Comparison of Two Commercially Available Dengue Virus (DENV) NS1 Capture Enzyme-Linked Immunosorbent Assays Using a Single Clinical Sample for Diagnosis of Acute DENV Infection

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Dengue virus (DENV) nonstructural protein 1 (NS1) has shown promise as a novel diagnostic marker of acute DENV infection. Current techniques used to diagnose acute DENV infection, including virus isolation and reverse transcription-PCR (RT-PCR), are costly and difficult to perform, while traditional serological assays have low sensitivities during the acute stage of infection. Two commercially available NS1 antigen capture enzyme-linked immunosorbent assays (ELISAs), the Platelia dengue NS1Ag test (Bio-Rad Laboratories, Marnes La Coquette, France) and the Pan-E dengue early ELISA test (Panbio Diagnostics, Brisbane, Australia), were evaluated against a well-characterized panel of 208 real-time RT-PCR- and virus isolation-positive sera, as well as 45 real-time RT-PCR- and serologically negative sera from patients with other acute febrile illnesses. The overall sensitivities were 64.9% (95% confidence interval [CI95], 58.2 to 71.1%) for the Panbio test and 83.2% (CI95, 77.5 to 87.7%) for the Bio-Rad test, with interserotype variation, especially for DENV serotype 4. Predictive models were constructed to identify factors that had a significant influence on a test’s outcome with respect to this panel of samples in order to identify the conditions in which the test will be most effective as a diagnostic tool. The immunoglobulin G titer was found to be the only covariate that significantly influenced results in the Bio-Rad test, while serotype and the day postonset were found to significantly influence results in the Panbio test. We concluded that the NS1 capture ELISA is a useful tool that can improve testing algorithms to diagnose DENV infection in single samples from acute and early convalescent cases.

Dengue viruses (DENVs) are members of the genus Flavivirus in the family Flaviviridae and exist as four antigenically distinct viruses. While most infections result in asymptomatic responses or mild febrile illness (dengue fever), all four serotypes are capable of producing the more severe, and sometimes fatal, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (10, 12, 28). Over the last 50 years, factors such as rapid urbanization and failure to control vector mosquitoes have led to the emergence of endemic dengue in over 100 countries. With 2.5 billion people at risk for infection, DENV has become the most important arthropod-borne virus affecting humans (11, 13, 34).

Current diagnostic methods are often unable to recognize emerging epidemics in a timely manner or at a reasonable cost, drastically reducing the efficacy of control measures (13, 34). Virus isolation is a lengthy process which requires specialized laboratory equipment (29, 32), and rapid antibody responses to DENV in secondary infections may prevent successful isolation from serum drawn as few as 3 days after the onset of fever (17, 28, 32, 33). The development and modification of nested reverse transcription-PCR (RT-PCR) (16, 21) as well as real-time RT-PCR (5, 30, 31) techniques have significantly reduced processing times; however, these procedures remain expensive and technically difficult, and laboratory contamination can yield false-positive results. Serological diagnosis offers many advantages, including more flexible schedules for testing, a lower cost, and more widely available reagents (32). However, cross-reactivity between other flaviviruses and “original antigenic sin” complicate specific diagnosis of secondary flavivirus infections (15, 17, 28, 32). Additionally, antibody half-lives of up to 2 months confound diagnosis in cases where the date of onset of illness is unknown (17, 28, 29).

The inability of serologic methods to reliably diagnose acute infections has been recognized as a serious impediment to quick detection of epidemics and effective clinical case management (34). A number of options are being explored to overcome this obstacle; one of the most promising is the detection of DENV nonstructural protein 1 (NS1) in infected serum. NS1 is essential for viral replication (20, 24, 25), and the amount of secreted NS1 (sNS1) in the sera of individuals infected with DENV serotype 2 (DENV-2) has been shown to directly correlate with viremia (23). The protein is detectable by enzyme-linked immunosorbent assay (ELISA) as early as the first day of fever, and detection does not appear to be hindered by the presence of anti-dengue immunoglobulin M (IgM) antibodies (1, 35). Given these attributes and protein kinetics, NS1-based ELISAs may be an important diagnostic tool for those samples in which IgM is not detectable and for which PCR is not available. Moreover, DENV-2 sNS1 levels have been shown to be higher in patients with DHF (23),...
making diagnosis by NS1 a valuable predictive tool for severe disease.

