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ENZYME-LINKED IMMUNOSORBENT ASSAY-FORMAT MICRONEUTRALIZATION TEST FOR DENGUE VIRUSES

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Abstract. A microneutralization test that measures anti-dengue antibodies was developed. Serum dilutions, neutralization reactions, and virus growth were performed in 96-well plates. After incubation, an enzyme-linked immunosorbent assay that used mouse anti-dengue antibodies and an enzyme-conjugated anti-mouse antibody was used to measure cell-associated viral antigens. The resulting optical density readings were processed and graphed automatically by a spreadsheet program. This procedure provided results that are essentially the same as those from the plaque-reduction neutralization test for serum samples from primary dengue virus infections, but results correlated poorly with results from samples from people with secondary infections. The test offers the advantages of ease of performance, ease in the calculation of results, lower cost, and increased speed.

INTRODUCTION

The dengue viruses belong to the family *Flaviviridae,* which is composed of ~ 70 known members. Only a small number of these are significant from a public health standpoint, primarily dengue, yellow fever, St. Louis encephalitis virus, West Nile virus, and Japanese encephalitis virus, but these are some of the most important viral diseases affecting humans.[1] The antigens shared by this group of viruses can be problems for diagnostic and surveillance laboratories in areas where ≥2 flaviviruses are transmitted, such as dengue and yellow fever in Africa and South America, dengue and Japanese encephalitis in Asia, and, most recently, St. Louis encephalitis and West Nile viruses in North America.

Although immunity after infection with these viruses is serotype specific, standard serological tests may not distinguish among them, depending on the circumstances. The hemagglutination inhibition test[2] and the immunoglobulin (Ig) G enzyme-linked immunosorbent assay (ELISA)[3,4] are considered to be cross-reactive within the flavivirus group and may show higher antibody titers to homologous antigens only in carefully timed samples from primary infections.[5] The IgM capture ELISA, the most commonly used test for laboratory diagnosis of flavivirus infections, is also cross-reactive. Additionally, this test requires careful quantitation of antibodies to distinguish between dengue and Japanese encephalitis virus infections[6] and sometimes, but not always, shows higher optical densities to homologous antigens within the dengue group (Centers for Disease Control and Prevention, unpublished data). The complement fixation test[7,8] is considered to be serotype-specific within the dengue group, but this test relies on short-lived antibodies and is rarely used.

The virus neutralization test, usually performed as the plaque-reduction neutralization test (PRNT),[9] is the most reliable and frequently used serotype-specific test. In the PRNT, dilutions of serum are usually made and tested against a constant amount of the 4 dengue viruses calibrated to give a defined number of plaques. This procedure commonly requires separate dilution tubes and eight 6-well tissue culture plates for each serum sample tested, multiple overlays or other staining procedures, and a considerable amount of time for counting plaques and tabulating results. Previous attempts to develop microneutralization tests for dengue viruses have been reported.[10–14] These have involved counting fewer plaques or infected foci in small wells, with a corresponding loss in accuracy—and with little reduction in time or effort.

Microneutralization tests based on an ELISA format have been reported for other kinds of viruses.[15–19] In these procedures, a reduction in virus growth due to neutralization by antibody was measured optically. These tests had the advantage of an automated readout, and when coupled with a spreadsheet software program, an automatic and almost instantaneous processing of data and graph production may be obtained. We have developed and evaluated a microneutralization test to measure anti-dengue antibodies that uses 96-well plates and an ELISA to measure viral antigen production. When coupled to a template on a spreadsheet program, the data are processed and graphed automatically, and the results may be compared with results from the standard PRNT.

