Comparative Evaluation of Three Assays for Measurement of Dengue Virus Neutralizing Antibodies

J. Robert Putnak,* Rafael de la Barrera, Timothy Burgess, Jorge Pardo, Francis Dessy, Dirk Gheysen, Yves Lobet, Sharon Green, Timothy P. Endy, Stephen J. Thomas, Kenneth H. Eckels, Bruce L. Innis, and Wellington Sun

Division of Viral Diseases, Walter Reed Army Institute of Research, Silver Spring, Maryland; Naval Medical Research Center, Silver Spring, Maryland; Global Vaccine Development, GlaxoSmithKline Biologicals, Rixensart, Belgium; Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School, Worcester, Massachusetts; Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

Abstract. Plaque reduction neutralization tests (PRNTs) have been commonly used for measuring levels of dengue virus (DENV) neutralizing antibodies. However, these assays lack a standardized format, generally have a low sample throughput, and are labor-intensive. The objective of the present study was to evaluate two alternative DENV neutralizing antibody assays: an enzyme-linked immunosorbent assay–based microneutralization (MN) assay, and a fluorescent antibody cell sorter–based, DC-SIGN expresser dendritic cell (DC) assay. False-positive rates, serotype specificity, reproducibility, sensitivity, and agreement among the assay methods were assessed using well-characterized but limited numbers of coded test sera. Results showed that all three assays had false-positive rates of less than 10% with titers near the cut-off and generally below the estimated limits of detection. All three methods demonstrated a high degree of specificity and good agreement when used to assay sera and serum mixtures from monovalent vaccines and sera from patients after primary natural infection, with the only notable exception being moderate-to-high neutralizing antibody titers against DENV 2 measured by PRNT in a mixture containing only DENV 3 and DENV 4 sera. The MN and DC assays demonstrated good reproducibility. All three assays were comparable in their sensitivity, except that the PRNT was less sensitive for measuring DENV 4 antibody, and the MN and DC assays were less sensitive for measuring DENV 2 antibody. However, when used to test sera from persons after tetravalent DENV vaccination or secondary DENV infection, there was poor specificity and poor agreement among the different assays.

INTRODUCTION

Plaque reduction neutralization tests (PRNTs) have been used for more than 30 years for measuring dengue virus (DENV) neutralizing antibodies, which is thought to be an important mediator of protection against dengue fever.1 Therefore, the PRNT can be considered to be the current gold standard for determining immunity to DENV. The PRNT is performed by incubating a small number of plaque-forming units of a dose or challenge virus with serial dilutions of the serum or plasma to be tested, and then inoculating the mixture onto sensitive cell monolayers and counting the plaques that form after several days. Virus neutralizing antibody titers are calculated by an interpolative method such as linear regression (probit) analysis and are usually reported as the reciprocal of the serum dilution exhibiting a 50% (or less frequently a 70% or 90%) reduction in the number of viral plaques compared with the number of plaques on control monolayers inoculated with virus plus diluent or a non-immune serum. Because PRNTs are labor intensive, time-consuming, and have a relatively low sample throughput, faster and more efficient methods are desirable to support large dengue seroprevalence studies and vaccine trials. In addition, results generated by PRNTs are sometimes difficult to reconcile with virologic and epidemiologic data. Because of significant serotype cross-reactivity, it is not always possible to identify the infecting virus serotype after a secondary DENV infection,2 and there are reports of dengue fever in persons with pre-existing, high-titer, serotype-specific DENV neutralizing antibodies measured by PRNT.3

In the present study, two new DENV neutralizing antibody assays, an enzyme-linked immunosorbent assay (ELISA)–based microneutralization (MN) assay modified from the version originally described,4 and a fluorescent antibody cell sorter–based, DC-SIGN expresser dendritic cell (DC) assay,5,6 were evaluated with a well-established Vero cell–based PRNT to independently test well-characterized sera from DENV vaccine clinical trials.7 These included sera from flavivirus-negative persons that were used to estimate the rate of false-positives results, sera from recipients of monovalent live-attenuated DENV vaccines that were used to assess serotype specificity and estimate the limit of detection (LOD) for each serotype, and sera from persons vaccinated with tetravalent live-attenuated DENV vaccines that were used to estimate the degree of concordance with PRNT results. A small number of coded duplicate sera were used for assessing repeatability. Finally, paired acute-phase (day 1–3 of illness) and convalescent-phase (day 180) plasma samples were tested to determine the ability of the assays to specifically measure DENV neutralizing antibodies after both primary and secondary natural infections and to determine if the observed antibody titers correspond to the infecting virus serotype.