Two commercial tests, the Platelia dengue NS1 kit (Bio-Rad Laboratories, Marnes La Coquette, France) and the Pan-E dengue early ELISA kit (Panbio Diagnostics, Brisbane, Australia), are currently available for the detection of DENV NS1 in human serum. Independent evaluations of the Platelia (9, 18, 19) and Pan-E (3) systems have been published, but the performances of the two tests have never been evaluated against a common serum panel. We evaluated the sensitivities of both NS1 detection systems with a well-characterized panel of 208 samples determined to be DENV positive by virus isolation and real-time RT-PCR. Additionally, specificity was evaluated using a panel of 45 serum samples from patients with acute febrile illnesses that tested negative for DENV by real-time RT-PCR and serological methods. The sensitivity varied by serotype for both tests, with the highest rates of detection occurring with DENV-1-positive samples and the lowest rates occurring with DENV-4-positive samples. The specificity was excellent for both kits.

MATERIALS AND METHODS

Clinical samples. Human serum samples were obtained from the reference collection at the Centers for Disease Control and Prevention (CDC Dengue Branch in San Juan, Puerto Rico. All accompanying epidemiological data were obtained from the database maintained by the CDC Dengue Branch. The panel consists of real-time RT-PCR- and virus isolation-positive DENV1-4 samples gathered as the result of routine surveillance activities carried out at the facility between 1998 and 2005. All samples were collected in Puerto Rico and were taken from patients during the first 5 days post-onset of symptoms (DPO), corresponding to the acute period of infection, as defined by the CDC Dengue Branch testing algorithm (CDC, unpublished data). The panel consisted of 56 DENV-1, 45 DENV-2, 52 DENV-3, and 55 DENV-4 samples. Additionally, the panel included 45 samples collected from patients with acute febrile illness that tested negative for DENV by PCR and serology. Human surveillance data from 1998 to 2005 suggest that DENV was the only flavivirus circulating on the island of Puerto Rico (CDC, unpublished data).

Sample characterization. Serum samples were tested for the presence of DENV by a diagnostic multiplex real-time RT-PCR assay (6) and by viral isolation in Cx/36 cells as previously described (14). Anti-dengue IgG titers were determined for all virus-positive samples, using an IgG ELISA described elsewhere (4). Because all samples were from acute infections (≥5 DPO), any IgG titer of ≥1:80 was considered to be evidence of a secondary flavivirus infection, in accordance with WHO guidelines adapted for the IgG ELISA (26, 33), and DENV was the only flavivirus known to be circulating in Puerto Rico at the time of collection (1998–2005).

Additionally, a subset of 140 samples were tested by an IgM antibody capture ELISA (MAC ELISA) (4). Table 1 shows the composition of the evaluation panel according to primary or secondary infection and describes the subset of samples tested by MAC ELISA. IgG and IgM titers were used to evaluate the influence of possible antibody-NS1 complexes on antigen detection.

PCR-negative samples were further tested by MAC ELISA, and 45 samples that tested negative for DENV in both tests were selected to evaluate specificity.

Kit evaluation. The Platelia dengue NS1 and Pan-E dengue early ELISA systems are antigen capture ELISAs that provide qualitative, non-serotype-specific detection of DENV NS1 antigen. All testing was performed in duplicate in strict adherence to the manufacturers’ instructions provided in the kit inserts. All reagents and control sera were provided by the manufacturers. Prior to testing, samples were thawed and all samples and kit reagents were allowed to warm to ambient laboratory temperature. All microplate wash steps were carried out with a 96-well manifold automatic plate washer, and plates were read at a wavelength of 450 nm, with a 630-nm reference filter. All samples and controls were tested in duplicate unless otherwise noted. The testing protocols for each kit are described briefly below.

(i) Bio-Rad kit. Fifty microliters of patient serum, positive control, negative control, or cutoff control was diluted 1:2 in sample diluent and combined with 100 μl of diluted horseradish peroxidase (HRP)-labeled anti-NS1 monoclonal antibody (MAb). The diluted serum and conjugate were added to capture anti-NS1 MAb-coated microwells, and plates were incubated for 90 min at 37°C. The reaction was stopped with the addition of 100 μl of stop solution (1 N H₂SO₄), and the plate was read. A sample ratio was determined for each sample by dividing the average optical density (OD) of the test sample by the average OD of the cutoff control (tested in quadruplicate). Sample ratios of 0.5, 0.5 to <1.0, and ≥1 were indicative of negative, equivocal, and positive results, respectively.

