blood donors studied by MAC-ELISA and the AuBioDOT assay**

| Panel no. | Serum source              | No. of samples negative/total no. of samples (%) |
|-----------|---------------------------|-----------------------------------------------|
| 6         | Healthy donor             | 126/126 (100)                                 |
| 7         | Hepatitis Laboratory      | 20/20 (100)                                   |
| 8         | Influenza Laboratory      | 10/10 (100)                                   |
| 9         | Leptospiro Laboratory     | 30/30 (100)                                   |
| Total     |                           | 186/186 (100)                                 |
When the results obtained by the MAC-EIM (data not shown) donors (panel 6) were positive. Of these six samples, four were (95%) were also negative and 6 (5%) from healthy blood plate strips kept at 4°C for 1 week; columns A, B, C, D, and E show the results for the plate strips kept at 37°C for 1 week; dots A, B, C, D, and E show the results for the plate strips kept at 37°C for 1 week; and columns 2, 4, 6, 8, and 12 show the results for the plate strips kept at 37°C for 1 week. The color intensities of positive sera were similar in plate strips kept at both 4 and 37°C. No color developed for the negative sera.

**Comparison of MAC-ELISA and AuBioDOT assay with all 336 serum samples.** When the results obtained by the MAC-ELISA and the AuBioDOT assay for all 336 serum samples were compared, 127 serum samples were positive by both assays, 200 were negative by both assays, 6 were negative by the MAC-ELISA and positive by the AuBioDOT assay, and 3 were positive by the MAC-ELISA and negative by the AuBioDOT assay. A sensitivity of 97.7% (95% confidence interval [CI], 92.9 to 99.4) and a specificity of 97.1% (95% CI, 93.5 to 98.8) were obtained. The concordance was 95.5% (95% CI, 90.0 to 98.2), and the negative predictive value was 98.7% (95% CI, 95.4 to 99.9).

**Plate stability assay.** Figure 1 shows the results of the plate stability assay. Columns 1, 3, 5, 7, 9, and 11 show the results for the plate strips kept at 4°C for 1 week; and columns 2, 4, 6, 8, and 12 show the results for the plate strips kept at 37°C for 1 week. The color intensities of positive sera were similar in plate strips kept at both 4 and 37°C. No color developed for the negative sera.

**DISCUSSION**

The detection of IgM antibody to dengue virus by ELISA has become one of the most important and useful methods for the diagnosis of dengue virus infection (2, 10, 18). Anti-dengue virus IgM antibody is produced transiently during primary and secondary infections (16).

MAC-ELISA with a simple acute-phase serum sample is slightly less sensitive than the hemagglutination inhibition test (10, 29; Pelegrino et al., Tenth Annu. Clin. Virol. Symp. Annu. Meet. Pan Am. Group Rapid Diagnosis, 1994). The MAC-ELISA has become an indispensable tool for laboratory surveillance for dengue virus infections. However, simpler and faster systems for dengue virus IgM detection are still required.

The AuBioDOT assay, based on the IgM capture ELISA principle, is totally manually operated, and can be performed in less than 1 h.

Of the serum samples from 150 patients suspected of having dengue virus infection, 85% were positive by both tests. Three samples with discordant results were positive by the MAC-ELISA and negative by the AuBioDOT assay and showed high levels of IgG antibodies by EIM. These results suggest that these three serum samples were from patients with probable dengue virus infection. It is important to point out that the MAC-ELISA shows very few false-positive reactions. For example, false-positive reaction rates of 0.08 and 0.54% were observed in national dengue surveillance studies conducted in 1998 and 1999, respectively (13).

Sera from patients without dengue virus infection were negative with both systems. Six serum samples from healthy blood donors were positive by the AuBioDOT assay and negative by the MAC-ELISA. These results were considered false-positive reactions because the samples were collected in 1995 and 1996, when no dengue virus circulation was demonstrated in Cuba (12). In addition, none of these individuals had traveled to areas where dengue virus is endemic. These false-positive results correspond to 3% of the group of negative sera (panels 6, 7, 8, and 9). Lam et al. (19) and Cuzzubbo et al. (4) obtained false-positive reaction rates of 6 and 5%, respectively, when they tested sera from patients without dengue virus infection.

The AuBioDOT assay is a new assay for the detection of dengue virus IgM. The test has high sensitivity, specificity, and concordance values compared with the results of the MAC-ELISA. Therefore, its characteristics of ease of performance, visual reading of reading of results, and rapidity allow us to recommend it for use for the diagnosis of dengue virus infection for both clinical diagnosis and seroepidemiological surveillance. Finally, the system is useful under field conditions and in laboratories with little equipment.

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