MATERIALS AND METHODS

Cells. The Vero cells used initially for the PRNTs were from an in-house cell bank (Vero–Walter Reed Army Institute of Research [WRAIR], passage-254). Because this subline lacked suitable documentation concerning its origin and sterility, it was replaced in subsequent assays by the better-characterized Vero-81 cell line (passage-123) from the American Type Culture Collection (Manassas, VA). Cells were propagated in 150-cm² (T150) flasks (Corning, Corning, NY) in 50 mL of Eagle’s minimum essential medium (EMEM) (Cambrex, Rockland, ME) with 10% heat-inactivated fetal...
bovine serum (FBS) (Cambrex), L-glutamine (Cambrex), non-essential amino acids (Gibco/Invitrogen, Grand Island, NY), and penicillin and streptomycin (Cambrex), at 35°C in an atmosphere of 5% CO₂ in closed or vent-capped flasks. The cells were subcultured weekly, one confluent T150 flask into five fresh flasks, by washing the cell sheet once with phosphate-buffered saline (PBS), pH 7.4, once with 5 mL of 0.25% trypsin-versene solution, and then removing the cells by incubation at 35°C with 1 mL of fresh 0.25% trypsin-EDTA (Gibco/Invitrogen) followed by gentle trituration. Subculturing was performed a maximum of 20 times before fresh cultures were started from a frozen cell bank. Six-well plates (60 cm²) (Corning/Costar, Corning, NY) for the assays were prepared from confluent T150 flasks with 8–10 six-well plates from one T150 flask. The plates were incubated at 35°C in an atmosphere of 5% CO₂ until the cell monolayers reached confluency (approximately 2–3 days) and were then used for the assays within 4 days.

The Vero cells used for the MN assay were from a WRAIR in-house cell bank from cells originated by the World Health Organization (WHO) (WHO DEN Vero Cells, National Institute for Biological Standards and Control [NIBSC]-011038, or Vero WHO) and were established, maintained, and distributed by the NIBSC (Potter’s Bar, Hertfordshire, United Kingdom). They were split weekly by trypsinization into T150 flasks containing 50 mL of growth medium composed of EMEM supplemented with 10% heat-inactivated FBS (Gibco/Invitrogen), 3% L-glutamine (200 mM), 1% streptomycin (10 mg/mL), and 0.5% neomycin (10 mg/mL), and incubated at 35°C in an atmosphere of 5% CO₂ at a relative humidity ≥ 95%. Flat bottom 96-well microplates (Corning/Costar) for the assay were prepared from confluent T150 flasks, and seeded with 0.1 mL/well of a cell suspension (5.0 × 10⁶ cells/mL in growth medium). The microplates were incubated at 35°C in an atmosphere of 5% CO₂ and a relative humidity ≥ 95% until the monolayer was confluent for use in the assay (up to 48 hours). The DC-SIGN–transfected Raji cells for the DC assay have been described in detail.5

Viruses. The challenge (dose) viruses used for the assays were near wild-type isolates of DENV 1 (strain WestPac-74), DENV 2 (strain S16803), DENV 3 (strain CH53489), and DENV 4 (strain TVP-360).3 Viruses were propagated in Vero cells cultured at 35°C in EMEM and infected at a multiplicity of infection of 0.01. Virus working stocks were produced at Vero passage level 4. Culture supernatants were harvested 5–7 days post-infection, filtered through a 0.22-µm filter, divided into 1-mL aliquots, freeze-dried, and stored at −20°C. Immediately prior to use, aliquots were rehydrated with 1 mL of sterile water and placed on ice.