(ii) Panbio kit. One hundred microliters of diluted (1:10 in serum diluent) patient serum, positive control, negative control, or calibrator was added to microwells precoated with a polyclonal capture anti-NS1 antibody and then incubated for 1 hour at 37°C. The plates were washed six times and incubated for an additional 1 h at 37°C following the addition of HRP-conjugated anti-NS1 MAb. After an additional six washes, antibody complexes were detected by adding TMB and incubating samples for 10 min at room temperature. The reaction was stopped by adding stop solution (1 M H₃PO₄), and the plates were read. The cutoff value was determined by multiplying the average OD of the calibrator (tested in triplicate) by the lot-specific calibration factor (provided in the kit insert). An index value was calculated by dividing the average OD of each sample by the cutoff value. Index values of <0.9, 0.9 to 1.1, and ≥1.1 were considered negative, equivocal, and positive, respectively.

Statistical analysis. Sensitivity was calculated as the ratio of samples correctly identified as positive to the total number of samples in the panel that were positive by tissue culture and real-time RT-PCR (n = 288). Similarly, specificity was calculated as the ratio of samples correctly identified as negative to the total number of samples in the negative samples panel (n = 45). Positive and negative predictive values were defined for each test as the ratio of true positive (TP) results to the sum of TP and false-positive (FP) results [TP/(TP + FP)]. Negative predictive value was defined as the ratio of true negative (TN) results to the sum of TN and false-negative (FN) results [TN/(TN + FN)].

Additional statistical analysis was carried out using R, version 2.3. A cross-tabulation of test results for both kits was conducted to assess agreement between the two tests. The kappa statistic for intraclass correlation and McNemar’s chi-square test for matched pairs were computed for the cross-tabulated data. Because one goal of the study was to identify factors that are most closely related to sensitivity, we collapsed the NS1 antigen capture ELISA results for the PCR virus isolation-positive panel into “positive”/“not positive” form (where equivocal results were considered not positive). Logistic regression was then used to model the positive/not positive response as a function of the covariates DPO, IgG titer, IgM titer (for the 140-sample subset), viremia (as measured by real-time RT-PCR), infection status, and DENV serotype. The best-fitting model was selected using a likelihood ratio test.

RESULTS

Sensitivity and specificity. Sensitivity and specificity were evaluated in order to gauge the tests’ ability to correctly diagnose a DENV infection. The Panbio test correctly identified 135 of 208 samples as positive, and the Bio-Rad kit correctly
identified 173 of 208 samples as positive. The overall sensitivities were 64.9% (95% confidence interval [CI95], 58.2 to 71.1%) for the Panbio kit and 83.2% (CI95, 77.5 to 87.7%) for the Bio-Rad kit. However, sensitivity varied between serotypes, with DENV-1 being the most readily detected serotype (78.6% [Panbio kit] and 92.9% [Bio-Rad kit]) and DENV-4 being the least detected serotype (36.4% [Panbio kit] and 70.9% [Bio-Rad kit]). Table 2 provides a breakdown of sensitivity by serotype for the two tests.

Of the 45 negative samples, the Panbio test identified 44 samples as negative and 1 sample as equivocal, while the Bio-Rad kit identified all 45 samples as negative. The overall specificities were 97.8% (CI95, 88.4 to 99.6%) for the Panbio kit and 100% (CI95, 92.1 to 100%) for the Bio-Rad kit. Table 2 shows the specificities of both tests. The positive predictive values for both kits were 100% (CI95, 97.2 to 100% for Panbio kit and 97.8 to 100% for Bio-Rad kit), and the negative predictive values were 39.3% (CI95, 30.7 to 48.5%) for the Panbio test and 62.5% (CI95, 51.0 to 72.8%) for the Bio-Rad test.

Agreement of kits. Statistical analysis was conducted to determine if the results of the tests were significantly different. Cross-tabulation of test results with the Panbio kit and test results with the Bio-Rad kit (Table 3) showed an overall agreement of nearly 78% between the two kits. The largest discrepancy occurred in the 40 samples identified as positive with the Bio-Rad kit but negative with the Panbio kit. The intracllass correlation between the tests was moderate (0.56; standard error = 0.05). The P value from McNemar’s test (<0.01) suggests that the two kits do not give the same result. When the data were collapsed into “positive”/“not positive,” the intracllass correlation remained about the same (0.59; standard error = 0.06), and the P value from McNemar’s test was still <0.01.

Predictive models for the positive panel. Predictive models were constructed using just the positive sample data to identify factors that had a significant influence on a test’s outcome in order to determine the conditions in which the test will be most effective as a diagnostic tool. The influence of a number of different covariates on the result of each test was examined to identify factors associated with the detection of NS1 in positive samples. Test results for the Panbio kit were found to be most closely related to IgG titer (P < 0.01) and DPO (P = 0.01). Test results for the Bio-Rad kit were found to be most closely related to IgG titer (P < 0.01).

