RAPID SEROLOGIC DIAGNOSIS OF DENGUE VIRUS INFECTION USING A COMMERCIAL CAPTURE ELISA THAT DISTINGUISHES PRIMARY AND SECONDARY INFECTIONS

DAVID W. VAUGHN, ANANDA NISALAK, TOM SOLOMON, SIRIPEN KALAYANAROOJ, NGUYEN MINH DUNG, RACHEL KNEEN, ANDREA CUZZUBBO, AND PETER L. DEVINE

United States Army Medical Component-Armed Forces Research Institute of Medical Science (USAMC-AFRIMS), Bangkok, Thailand; Wellcome Trust Clinical Research Unit, and Pediatric Intensive Care Unit, Center for Tropical Diseases, Cho Quan Hospital, Ho Chi Minh City, Vietnam; Queen Sirikit National Institute of Child Health (Bangkok Children’s Hospital), Bangkok, Thailand; Cho Quan Hospital, Ho Chi Minh City, Vietnam; PanBio Pty. Ltd., Brisbane, Queensland Australia

Abstract. A commercial capture ELISA for specific IgM and IgG antibodies produced during dengue infection (PanBio Dengue Duo) showed excellent sensitivity (99%, n = 78) using sera collected at hospital discharge compared with established ELISA and hemagglutination inhibition (HAI) assays. Furthermore, the ELISA was able to diagnose 79% of the dengue cases using sera collected at hospital admission. The ELISA also showed high specificity (92%) in paired sera from patients without flavivirus infection (n = 26), although 45% of the patients with Japanese encephalitis (n = 20) showed elevation of IgG but not IgM. The IgG capture ELISA showed good correlation with the HAI assay (r = 0.83, P < 0.0001), and IgG levels could be used to distinguish between primary and secondary infection, with 100% of primary infections and 96% of secondary infections being correctly classified. This ELISA should prove useful in the clinical diagnosis of dengue infections.

In terms of morbidity and mortality, dengue is the most important arboviral disease with 2.5 billion people living in areas of risk and many tens of millions of cases occurring each year. Infection with one of the four dengue virus serotypes can lead to dengue fever (a self-limiting flu-like illness with very low mortality) or dengue hemorrhagic fever (DHF; characterized by increased vascular permeability, thrombocytopenia, and hemorrhagic manifestations, with an untreated mortality rate exceeding 10%). Primary infection with one of the four serotypes confers lasting immunity to that serotype. Secondary infection with a different serotype is associated with an increased risk of DHF: enhanced viral entry into macrophages due to sub-neutralizing cross-reactive antibodies is postulated as an important pathophysiological mechanism. In addition to the classic syndromes of dengue fever and DHF, unusual clinical presentations are also being recognized, including neurologic manifestations and dengue hepatitis.

Traditionally, the hemagglutination inhibition (HAI) assay has been used to classify dengue infections as primary (gradual increase in antibody to moderate titer) or secondary (rapid increase to high titer). However, the HAI assay has various practical limitations, and since it usually requires paired sera, it cannot give an early diagnosis. Furthermore, the variable potency of hemagglutinins made in different laboratories has compromised the general applicability of this assay.

In the 1980s, an antibody-capture radioimmunoassay was developed that was soon replaced by simpler ELISAs. Some of these assays detect IgM and IgG in the serum, and thus are able to distinguish primary infections (IgM elevated early, IgG later to low titer) from secondary infections (IgG also elevated early to higher titer than IgM). However, they too have only been used at larger centers because they require preparation of dengue antigen and anti-dengue antibodies as preliminary steps. Although they give a diagnosis on the acute admission samples in the majority of patients, their usefulness in acute management is limited by the need for overnight incubation.

Recently, IgM and IgG capture ELISAs have been modified into immunochromographic formats in which the result of the assay is a color change visible to the naked eye. The most rapid of these gives a diagnosis within 7 min. However, because of their format, these kits are most suitable for individuals or small numbers of patients. There is a need for a standardized ELISA kit for diagnosing large numbers of patients at hospitals in endemic areas that do not have facilities for preparing their own reagents. We report here the evaluation of such a kit (Panbio Dengue Duo), with the added advantage of an operational time of less than 3 hr made possible by simultaneous incubation of antigen and peroxidase-labeled anti-dengue monoclonal antibody.