Serum panels. All assays were performed in a blinded fashion using numerically encoded serum aliquots. Panel one was composed of 95 sera collected from volunteers who received candidate live-attenuated tetravalent DEN vaccines, and included pre-vaccination (day 0) specimens (n = 32), specimens collected 30 days post-dose one (day 30) and 30 days post-dose two (day 210), and represented seroconversions against 0, 1, 2, 3, or all 4 DENV serotypes. This panel also contained four coded duplicate sera. Sera for panel two were obtained from volunteers who received candidate monovalent (monotypic) live-attenuated DEN vaccines. One monovalent DENV antisera was prepared for each DENV serotype (1, 2, 3, and 4) by pooling appropriate individual serum specimens. Each monotypic serum pool was adjusted to a volume of 10 mL with normal (non-flavivirus immune) serum. Multivalent (multitypic) antisera mixtures were then prepared by mixing equal volumes of the monovalent serum pools to reconstitute all possible combinations of mono-, di-, tri-, and tetra-typic DENV antisera, which were aliquoted and numerically recoded. Panel three was prepared from plasma samples collected from study subjects with natural dengue virus infections admitted to the dengue ward of the Queen Sirikit National Institute of Child Health (Bangkok, Thailand).9 Subjects were enrolled if they had fever for less than 72 hours and no localizing signs or symptoms.

Acute-phase (illness days 1–3) and convalescent-phase (day 180) samples were used for testing. Serotype specificity was determined by nested or quantitative reverse transcription–polymerase chain reaction assays and viral culture.10,11 Primary or secondary infection status was determined by hemagglutination inhibition titer and ELISA IgM/IgG ratios.12–14 All aliquots for testing were numerically recoded and frozen at −80°C. Immediately prior to testing, the sera were thawed and heat-inactivated at 56°C for 30 minutes. The samples used in this comparative study were previously collected under the following human use protocols: WRAIR #623, WRAIR #877, WRAIR #937 (from adult volunteers with informed consent), and dengue hemorrhagic fever (DHF) Project I (from children and adults with informed or parental consent), with approvals from the following institutional review boards (IRBs): United States Army Surgeon General, United States Navy, Thai Ministry of Public Health, and University of Massachusetts Medical School.

Plaque reduction neutralization test. Sera for testing were diluted in EMEM with 2% heat-inactivated FBS to make serial four-fold dilutions ranging from 1:10 to 1:640 or higher as required to reach an endpoint. The tubes were kept on ice. An equal volume of DEN dose virus of the appropriate serotype, diluted to a virus infectivity concentration of 500 plaque-forming units/mL, was added to each serum serial dilution tube. The tubes were mixed, incubated at 35°C for 30 minutes, and returned to an ice bath. Six-well plates were prepared for inoculation by removing all but 0.1–0.2 mL of the culture medium. Each virus plus serum dilution was inoculated onto duplicate or triplicate wells (0.2 mL/well). A virus dose control (virus plus diluent only) was added to 12 replicate wells. The plates were rocked gently to distribute the inoculum and incubated at 35°C in an atmosphere of 5% CO₂ for 60 minutes. After incubation, the cell monolayers were overlaid with nutrient agarose consisting of 0.9% Sea-Plaque low-melting agarose (SeaPlaque/Cambrex, Rockland, ME), EMEM, L-glutamine, non-essential amino acids, and penicillin, streptomycin, and amphotericin B (Cambrex). The plates were kept at room temperature for 20–30 minutes to enable the agarose to solidify and then incubated at 35°C in an atmosphere of 5% CO₂. After 4–5 days of incubation, the viral plaques were visualized by staining with neutral red (Sigma, St. Louis, MO) (3.5 mL of a 0.33% [w/v] neutral red solution and 0.9% low-melting agarose in 100 mL of PBS), and incubated at 35°C in an atmosphere of 5% CO₂ for 18–24 hours. Plaques were counted and plaque reduction 50% endpoint (PRNT₅₀) titers were calculated by probit analysis implemented on a microcomputer with the SPSS statistical software package (SPSS Inc., Chicago, IL). A positive antibody control (pooled, tetravalent DENV neutralizing anti-
body–positive human sera) and a virus dose control (virus plus diluent without serum) were included in each assay run. For a valid assay, the number of viral plaques in the virus dose control wells was required to be in the range of 20–100.