MATERIALS AND METHODS

Serum samples. Serum samples were obtained from the collection of Centers for Disease Control and Prevention's Dengue Branch laboratory in San Juan, Puerto Rico. Primary or secondary infection status was determined by the absence or presence of IgG antibody in an acute phase sample or by IgG titers characteristic of a secondary anamnestic immune response as determined by an IgG ELISA.[4]

Cell lines. C6/36 cells were maintained in minimum essential medium supplemented with 5% heat inactivated fetal bovine serum (Hyclone, Logan, UT), 2× vitamins, 2× L-glutamine, 2× nonessential amino acids, and 3.5% sodium bicarbonate (Invitrogen, Carlsbad, CA). The Vero cells used in both neutralization assays were split once a week by trypsinization. They were grown at 37°C in M199 medium containing 5% heat-inactivated fetal calf serum, 3.5% sodium bicarbonate, and HEPES buffer (Invitrogen).

Virus seed production. Dengue viruses used in both the PRNT and microneutralization tests were as follows: dengue (DEN)-1, Hawaiian; DEN-2, New Guinea C; DEN-3, H87; and DEN-4, H241. Virus suspensions were prepared by growth in C6/36 (*Aedes albopictus*) cells at 33°C for 5 days. Tissue culture supernatants were formed into aliquots and stored at −70°C.

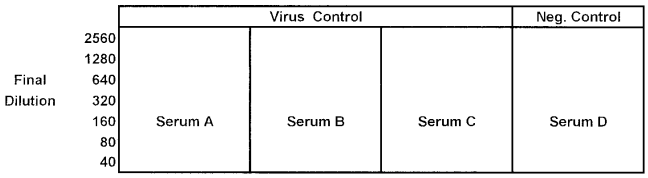

FIGURE 1. Diagramatic representation of the microtiter plate layout used for the microneutralization test.

Plaque reduction neutralization test. The PRNT test was performed as previously described.[9] Heat-inactivated serum specimens were diluted 2-fold (1:20 to 1:1,280) in tubes, with M199 (30% fetal bovine serum) used as a diluent, mixed with an equal volume of virus suspension (diluted to give 40–60 plaque-forming units [pfu]/well) and incubated for 2 h at room temperature. The serum-virus mixture (150 μL/well) was inoculated in duplicate onto Vero cells in 6-well plates. After adsorption for 1 h, 4 mL of enhanced medium (E-M199; M199 containing 5% fetal bovine serum, 2× vitamins, essential amino acids, L-glutamine, 4% sodium bicarbonate, and gentamicin plus 1% agar) was added to each well. Plates were incubated in 10% CO_2 at 37°C for 5 days. The plates were stained with 3.2% neutral red solution in phosphate-buffered saline (PBS). Plaques were counted after 24 h. A 50% reduction in plaque count was used as the end point of the titration.

Microneutralization test. A microneutralization assay in which an ELISA was used to measure virus neutralization was performed in 96-well, flat-bottomed tissue culture plates (Corning Costar, Wilkes Barre, PA). To prepare plates, Vero cells were suspended at 2×10^5 cells/mL in E-M199 medium. The cell suspension (100 μL/well) was dispensed into microtiter plate wells and incubated for 48 h at 37°C with 10% CO_2 until the monolayer was confluent. On the day of the test, 150 μL of serum sample was diluted 1:20 in E-M199 and inactivated at 56°C for 30 min. Microtiter plates for dilutions and incubations were prepared by adding 200 μL of inactivated serum to triplicate wells and serially diluting (2-fold up to 1:1,280) with a multichannel pipette. An equal volume (100 μL) of virus suspension in E-M199, diluted to contain 1×10^4 pfu/mL (previously determined by plaque titration), was added to each serum dilution well. This is approximately the highest dilution of virus that will produce an optical density (OD) of 1.0–1.5 in the ELISA after 5 days of incubation. The mixtures of serum and virus were incubated for 2 h at room temperature, then transferred to plates containing the Vero cells. The plates were incubated for 5 days at 37°C with 10% CO_2. Each plate included both uninfected and infected cell controls (Figure 1).

After incubation, the culture medium was removed from the plates, and the cells were fixed by filling the wells with a cold 1:1 methanol-ethanol solution. Plates were incubated at −20°C for 30 min and then washed 3 times with PBS. The ELISA was performed on the same day as the fixation; alternatively, the fixed plates may be stored covered at −20°C for at least 3 days.