MATERIALS AND METHODS

Case definitions. Dengue infection was diagnosed in children experiencing a febrile illness consistent with dengue fever or DHF if dengue virus was isolated, or IgM to dengue virus (as opposed to IgM to Japanese encephalitis virus [JEV]) was detected, or a sustained elevation (> 1:2,560) or four-fold increase in dengue virus HAI titer occurred. Japanese encephalitis (JE) was defined as a febrile illness associated with an altered level of consciousness and the presence of IgM to JEV in the cerebrospinal fluid. Dengue infection was categorized as primary or secondary using the HAI assay results according to the World Health Organization criteria and the standard operating procedure for the reference ELISA.

Serum samples. Serum was collected from patients at the time of hospital admission and discharge at either the Queen Sirikit National Institute of Child Health (Bangkok Children’s Hospital) or the Center for Tropical Diseases (Ho Chi Minh City, Vietnam) and frozen at −70°C prior to assay. In this study, diagnostic specimens were obtained from a freezer collection at AFRIMS and tested under code (no personal identifiers) to evaluate the new diagnostic assay. This study was approved as exempt from the need for informed consent by our Institutional Review Board on the basis of Appendix
other containing either anti-human IgM or anti-human IgG. Australia) came as two microtiter plate sets with one plate for IGM and IgG capture ELISA (PanBio, Pty., Ltd., Brisbane, Australia) showed elevation of IgM. Consequently, when IgM was used alone, 61 of 78 patients with dengue infection were diagnosed through the use of IgG antibody against dengue virus. Peroxidase-labeled anti-dengue monoclonal antibody (125 μl/well) was added to the antigen plate to solubilize the antigens and to form antibody-antigen complexes. Concurrently, 100 μl/well of patient sera, diluted 1:100 in the diluent provided, was added to the assay plate containing either bound anti-human IgM or anti-human IgG to capture the IgM or IgG in the patient’s sera. Both plates were incubated for 1 hr at room temperature (antigen plate) or 37°C (assay plate), after which time the assay plate was washed and 100 μl/well of antibody-antigen complexes was transferred from the antigen plate to the assay plate. These complexes were then captured by dengue-specific IgM or IgG during an incubation of 1 hr at 37°C. The plate was then washed and bound complexes were visualized through the addition of a 100 μl/well of tetramethylbenzidine substrate. After 10 min, the reaction was stopped by the addition of 100 μl/well 1 M phosphoric acid, and the wells were read at 450 nm using a microtiter plate reader. Results were determined by comparison to IgM and IgG reference sera provided (cut-off calibrators). A positive sample was defined as having a sample:calibrator absorbance ratio ≥ 1.0 and a negative sample with ratio < 1.0. Dengue infection was characterized by the elevation of either IgM or IgG, with a negative sample defined as having both IgM and IgG ratios < 1.0.

Analysis. The proportion of patients with antibody levels above the designated sample:calibrator absorbance ratio for ELISA was determined. Fisher’s exact test was performed to compare sensitivity and specificity. Pearson’s correlation analysis was performed to compare ELISA ratios and HAI titers in individual sera. Analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test were used to compare the mean IgG ELISA ratios for different HAI titers. The Student’s t-test was used to compare mean ELISA ratios. Statistics were performed using using SPSS for Windows version 8.0 (SPSS, Inc., Chicago, IL) and Instat® (Graphpad Software, Inc., San Diego, CA).

RESULTS

Sensitivity and specificity of the IgM capture ELISA. The performance of the ELISA in sera collected at hospital discharge from patients with dengue virus infection, JEV infection, or without flavivirus infection is shown in Table 1. Using paired sera, 30 (100%) of 30 patients with primary dengue infection and 31 (65%) of 48 patients with secondary dengue infection were diagnosed through the use of IgM alone (sample:calibrator absorbance ratio ≥ 1.0), while no patients with JE (n = 20) or nonflavivirus infections (n = 26) showed elevation of IgM. Consequently, when IgM was used alone, 61 of 78 patients with dengue infection were diagnosed (sensitivity = 78%), and the corresponding specificity was 100%.