**Microneutralization assay.** The MN assay has been described in detail and modified for the current study. Serum samples to be assayed were diluted 1:10 in EMEM containing 10% FBS and heat-inactivated at 56°C for 30 minutes. The heat-inactivated sera were then serially diluted three-fold (50 μL/H9262L serum plus 100 μL/H9262L diluent) to a dilution of 1:7,290 in round-bottom, 96-well microplates (Corning/Costar). One hundred microliters of the calibrated virus dose (the highest dilution of each serotype, DENV 1-4, that produced 1.0 ELISA optical density units at 405 nm [OD 405] after a five-day incubation) was added to 100 μL/H9262L of each serum dilution and incubated at room temperature (23 ± 2°C) for 2 hours. After incubation, the contents of the round bottom, 96-well plates were transferred to flat bottom microplates containing Vero cell monolayers from which the growth medium had been decanted. The microplates were formatted to test four serum samples with three wells for each serum dilution, nine virus-only control wells, and three blank wells containing growth medium alone. After inoculation, the plates were incubated for 5 days at 35°C in an atmosphere of 5% CO2, and a relative humidity ≥95%. After incubation, the media was removed and the cells were fixed with 300 μL/well of absolute ethanol:methanol (1:1) for 1 hour at −20°C. For the ELISA, the fixative was removed and the plates were washed three times with PBS. After washing, a 1:16,000 dilution of anti-DEN monoclonal antibody 4G2 (Centers for Disease Control and Prevention, Atlanta, GA) in antibody sample diluent (0.5% casein, 0.5% bovine serum albumin, 0.5% normal goat serum in deionized water) was added to the plates (100 μL/well), incubated at 35°C for 2 hours, then washed 5 times with PBS (350 μL/well). Goat anti-mouse antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) diluted 1:4,000 in antibody sample diluent was added to the plates (100 μL/well) and the plates were incubated at 35°C for 1 hour. After incubation, the plates were washed 5 times with PBS (350 μL/well) and 100 μL/well of horseradish peroxidase substrate (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was

![Figure 1](image-url)
added to each well. The OD_{405} was read on a microplate reader (Titertek-Ascent) 55 ± 5 minutes after the addition of substrate. For a valid assay, the average OD_{405} of the three non-infected control wells of each microplate must be ≤ 0.4, and this value was used to normalize the entire microplate. The nine virus-only (dose) control wells must have an OD_{405} ≥ 1.0. The normalized OD values were then transformed to a four-parameter logistical model using Graph Pad Prism version 4.0 software for Windows (Graph Pad Software, San Diego, CA) to calculate 50% MN (or 50% effective concentration) titers by the use of a sigmoidal dose response (variable slope) formula. The virus neutralization titer was defined as the reciprocal of the serum dilution giving 50% reduction in the absorbance readout of the assay when compared with the virus dose control without serum. For a valid 50% MN titer, the R^2 value for the fitted sigmoidal curve was required to be ≥ 0.80.

**Fluorescent antibody cell sorter–based DC-SIGN expr**essor cell (DC) assay. The DC assay was performed as described in detail.5

**Data analysis.** The lower limit for a positive result (assay cut-off value) was set by convention at a reciprocal serum dilution of 10 for all three assays. The LOD for each assay with each DENV serotype was then independently estimated by assaying monotypic serum pools, which were serially diluted three-fold to beyond the point of the last positive result. The LOD was defined as the midpoint between the lowest positive (non-negative) titer ≥ 10 and the titer obtained for the next higher serum dilution (i.e., a titer < 10). The amount of disagreement among assay methods was determined by a non-parametric test, McNemar’s association test,15 implemented on Graph Pad Prism version 4.0 for Windows. Assay repeatability was determined using coded duplicate serum specimens and the percent coefficient of variation (% CV) between repeats was calculated. Percent recoveries were used for comparing assay results with dilutions of monovalent DENV serum and polyvalent serum pools.