The ELISA was performed by adding 100 μL/well of mouse hyperimmune ascitic fluid (an equal mixture for the 4 serotypes), diluted 1:4,000 in PBS, plus 10% normal goat serum. After incubating for 2 h at 37°C, the plates were washed 5 times with PBS. Peroxidase-conjugated goat anti-mouse IgG (Kirkegaard and Perry, Gaithersberg, MD; 100 μL/well) diluted 1:2,000 in PBS–10% normal goat serum was added, and the plates were incubated for 1 h at 37°C. Finally, the plates were washed 5 times with PBS, and 100 μL/well of ABTS substrate solution (Kirkegaard and Perry) was added. Color was developed at room temperature for 40–60 min.

The optimum dilutions of mouse ascitic fluid and goat anti-mouse IgG used throughout the study were determined by box titration. Optical density readings were obtained with a BioTek microplate reader at 405 nm and transformed for computer analysis with KC4 software (BioTek, Winooski, VT). Transformed data were processed and graphed by means of a spreadsheet (Excel; Microsoft, Redmond, WA) containing 3 templates. The first template accepts the unprocessed OD readings from the plate reader as a formatted DOS text file. The second template calculates the mean of each triplicate dilution and the cutoff value. The cutoff value was established as the mean OD of virus controls minus 2 standard deviations. After running a battery of 8 normal hu-

TABLE 1

Mean optical densities from triplicate dilutions calculated from the initial microplate reading*

DEN-1	Mean OD = 1.357	Cut-off = 1.097		
DILUTION	Serum A	Serum B	Serum C	Serum D
40	1.126	0.064	1.593	0.026
80	1.392	0.963	1.414	0.313
160	1.546	1.290	1.365	1.177
320	1.456	1.343	1.378	1.284
640	1.362	1.358	1.334	1.277
1280	1.367	1.373	1.331	1.346
2560	1.451	1.407	1.346	1.342
DEN-2	Mean OD = 1.514	Cut-off = 1.254		
DILUTION	Serum A	Serum B	Serum C	Serum D
40	1.053	1.257	1.947	0.022
80	1.296	1.666	1.800	0.007
160	1.396	1.553	1.645	−0.013
320	1.469	1.512	1.685	−0.002
640	1.476	1.643	1.517	0.048
1280	1.445	1.532	1.489	0.248
2560	1.490	1.593	1.628	1.474
DEN-3	Mean OD = 1.559	Cut-off = 1.299		
DILUTION	Serum A	Serum B	Serum C	Serum D
40	0.555	0.039	0.206	0.180
80	0.953	0.098	0.384	0.339
160	1.206	0.301	0.987	0.684
320	1.238	0.848	1.285	1.245
640	1.304	1.439	1.464	1.479
1280	1.360	1.364	1.512	1.462
2560	1.387	1.487	1.492	1.427
DEN-4	Mean OD = 1.378	Cut-off = 1.118		
DILUTION	Serum A	Serum B	Serum C	Serum D
40	0.468	1.539	1.307	0.056
80	0.824	1.495	1.261	0.053
160	1.124	1.419	1.227	0.153
320	1.261	1.321	1.278	0.484
640	1.328	1.428	1.156	1.033
1280	1.296	1.445	1.235	1.240
2560	1.318	1.451	1.208	1.274

* See Figure 2. The mean optical density (OD) value at the top of each table is for the virus control wells. The cutoff value is the mean of the virus control OD values minus 2 standard deviations.