(i) Bio-Rad kit. The best model for the Bio-Rad test used IgG titer as the only predictor. While both serotype and DPO were included in the model for the Panbio data, after adjusting for the effects of IgG titer, neither serotype nor DPO was statistically significant in the model for the Bio-Rad data. In general, as IgG titer increased, the frequency of a positive result (detection of NS1) with the Bio-Rad kit decreased (Fig. 1). However, this inverse relationship does not hold true in our sample for IgG titers of 1:163,840 and 1:655,360, for which there were three observations and one observation, respectively. The relatively small sample sizes at these titers mean class correlation remained about the same (0.59; standard error = 0.06), and the P value from McNemar’s test was still <0.01.

(ii) Panbio kit. The best model for the Panbio test used only serotype and DPO, as all other covariates were highly nonsignificant. In contrast to the case for the Bio-Rad kit, the covariate IgG titer was nonsignificant (P = 0.853). Generally, for the Panbio kit, as DPO increased, the frequency of a positive result also increased (Fig. 2). This direct relationship was observed.

![FIG. 1. Predictive model for the Bio-Rad kit based on the covariate IgG titer. IgG titer is represented as an integer value along the x axis, where 0 is <1:40, 1 is 1:40, 2 is 1:160, 3 is 1:640, 4 is 1:2,560, 5 is 1:10,240, 6 is 1:40,960, 7 is 1:163,840, and 8 is ≥1:655,360. The frequency of a positive result is plotted along the y axis. The solid line represents the frequency of a positive result, as predicted by the model, and the dotted line represents the actual data. Generally, as the IgG titer increases, the frequency of a positive result decreases.](http://cvi.asm.org/.../figures/figure1.png)
and a monoclonal detection antibody. Our results suggest that the MAb used in the Bio-Rad kit is a more efficient capture antibody than the polyclonal antibody used by the Panbio kit.

Both kits showed some variation in sensitivity between serotypes, and both tests were the least sensitive for DENV-4. However, intraserotype variability was statistically significant only for the Panbio kit and was an important covariate (along with DPO) for predicting the outcome of the test. The inconsistency across serotypes is most likely a result of the inherent difficulty in finding a capture and detection antibody with equally high affinities for all four DENV serotypes. The variation in sensitivity between serotypes may reduce the utility of these tests in outbreak investigations, especially where DENV-4 is suspected to be the major infecting serotype. The substantial reduction in sensitivity of both kits to DENV-4 samples may be due to variation in viral genome sequences between strains of different geographic origins (22). As a result of the proprietary nature of the commercial diagnostics evaluated in this study, we do not know the geographic origin of the samples used to develop and evaluate the antibodies in these tests. Consequently, phylogenetic data are not available to further explore this possibility.

Our study was in agreement with the only other published evaluation of the Panbio test, which found a 63% (CI95, 53.4 to 73.0%) overall sensitivity for acute-phase specimens (3). Both studies found the sensitivity of the test to be lower than the 90.7% (CI95, 77.9 to 97.4%) stated in the kit insert (27), underscoring the importance of independent testing of new diagnostics prior to use in a public health setting. Our results for the Bio-Rad test were in line with one other study that found an overall sensitivity of 88.7% (CI95, 84.0 to 92.4%) but did not show the same interserotype variation (9) and slightly lower than those of two studies by Kumarasamy et al. (18, 19) that found the sensitivity to be 93.4 and 93.3%. The panels used in these two studies were comprised mainly of samples from primary infections (184 of 213 and 192 of 224 samples), to which the test appears to be more sensitive (97.3% versus 70.0% and 97.4% versus 68.8% for primary versus secondary infections). We also observed a higher rate of sensitivity for primary infections (overall rate, 98.3%) than for secondary infections (overall rate, 77.3%) (data not shown) with the Bio-Rad kit (this difference was not as obvious with the Panbio test). This difference may not have appeared statistically significant ($P = 0.08$ for the Bio-Rad kit and 0.84 for the Panbio kit) due to the relatively small sample size ($n = 58$) of primary cases in our panel. These findings contradict a previously published study employing a noncommercial NS1 capture ELISA which found that NS1 levels were detectable only in secondary infections (36).

IgG titer was an important predictor of outcome with the Bio-Rad kit, where an inverse relationship was observed between IgG titer and the frequency of a positive test outcome, but no such relationship was observed with the Panbio test. Unlike IgG titer, IgM titer did not appear to inhibit NS1 detection in either test. This observation is in agreement with other published data (1, 35). While the majority of the samples in our evaluation panel were IgM negative (probably a result of all samples being obtained at ≤5 DPO), the combination of IgM and NS1 testing, especially for samples taken after 4 DPO, has been shown to be more sensitive than either method alone.