**RESULTS**

**False-positive rate and LOD for each DENV serotype.** Thirty-two sera from flavivirus-negative, antibody-negative persons were used to determine the rate of false-positive results for the assays. As shown in Table 1, the MN assay exhibited a low rate of false-positive results only for DENV 1 and DENV 2, whereas, the DC assay exhibited a low rate of false-positive results only for DENV 3 and DENV 4. All false-positive titers were < 15. The geometric mean titers of false-positive results were below the estimated assay LOD, (see below) except for DENV 1 neutralizing antibody measured by the MN assay and DENV 4 antibody measured by the DC assay.

The assay sensitivity and LOD for each DENV serotype were estimated using monosero type-specific (monovalent) antiserum pools for DENV 1, 2, 3, and 4, which were diluted serially three-fold to beyond the dilution at which neutralizing antibody could be detected (Figure 1 and Table 1). With the DENV 3 antiserum pool, all three assays generated similar neutralizing antibody titers across the range of serum dilutions tested and gave similar LODs. With the DENV 1 antiserum pool, the MN assay tended to give higher titers than the PRNT and DC assay and exhibited a lower LOD than the DC assay. With the DENV 2 antiserum pool, the PRNT gave consistently higher neutralizing antibody titers than the MN and DC assays. However, with the DENV 4 antiserum pool, this assay gave lower neutralizing antibody titers compared with the other two assays although the estimated LOD was not much lower.

**Serotype specificity.** To assess the serotype specificity of the assays, the DENV monovalent antiserum pools were combined to produce all possible combinations of di-, tri-, and tetravalent antisera. The resulting multivalent antiserum mixtures were then assayed for DENV 1, 2, 3, and 4 neutralizing antibodies with all three tests. The PRNT exhibited nonspecificity (i.e., serotype cross-reactivity) only for DENV 2, which was associated with moderate-to-high neutralizing antibody titers against DENV 2 in a mixture containing only DENV 3 and DENV 4 antisera (Figure 2). The MN assay exhibited nonspecificity only for DENV 1, which was associated with low titers of DENV 1 antibody in a mixture containing only DENV 2 and DENV 3 antisera (Figure 3). The DC assay exhibited some nonspecificity for all four serotypes, but the antibody titers were low (Figure 4). In most

![Figure 2. Plaque reduction neutralization assay used to test dengue virus (DENV) 1, 2, 3, and 4 monotypic antiserum pools (A), and reconstituted bivalent (B) and trivalent and tetravalent (C) antiserum pools. Reciprocal neutralizing antibody titers obtained for each DENV serotype are represented by bars with positive error bars representing one standard deviation.]
cases, the recovery remained within 50–200% of the expected value, which is consistent with the historically observed inter-assay variation.

The relationships among the three different neutralization assays were evaluated using the data collected with the monotypic and reconstituted multivalent sera. Samples generating a negative test result were discarded from the evaluation because they would have introduced a bias leading to an overestimation of the agreement between the different assay methods. The PRNT and MN assay showed overall good agreement except for DENV 2. This lack of agreement for serotype 2 might be explained by the narrow distribution of the antibody titers, which were all within a three-fold range. The DC assay also showed a good agreement with the PRNT and MN assay except for DENV 2 for the same reason. When the DC assay was compared with the MN assay and the PRNT, the slope of the regression curves differed greatly among the four serotypes, which suggests that the absolute neutralizing antibody titers might not be comparable among the DENV serotypes.

**Assay results concordance and repeatability.** Sera collected after tetravalent DENV vaccination were assayed using the MN and DC assays and the results were compared with historical results obtained with the PRNT. McNemar’s association test (see Materials and Methods) was used to test for significant disagreement among the different assay methods. The PRNT and MN assay demonstrated significant disagreement only for DENV 3 antibody (Table 2), whereas, the PRNT and DC assay demonstrated disagreement for both DENV 3 and 4 antibodies (Table 3), and the MN and DC assays demonstrated disagreement for DENV 1, 2, and 3 antibodies (Table 4).