Sensitivity and specificity of the IgG capture ELISA. Using discharge sera, six (20%) of 30 patients with primary dengue infection and 46 (96%) of 48 patients with secondary dengue infection were diagnosed through the use of IgG alone (ratio ≥ 1.0). Only two (8%) of 26 patients without flavivirus infections showed elevation of IgG, although nine
of 20 patients with JE showed elevation (Table 1). Consequently, when IgG was used alone, 52 of 78 patients with dengue infection were diagnosed (sensitivity = 67%), and the corresponding specificities in nonflavivirus infections and JEV infections were 92% and 55%, respectively (Table 1). When an IgG sample:calibrator absorbance ratio of 3.0 was used as the cut-off, sensitivity in secondary dengue was unchanged while specificity in nonflavivirus and JEV infections increased to 100% and 80%, respectively (Table 1).

**Combined use of the IgM and IgG ELISAs.** Since most IgM negative cases of dengue infection showed a positive IgG response and vice versa, the combined use of IgM and IgG was investigated. When either IgM ≥ 1.0 or IgG ≥ 1.0 was used to define dengue infection, sensitivity was improved significantly ($P < 0.0001$, by Fisher’s exact test). Sensitivity was 100% for primary infections (30 of 30), 98% for secondary dengue infections (47 of 48), and 99% for all dengue cases (77 of 78) (Table 1). The specificity was 92% for nonflavivirus infections and 55% for JEV infections. When an IgG sample:calibrator absorbance ratio of 3.0 was used as the cut-off in combination with the IgM test, sensitivity in primary and secondary dengue was unchanged while specificity in nonflavivirus and JE infections increased to 100% and 80%, respectively (Figure 1). Furthermore, the IgG positive cases of JE showed lower IgM ratios than IgG positive cases of dengue (Figure 1).

**Early diagnosis of dengue infection.** The performance of the Dengue Duo ELISA in the first sera of the pair was also investigated to determine the utility of this test in the early diagnosis of dengue infection (Table 2). The IgM ELISA diagnosed 33 (42%) of 78 cases of dengue in the first sera, while the IgG ELISA detected 40 (51%) of 78 cases. When the combined use of IgM and IgG was used to diagnosed dengue infection, the sensitivity was increased to 79% (62 of 78), which was significantly higher than the use of IgM or IgG alone ($P < 0.0001$, by Fisher’s exact test). Among the 16 admission sera that did not have diagnostic levels of antibody by the Dengue Duo ELISA, 15 were also negative by the reference ELISA and a dengue virus was isolated from 15 (94%). The dengue virus isolation rate was less for the specimens with diagnostic levels of antibody by the Dengue Duo ELISA (31%; $P < 0.0001$).

The sensitivity of the Dengue Duo ELISA in primary and secondary dengue infection for up to eight days after the onset of clinical symptoms was also investigated (Figure 2). Ninety-four percent of patients with primary dengue were detected by day 5 of illness and 94% of those with secondary dengue by day 4; all patients were serologically diagnosed by day 6.

**Distinction between primary and secondary infection.** The IgG capture ELISA showed good correlation with HAI titer (Pearson’s $r = 0.82$, $P < 0.0001$) (Figure 3). The mean ELISA ratio was significantly correlated with increasing HAI titer ($P < 0.0001$, by ANOVA) and there was a significant increase in the proportion of patients showing elevation of IgG ratio ($> 1.0$) with increasing HAI titer ($P < 0.0001$, by Fisher’s exact test). The best distinction between primary and secondary dengue was observed when an IgG cut-off value of 3.0 was used, with 30 (100%) of 30 primary infections and 46 (96%) of 48 secondary infections being correctly classified (Figure 1). Indeed, this cut-off value correlated well with an HAI titer of 1:2,560 (Figure 3). Ninety-one percent of sera with an HAI titer > 1:2,560 had IgG levels > 3.0, while 95% of sera with an HAI titer < 1:2,560 had an IgG level < 3.0.