**FIG. 2.** Predictive model for the Panbio kit based on the covariates DPO and serotype. DPO is plotted along the x axis, with the frequency of a positive result plotted along the y axis. The solid lines represent the frequencies of positive results for DENV-1, DENV-2, DENV-3, and DENV-4 predicted by the model for each DPO. The dotted lines represent the actual data. In general, as DPO increases, so does the frequency of a positive result. The frequency of a positive result was significantly lower for DENV-4 samples than for DENV-1 to -3 samples.
Chuansumrit et al. demonstrated a range of positive rates of detection for 4 to 7 DPO, from 83.3% to 95.6% for the combined methods and from 29.8% to 76.9% for NS1 detection alone (7). A separate evaluation of diagnostics for acute DENV infection found an overall positive detection rate of 66.7% for RT-PCR (18), making the MAC ELISA/NS1 capture ELISA a very attractive alternative testing algorithm for the diagnosis of acute DENV infection, especially when paired sera are not available.

NS1-based testing may reduce the uncertainties associated with serological diagnostic methods such as the MAC ELISA. The antigen has been shown to be present as early as 0 or 1 DPO, typically peaks around 3 to 5 DPO, and subsides around 9 DPO (although one study detected NS1 until day 18) (1, 23, 35). In our study, the Bio-Rad kit detected NS1 in positive samples with a higher frequency for each time point (DPO 1 to 5) than that for the Panbio kit (Fig. 3). However, the increase in detection of NS1 over time was statistically significant only for the Panbio model; it was marginally nonsignificant ($P = 0.056$) for the Bio-Rad model. The peak in serum sNS1 concentration tends to correlate with defervescence (the point at which the patient is at the greatest risk of developing DHF) (7), and a test that can accurately diagnose a DENV infection during the acute phase may prove to be an invaluable clinical triage tool.

Moreover, the peak level of NS1 concentration (3 to 5 DPO) occurs around the time when viremia is typically waning and IgM has yet to rise (in primary infections) (28). Additionally, NS1 testing may be very useful in diagnosing secondary DENV infections, where IgM production is reduced or completely absent (17). Finally, the extended duration of IgM antibodies in serum (up to 3 months postinfection in patients with primary infections) (17) limits the MAC ELISA’s utility as a real-time DENV diagnostic tool. NS1 levels rise before IgM titers and subside much more quickly (1, 23, 35), greatly increasing the probability that a positive result is from the current infection, not a recent one (as is sometimes the case with IgM-based diagnoses).

In addition to affording early and specific detection, sNS1 may be an indicator of disease severity. Libraty et al. showed a correlation between viremia and sNS1 levels as well as between sNS1 concentration and frequency of DHF (23). In our study, levels of virus in the serum (as measured by real-time RT-PCR) did not correlate with the outcome for either test (data not shown). This was also observed by Alcon et al., who noted that none of the samples in the study were from patients who met WHO criteria for DHF/DSS and that the patients may have had relatively low levels of sNS1 in their sera (none of our samples were from DHF/DSS cases either). Alcon’s group also noted that sNS1 levels vary significantly depending on the individual infected, the phenotype of the infecting virus, and even storage conditions following specimen collection (2).

Our study revealed specificities of 97.8% and 100% for the Panbio and Bio-Rad tests, respectively. These findings were in line with other published studies that found 100% specificity for both tests (Panbio [3] and Bio-Rad [9, 18]). The excellent specificities of both kits suggest that NS1-based diagnostics may not be subject to the problems of cross-reactivity traditionally observed in secondary flavivirus infections. Consequently, NS1 may prove to be a useful differential diagnostic tool in areas where multiple flaviviruses are endemic. A small number of samples from patients with acute West Nile virus infection were tested with both kits and found to be negative (data not shown). However, in order to maximize the utility of the NS1 capture ELISA as a differential diagnostic tool, the kits still need to be evaluated against a comprehensive panel of sera from acutely ill patients positive for other flaviviruses. Additionally, the performances of both kits should be evaluated against samples from primary infections in order to determine the kits’ utility as a diagnostic tool in these situations.

In conclusion, the idea of an NS1 capture ELISA for the
diagnosis of acute DENV infection holds a great deal of promise for improving DENV diagnostics. Used in combination with current diagnostic methods, such as MAC ELISA, the test has the ability to drastically improve current diagnostic algorithms. Moreover, the easy-to-use ELISA format requires minimal technologically advanced equipment and has the potential to deliver sensitive, specific, and timely dengue diagnoses in areas where they were never before available.

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