The repeatability of the MN and DC assays was determined using four coded duplicate sera, which were assayed by both assays in a one assay run. The % CV between the results for each coded duplicate sample was then determined. With the MN and DC assays, the titers obtained for the coded duplicate samples fell within two-fold of each other with a % CV ≤ 40%, except for one duplicate sample where the DENV 2 titers measured by the DC-SIGN assay exhibited a % CV of 67%.
Measurement of DENV neutralizing antibodies after natural infection. The ability of the three different assay methods to specifically detect and measure DENV neutralizing antibodies after natural infection was evaluated using paired acute-phase (days 1–3 of illness) and convalescent-phase (day 180) plasma samples from persons clinically diagnosed with primary or secondary DENV 1, 2, 3, or 4 infection. The clinical diagnosis was compared with the neutralizing antibody titers obtained for the convalescent specimens (in cases of presumptive primary infection) or the fold-increase in titer from the acute-phase to the convalescent-phase specimen pairs (in cases of presumptive secondary infection). The results are summarized in Table 5. In four cases of primary DENV 1 infection, the PRNT results were in agreement with the clinical diagnosis in three of the four cases, and the MN and DC assays results agreed in all four cases. In five cases of primary DENV 3 infection, the PRNT and MN assay results were in agreement with the clinical diagnosis in all cases, and the DC assay results agreed in three of the five cases. In five cases of secondary DENV 2 infection, results with each assay method agreed with the clinical diagnosis in only three of the five cases; furthermore, agreement among results with the three assay methods was also poor. Similarly, in cases of secondary infections with other DENV serotypes, there was poor agreement among results with the different assay methods and poor agreement with the clinical diagnosis.

DISCUSSION

A PRNT format is currently the most widely used and accepted method for measuring DENV neutralizing antibodies. Although PRNT results are considered by many to be the gold standard, is it possible that other assays will give results that better correlate with protection. Also from a practical perspective, assays that are faster, more efficient, and amenable to automation will be required for the testing of large numbers of specimens that will be generated from anticipated Phase 3 clinical trials of several candidate DENV vaccines currently under development.

Two new assays to replace the PRNT have recently been described: a microneutralization ELISA in Vero cells and a flow cytometry–based assay performed in DC-SIGN–bearing cells. The goal of this study was to compare the performance of the two new assays with the PRNT in measuring DENV neutralizing antibodies using well-characterized sera from DENV vaccine recipients and plasma collected from patients with naturally acquired DENV infections.

Prior to conducting the evaluation, criteria were established to enable comparison among the different assays. These criteria were 1) the rate of false-positive results, 2) serotype specificity and sensitivity to detect neutralizing antibodies in low-titer specimens, and 3) repeatability. Finally, agreement with PRNT results and the ability of the assays to measure antibodies after primary and secondary DENV infection were considered with respect to the performance of each assay.

The frequency of false-positive results was low (0–6%) for the two new assays and the false-positive reciprocal titers were less than 15, with an assay cut-off value of 10. For the two new assays, the level of serotype specificity in measuring DENV neutralizing antibodies in monovalent (monotypic) antisera and reconstituted polyvalent mixtures was similar and at least as good as that observed with the PRNT.

The sensitivities of the MN and DC assays were globally similar to each other in measuring virus neutralizing antibodies in diluted monovalent DENV antisera. These assays were more sensitive than the PRNT in measuring DENV 4 neutralizing antibodies, but less sensitive in measuring DENV 2 neutralizing antibodies. The higher sensitivity of the DENV 2 PRNT and DENV 1 MN assays should be contrasted with

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DENV 1</th>
<th>DENV 2</th>
<th>DENV 3</th>
<th>DENV 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. (%) discordant results</td>
<td>15 (25)</td>
<td>19 (32)</td>
<td>27 (46)</td>
<td>8 (14)</td>
</tr>
<tr>
<td>PRNT positive and DC negative</td>
<td>11</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>DC positive and PRNT negative</td>
<td>4</td>
<td>14</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>Significance level (two-tailed)</td>
<td>Not significant (P = 0.121)</td>
<td>Not significant (P = 0.067)</td>
<td>Significant (P = 0.001)</td>
<td>Significant (P = 0.013)</td>
</tr>
</tbody>
</table>