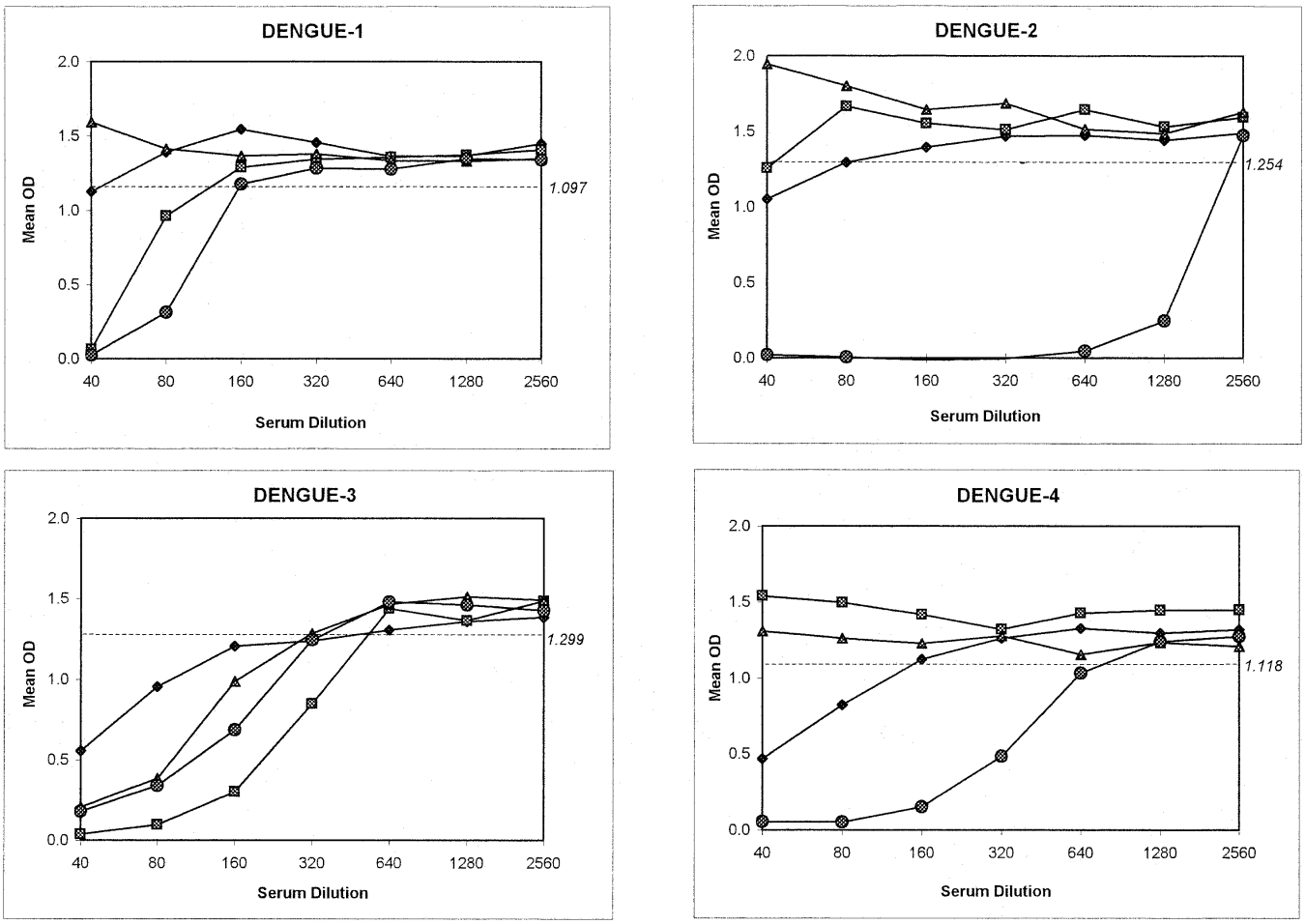

FIGURE 2. Graphical representation of the neutralization test results for 4 serum samples. In each graph, 4 serum samples are tested against the indicated dengue serotype. The cutoff value for neutralization is indicated by the dashed line. Serum samples A–D are represented by diamonds, squares, triangles, and circles, respectively. The data are from Table 1.

man serum specimens, the 2 standard deviation value was determined to be 0.26. For all subsequent experiments, the cutoff value for positivity (neutralization) was calculated by subtracting 0.26 from the mean OD of the virus controls. The neutralization titer was the highest serum dilution with a mean OD below the cutoff value. The third template graphs the processed data from the second template.