**Comparison of the PanBio and AFRIMS IgM capture and IgG capture ELISAs.** The PanBio and AFRIMS

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### Table 1

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<th>Dengue Duo ELISA method of interpretation†</th>
<th>AFRIMS serologic diagnosis*</th>
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<td></td>
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* AFRIMS = Armed Forces Research Institute of Medical Science. The AFRIMS serologic diagnosis was used as the criterion standard based on virus isolation and results from both a hemagglutination-inhibition assay using the World Health Organization criteria and an in-house enzyme immunoassay. † Interpretation of Dengue Duo ELISA was based on IgM or IgG antibody levels (sample:calibrator absorbance ratio) alone or in combination. Two sample:calibrator absorbance ratios were used as the cut-off value for dengue IgG (≥ 1.0 or ≥ 3.0).

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**Figure 1.** Comparison of the Dengue Duo IgM and IgG ELISA sample:calibrator absorbance ratios in hospital discharge sera from 124 patients. Based on viral isolation, a hemagglutination inhibition assay, and an in-house ELISA, 30 patients were diagnosed with primary dengue (closed circles), 48 with secondary dengue (open triangles), 20 with Japanese encephalitis (closed triangles), and 26 had no evidence of dengue infection (open circles). Broken lines show ELISA ratios of 1.0 for IgM and 3.0 for IgG.
Table 2: Performance characteristics of the Dengue Duo ELISA using 124 sera collected at hospital admission

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* AFRIMS = Armed Forces Research Institute of Medical Science. The AFRIMS serologic diagnosis was used as the criterion standard based on virus isolation and results from both a hemagglutination-inhibition assay using the World Health Organization criteria and an in-house enzyme immunoassay. JE = Japanese encephalitis; 1st Den = primary dengue virus infection; 2nd Den = secondary dengue virus infection.
† Interpretation of Dengue Duo ELISA was based on IgM or IgG antibody levels (sample:calibrator absorbance ratio) alone or in combination. Two sample:calibrator absorbance ratios were used as the cut-off value for dengue IgG (≥1.0 or ≥3.0).

ELISAs showed an excellent correlation in the 248 sera tested (Pearson’s r = 0.92 for IgM and r = 0.91 for IgG; P < 0.0001) (Figure 4).

**DISCUSSION**

Although epidemics attributed to dengue have been described for more than 200 years, it is only in the latter half of this century that the disease has taken on major importance. The spread of dengue since World War II has been linked to a worldwide resurgence of *Aedes aegypti*, increased densities of human populations, and increased travel.

In endemic areas, the clinical diagnosis of older children and adults presenting with the classical features of dengue fever is often possible. However, many other patients, especially young children, present with undifferentiated fever or symptoms of an upper respiratory tract infection. Likewise, the differential diagnosis of the more severe DHF is wide and includes meningococcemia, rickettsioses, leptospirosis, fulminating hepatitis, and depending on geographic location, other viral hemorrhagic fevers. Etiology is not only of epidemiologic interest but determines treatment options including those for DHF in which special treatment regimens are required due to the rapid and marked changes in vascular permeability.

The serologic diagnosis of dengue infection has advanced considerably over the last 20 years (Gubler DJ, Sather GE, 1988, unpublished data). The IgM and IgG capture ELISAs overcame many of the problems associated with hemagglutination inhibition, namely the need for paired sera, acetone extraction of serum, serial dilutions, and the lack of specificity among flavivirus infections. However, since ELISAs require the preparation of dengue antigen and anti-dengue antibody as preliminary steps, their use has largely been confined to specialized virology centers. With the advent of a commercial kit that incorporates all these reagents, diagnosis of dengue in routine hospital diagnostic laboratories becomes possible.

The ELISA evaluated in this report (PanBio Dengue Duo) proved to be a reliable test to diagnose dengue virus infection. By combining the results of the IgM and IgG ELISA on paired sera, 99% of the dengue patients were diagnosed and 79% were diagnosed using the first sample alone (58% using an IgG sample:calibrator absorbance ratio of 3.0 as the cut-off value). Eighty-two percent of the patients with dengue were positive on day 5 of illness (95% confidence interval = 64–99%). With a cut-off ratio of 3.0 for the IgG portion of the assay, 100% of primary and 96% of secondary infections were correctly identified.

![Figure 2](image-url) Comparison of Dengue Duo ELISA sensitivity in primary and secondary dengue infections and days after onset of infection. Sensitivity for primary dengue is shown by triangles, while sensitivity for secondary dengue is shown by the circles.