*PRNT = plaque reduction neutralization test; DC = dendritic cell; DENV = dengue virus. Significance level is a statistical measure of the assay disagreement determined using McNemar's test (see Materials and Methods).
**TABLE 4**

Assay disagreement by McNemar’s test for MN assay versus DC assay (n = 63 samples)*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DENV 1</th>
<th>DENV 2</th>
<th>DENV 3</th>
<th>DENV 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. (%) of discordant results</td>
<td>17 (27)</td>
<td>17 (27)</td>
<td>35 (56)</td>
<td>6 (10)</td>
</tr>
<tr>
<td>MN positive and DC negative</td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>DC positive and MN negative</td>
<td>2</td>
<td>14</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>Significance level (two-tailed P)</td>
<td>Significant (P = 0.004)</td>
<td>Significant (P = 0.015)</td>
<td>Significant (P = 0.0001)</td>
<td>Not significant (P = 0.221)</td>
</tr>
</tbody>
</table>

* Only 62 samples were run for comparison for dengue virus (DENV) type 1. MN = microneutralization; DC = dendritic cell. Significance level is a statistical measure of the assay disagreement determined using McNemar’s test (see Materials and Methods).

Their lower specificity. This observation suggests that a slight upward adjustment in the cut-off values for a positive result might make all three tests more similar in their sensitivity and also increase the specificity of the PRNT for DENV 2 antibody. Both the MN and DC assays exhibited repeatability superior to that observed historically with the PRNT, with the titers for a small number of coded duplicate samples mainly falling within a two-fold range (i.e., a % CV ≤ 40%).

When used to measure DENV neutralizing antibodies in plasma samples from patients with a clinical diagnosis of primary DENV natural infection, all three assays exhibited a generally high degree of serotype specificity. However, in a few cases, the PRNT measured more heterologous than homologous neutralizing antibody in the convalescent-phase specimen, which is suggestive of a secondary infection. In cases where the clinical diagnosis was one of secondary DENV infection, none of the assays exhibited a high degree of serotype-specificity for the second infecting serotype, although in such cases it is not uncommon to observe the highest antibody titer and titer increase against the initial infecting serotype.

Although the PRNT has not been validated by most laboratories, it has been in use for a long time and is widely accepted. Therefore, we felt that it was important to determine how the results obtained with the two new assays agreed with those obtained with the PRNT. This was done by testing sera from DENV tetravalent vaccine recipients by all three methods and using McNemar’s association test to estimate the amount of disagreement between assays. The MN assay demonstrated significant disagreement with the PRNT only for DENV 3 antibodies. The DC assay demonstrated disagreement with the PRNT for DENV 3 and 4 antibodies. The MN and DC assays demonstrated disagreement for DENV 1, 2 and 3 antibodies. It was not surprising that some disagreement among the assay methods was seen, given the differences in the methods and cell substrates used. For example, the use of different receptors for DENV attachment to the Vero cells used in the PRNT and MN assays and the DC-SIGN-expressing Raji cells used in the DC assay could change the viral epitopes involved in neutralization on the respective cell substrates.

One additional criterion, assay throughput, was not directly measured in this study, but it was estimated that the MN and DC assays have the capability to exceed the throughput of the PRNT by at least five-fold. The larger number of samples that can be accommodated per assay run with the MN and DC assays and the shorter incubation time of the DC assay are clear advantages over the PRNT. In addition, there may be a smaller but still important cost-savings over the PRNT, which is more labor-intensive than the MN and DC assays.

At the conclusion of the present study, no new assay was clearly superior and both remain candidates to replace the PRNT. The MN assay was recently successfully qualified (de la Barrera and others, unpublished data) prior to its validation as a necessary step for its use in Phase 3 vaccine clinical trials. A future goal is to further evaluate both new assays by testing of additional serum specimens including human anti-DENV neutralizing antibody standards, which will also be useful for assessing the accuracy of the titers obtained. It is perhaps even more important to know how DENV neutralizing antibody titers obtained with the different assays correlate with protection to determine the biological and clinical relevance of the results. This question may only be answered by prospective evaluation of all assays in an endemic clinical setting for dengue, perhaps in conjunction with vaccine field trials.