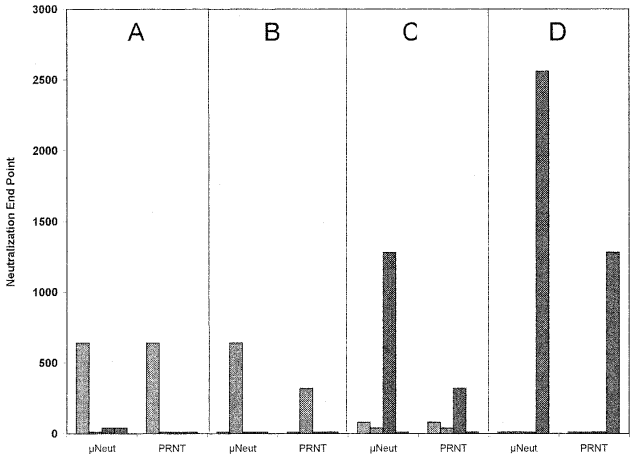

FIGURE 3. Antibody titers by microneutralization (µNeut) and plaque-reduction neutralization test (PRNT) to the 4 dengue serotypes by use of samples from primary dengue virus infections.

RESULTS

Serum samples from both primary and secondary dengue infections were evaluated by the microneutralization test. The neutralization curves generated by plotting the OD readings obtained for each serum dilution (Table 1) are shown in Figure 2. In this example, serum C shows a neutralization titer of 1:320 against DEN-3 only, typical of a primary immune response. Serum samples A and B demonstrated cross-reactivity of varying degrees, but in each case, the titer to one serotype was 4-fold more than titers to other serotypes. This degree of cross-reactivity is also typical of primary immune responses. Serum D, on the other hand, demonstrated higher neutralization titers of 1:80, 1:1,280, 1:320, and 1:640 against DEN-1 through DEN-4, with no titer being 4-fold or higher against all other titers. This broad cross-reactivity is characteristic of secondary flavivirus infections.

We compared the cross-reactivity patterns of both primary and secondary dengue serum samples by titration in both the PRNT and microneutralization tests. In Figure 3, the results

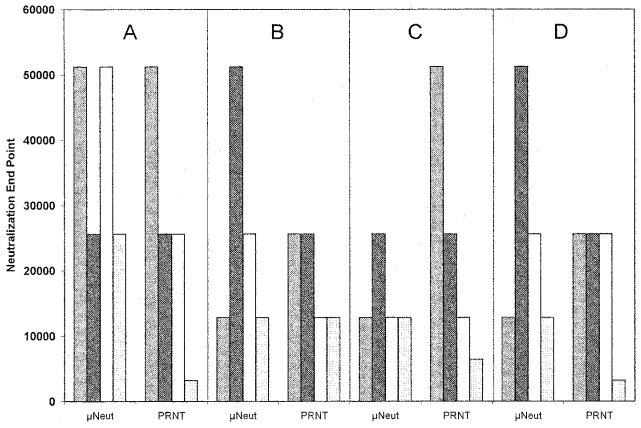

FIGURE 4. Antibody titers by microneutralization (μNeut) and plaque-reduction neutralization test (PRNT) to the 4 dengue serotypes by use of samples from secondary dengue virus infections.

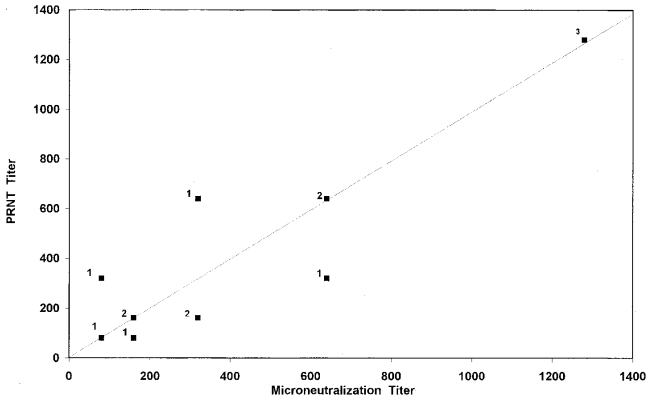

FIGURE 5. Scatter plot of end-point titers by plaque-reduction neutralization test (PRNT) and microneutralization test using primary dengue virus infections. Numbers indicate data points under each mark. The dashed line indicates complete correlation.