![Figure 3](image-url) Comparison of IgG ELISA sample:calibrator absorbance ratio and hemagglutination inhibition assay (HAI) titer in 248 sera. The broken lines represent HAI titers of 2,560 and ELISA ratios of 1.0 and 3.0. The horizontal bar represents the mean ELISA ratio for each HAI titer.
Correlation between PanBio and Armed Forces Research Institute of Medical Science (AFRIMS) capture ELISAs for IgM and IgG. Broken lines show sample:calibrator absorbance ratios of 1.0 (PanBio IgM), 1.0 and 3.0 (PanBio IgG), and ELISA unit values of 40 (AFRIMS IgM), and 100 (AFRIMS IgG).

Figure 4. Correlation between PanBio and Armed Forces Research Institute of Medical Science (AFRIMS) capture ELISAs for a, IgM and b, IgG. Broken lines show sample:calibrator absorbance ratios of 1.0 (PanBio IgM), 1.0 and 3.0 (PanBio IgG), and ELISA unit values of 40 (AFRIMS IgM), and 100 (AFRIMS IgG).

Dengue infections were correctly classified. Our data suggest that the assay can diagnose acute dengue virus infection in patients infected by any of the four dengue serotypes since all four dengue virus serotypes were represented in our study population. None of the negative control patients had elevated IgM levels on admission or discharge. While our sample size for non-dengue patients was relatively small, our data were consistent with previous studies. We found that 8% of the patients without acute flavivirus infection and 45% of those with acute JE had moderately elevated levels of IgG (sample:calibrator absorbance ratio > 1.0). However, with the higher cut-off of 3.0, only 20% of those with JE and none of those without a recent flavivirus infection were positive for IgG. Most cases of JE can be differentiated from dengue virus infection on clinical grounds, although there may be unusual cases of dengue virus encephalopathy. In areas where JEV and dengue viruses cocirculate, an assay that detects the virus (isolation or reverse transcriptase–polymerase chain reaction), measures neutralizing antibodies, or compares IgM specific to dengue viruses versus JEV may be needed to distinguish the infecting flavivirus. Although JEV is not found in Africa or the Americas, other flaviviruses occur there, and the specificity of the Dengue Duo ELISA will need to be evaluated in these regions.

The assay described was rapid, requiring just 3 hr to complete, and large numbers of specimens could be tested simultaneously. However, results from any dengue diagnostic assay based on antibody response must be interpreted with care early in the course of dengue illness. Failure to identify dengue-specific IgM or IgG antibody during the first 5–7 days of illness (approximately two days following defervescence) does not eliminate dengue virus as the etiology of the illness and follow-up testing should be performed. Although nearly all dengue patients in this study produced dengue-specific antibodies by day 5 of illness, before day 5 many dengue-infected patients had not produced antibodies. Thus, any antibody detection test, including the PanBio Dengue Duo, may give false-negative results early in the course of the illness. A rapid, simple assay for dengue virus genome or antigen is needed to complement antibody-based diagnostics; in this study, dengue virus was isolated from 15 of the 16 admission specimens that were negative by the Dengue Duo assay. Additionally, dengue infection among children in endemic areas is often subclinical and the presence of anti-dengue antibody does not guarantee that the febrile illness under consideration is due to dengue virus infection. The history, physical examination, and clinical laboratory results (e.g., leukopenia and thrombocytopenia) provide the basis for diagnosis.

In summary, the Dengue Duo ELISA is a reliable, rapid, sensitive, and specific diagnostic test to diagnose primary and secondary dengue infections based on antibody responses to support or refute the clinical suspicion of dengue.

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Authors’ addresses: David W. Vaughn, Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, DC 20307-5100. Ananda Nisalak, U.S. Army Medical Component, Armed Forces Research Institute of Medical Sciences, 315/6 Rajvithi Road, Bangkok 10400, Thailand (from the United States: USAMC-AFRIMS, APO AP 96546). Tom Solomon and Rachel Kneen, Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom. Siripen Kalayanarooj, Queen Sirikit National Institute of Child Health (Bangkok Children’s Hospital), Bangkok, 10400, Thailand. Nguyen Minh Dung, Pediatric Intensive Care, Center for Trop-
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