**TABLE 5**

Agreement between clinical diagnosis and neutralizing antibody assay results in cases with primary and secondary natural infections with dengue virus (DENV)*

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>PRNT results</th>
<th>MN assay results</th>
<th>DC assay results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agree</td>
<td>Disagree</td>
<td>Agree</td>
</tr>
<tr>
<td>Primary DEN 1</td>
<td>3</td>
<td>1 (DEN 3)</td>
<td>4</td>
</tr>
<tr>
<td>Primary DEN 3</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Secondary DEN 2</td>
<td>3</td>
<td>2 (DEN 3, 4)</td>
<td>3</td>
</tr>
<tr>
<td>Secondary DEN 3</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

*PRNT = plaque reduction neutralization test; MN = microneutralization; DC = dendritic cell. Plasma samples from four patients with primary DENV 1 infections, five patients with primary DENV 3 infections, and five patients with secondary DEN 2 infections previously diagnosed clinically by virus isolation, serotype identification, hemagglutination inhibition assay, and enzyme-linked immunosorbent assay (IgM/IgG ratios) were analyzed. Results were tested by each virus neutralizing antibody assay in a blinded fashion. In cases of primary DENV infection, there was agreement if the highest fold-increase in neutralizing antibody titer from the acute-phase to the convalescent-phase specimen was against the isolated serotype. In cases of secondary DENV infection, there was agreement if the highest fold-increase in neutralizing antibody titer in the convalescent-phase specimen was against the isolated serotype. Shown are the number of cases of agreement and disagreement. In cases where there was disagreement, the DENV serotype(s) that elicited the highest antibody titer or titer increase are shown in parentheses.
Received August 27, 2007. Accepted for publication April 27, 2008.

Acknowledgments: We thank Dr. Mammen Mammen for providing sera from persons with naturally acquired dengue infections; and Michelle Eustache, Tshay Aberra, and David Barvir for performing the PRNTs.

Financial support: This study was partially supported by the U.S. Military Infectious Disease Research Program. Dengue vaccine trials were jointly supported by the U.S. Army Medical Research and Material Command and GlaxoSmithKline Biologicals. Samples from the Queen Sirikit National Institute of Child Health were collected as part of a collaborative study between the Armed Forces Research Institute of Medical Sciences, University of Massachusetts Medical School, and Queen Sirikit National Institute of Child Health, which was supported by the U.S. Army Military Infectious Diseases Research Program and the National Institutes of Health (P01 AI134533).

Disclaimer: This material has been reviewed by the Walter Reed Army Institute of Research and GlaxoSmithKline. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

Disclosure: The Walter Reed Army Institute of Research and GlaxoSmithKline have a Cooperative Research and Development Agreement for development of a dengue vaccine. J. Robert Putnak is currently conducting research sponsored by GlaxoSmithKline. This statement is made in the interest of full disclosure and not because the author considers this to be a conflict of interest.

Authors’ addresses: J. Robert Putnak and Stephen J. Thomas, Division of Viral Diseases, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Suite 3A12, Silver Spring, MD 20910, E-mails: robert.putnak@na.amedd.army.mil and stephen.thomas@na.amedd.army.mil. Rafael de la Barrera and Kenneth H. Eckels, Pilot Bio-Production Facility, Division of Regulated Activities, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910, E-mails: rafael.delabarrera@na.amedd.army.mil and kenneth.eckels@amedd.army.mil. Timothy Burgess, Viral Diseases Program, Naval Medical Research Unit 2, Jakarta; FPO, AP 96520, kenneth.eckels@amedd.army.mil. Rafael de la Barrera and Kenneth H. Eckels, Pilot Bio-Production Facility, Division of Regulated Activities, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910, E-mails: rafael.delabarrera@na.amedd.army.mil and kenneth.eckels@amedd.army.mil. Timothy Burgess, Viral Diseases Program, Naval Medical Research Unit 2, Jakarta; FPO, AP 96520, kenneth.eckels@amedd.army.mil. Timothy Burgess, Viral Diseases Program, Naval Medical Research Unit 2, Jakarta; FPO, AP 96520, kenneth.eckels@amedd.army.mil.

REFERENCES