from 4 serum samples from primary dengue virus infections are shown. The results demonstrate a similar pattern of neutralizing antibody titer by both tests. Serum samples E–H are from patients with primary DEN-1–4 cases, respectively. In each example, the titers indicate that either monospecific reactions occurred or that there was minimal cross reaction to other serotypes. In each of the latter cases, the highest titer was at least 4-fold higher than the other titers.

In a similar manner, results obtained with serum samples from patients with secondary dengue infections are shown in Figure 4. A broader pattern of cross-reactivity with higher titers characteristic of secondary flavivirus infections was seen. In none of these 4 samples was the titer to any one serotype 4-fold or higher than titers to the other 3 serotypes by either the PRNT or the microneutralization test.

The cross-reactivity of the microneutralization test was further evaluated by testing antisera from human cases of yellow fever, St. Louis encephalitis, and Japanese encephalitis viruses. The results were uniformly negative (data not shown). Likewise, serum samples from patients with dengue did not neutralize the 17D yellow fever virus strain in this test. The results obtained with the microneutralization test and the PRNT were compared by graphing the end-point titers from 17 primary dengue infections in a scatter plot (Figure 5). In 15 patients, the microneutralization titers fell within one dilution (2-fold) of the PRNT titer. The other 2 examples showed 4-fold differences in titer between the 2 tests, but in both of these patients, the highest titer was to the same serotype and 4-fold higher than to other serotypes. The Pearson correlation coefficient between the 2 data sets was 0.84. On the other hand, no meaningful correlation was seen in end-point titers when we used serum samples from patients with secondary dengue.

DISCUSSION

The serological diagnosis of dengue virus infections is usually performed by isotype-specific ELISAs or by the hemagglutination inhibition test. These tests are not considered to be reliably serotype specific, but this is usually acceptable because the documentation and treatment of dengue virus infections does not depend on knowing the serotype involved. On the other hand, for various research and epidemiologic investigations, a serotype-specific test is required. The PRNT has long been the standard assay for obtaining this information. This test has several drawbacks, however, including a requirement of separate tubes for dilutions, many plastic plates to accommodate replicate samples of multiple dilutions, and the counting and tabulating of plaques. The PRNT is therefore labor-intensive and unsuitable for large numbers of samples.

The ELISA-format neutralization tests avoid these problems and significantly reduced processing time. At least 4 serum samples can be tested against a virus on a single plate, and dilutions are performed via multichannel pipetting devices. In addition, results of each test are obtained as OD values and can be processed and graphed by a computer. These factors lead to a neutralization test that is less expensive, less time-consuming, and more conducive to testing large numbers of samples. A 5-day incubation period was chosen so that minimal amounts of virus could be used in the test. Higher concentrations of virus could reduce the incubation time of the test but might result in a loss of sensitivity.

The microneutralization test reported here produces results that are essentially the same as those obtained from the PRNT with serum samples from patients with primary dengue. End-point titration values with one test were found to be almost always within one 2-fold dilution of the other test even though, unlike the PRNT, no agar is used to inhibit spread of new virus in the culture. It may be that residual neutralizing antibody in solution performs the same function in the microneutralization test. It is unknown whether this antibody also inhibits cell-to-cell spread of virus. This clear correlation did not hold true with serum samples from secondary infections. In this case, neither test consistently produced titers either higher or lower than the other. The overall pattern was the same, however, in that each serum sample produced neutralization against all 4 serotypes of virus in both tests, and diagnostic 4-fold differences in titers were not obtained.

This outcome may be related to either greater cross-reactivity or higher avidity of the resulting antibody population in secondary infections, the effect of which is more efficient

neutralization for heterologous serotypes in the respective tests. As with the PRNT, we do not believe that a diagnosis of serotype can be made with the microneutralization test after secondary infections.

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