Utility of a Commercial Nonstructural Protein 1 Antigen Capture Kit as a Dengue Virus Diagnostic Tool†‡

Kovi Bessoff,1† Elena Phoutrides,1† Mark Delorey,2 Luz N. Acosta,1 and Elizabeth Hunsperger1*

Dengue Branch, Centers for Disease Control and Prevention, San Juan, Puerto Rico,1 and Arbovirus Diseases Branch, Centers for Disease Control and Prevention, Fort Collins, Colorado2

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Annually, over 2.5 billion people are at risk for infection with dengue virus (DENV), while between 50 and 100 million people contract the infection. There is an urgent need for alternative diagnostic tools that can detect DENV during acute infection. Recent studies have shown that DENV nonstructural protein 1 (NS1) is detectable in the blood as early as the onset of symptoms and persists well into the convalescent phase of the infection. We evaluated the utility of the Bio-Rad Platelia DENV NS1 antigen capture kit in combination with real-time reverse transcriptase PCR (RT-PCR) and an IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) for refining a new algorithm for the diagnosis of acute- or convalescent-phase DENV infection with a single clinical sample. We tested the Bio-Rad kit with three panels of sera. These panels were designed to evaluate the sensitivities of the NS1 kit for (i) early-convalescent-phase samples, (ii) acute-phase samples with false-negative PCR results, and (iii) IgM-negative convalescent-phase samples from patients with confirmed secondary DENV infections. Results show that NS1 can be detected in 22% of serum samples collected more than 10 days after the onset of illness and in 22% of samples that did not elicit an IgM response. Additionally, NS1 was detected in 37% of the tested acute-phase samples with false-negative PCR results, suggesting that NS1 detection may be valuable in increasing the sensitivity of current acute-phase diagnostics. These results will improve diagnosis with a single acute-phase or early-convalescent-phase sample for disease surveillance and clinical diagnosis.

Despite the importance of the dengue viruses (DENVs) as emerging pathogens, diagnostic tests remain inadequate for efficient and accurate identification of DENV infection. Annually, 50 to 100 million people are infected with one of the four DENV serotypes, while over 2.5 billion people are at risk for infection (6, 7). Dengue is endemic in over 100 countries worldwide, and the clinical course ranges from asymptomatic infection to fatal hemorrhagic fever or shock syndrome. Early diagnosis during acute infection is critical to clinically manage severe disease and to identify potential outbreaks in a timely manner (20).

Current dengue diagnostic methods have a number of serious limitations. The gold standard for diagnosis of acute DENV infection is viral isolation, but the procedure is costly, time-consuming, and technically difficult to perform (8). Reverse transcriptase PCR (RT-PCR) has been widely adopted as an alternative to viral isolation for the diagnosis of acute infection, but PCR is technically intensive and expensive, and sensitivity varies from 80 to 90% based on primer sets (4, 14). An IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) is useful primarily for diagnosing dengue infection in the late acute or early convalescent phase of the illness but is often insensitive for early-acute-phase infections (19). For the period between days 3 and 5 post-onset of illness, there is no available diagnostic test that is adequately sensitive. An ideal dengue diagnostic test would be fast, easy to perform, specific to DENV, sensitive during acute infection, and able to differentiate primary and secondary DENV infections.

Nonstructural protein 1 (NS1) of DENV has been shown previously to be useful as a tool for the diagnosis of acute dengue infections. NS1 has been detected in the sera of DENV-infected patients as early as 1 day post-onset of symptoms (DPO) and up to 18 DPO (22). Previous work evaluating commercially available NS1 detection systems has shown excellent sensitivity (83.2%) and specificity (100%) for the Platelia DENV NS1 antigen (NS1Ag) test (Bio-Rad Laboratories, Marnes-La-Coquette, France) (1). However, no studies to date have evaluated the ability of the kit to detect NS1 in late-convalescent-phase sera. Additionally, no specific analyses have been done to assess the sensitivity of NS1 detection in samples with false-negative PCR results. Finally, theoretical variations in antibody kinetics between primary and secondary flavivirus infections underscore the need to evaluate differences in sensitivity for primary and secondary infections (11, 18).

The present study addresses these areas using three panels of well-characterized sera, with the ultimate goal of improving the sensitivity and specificity of the testing algorithm in cases of suspected DENV infection. The Bio-Rad kit was tested against three panels of paired sera designed to determine the utility of the test under specific diagnostic conditions. These panels were designed to evaluate the sensitivity of the NS1 kit for (i) convalescent-phase samples taken 6 to 36 DPO, (ii) PCR-negative acute-phase samples from patients for whom infection was confirmed by seroconversion in the convalescent-phase sample, and (iii) IgM-negative convalescent-phase samples corre-

† These authors contributed equally to this work.
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TABLE 1. Numbers and proportions of samples in serum panels A, B, and C according to DPO*

| Sample type          | DPO  | A (% of samples in panel) | B (% of samples in panel) | C (% of samples in panel) |
|----------------------|------|---------------------------|---------------------------|---------------------------|
| Acute-phase samples  | 0    | 6 (9.5)                   | 10 (14.9)                 | 11 (27.5)                 |
|                      | 1    | 15 (23.8)                 | 11 (16.4)                 | 11 (27.5)                 |
|                      | 2    | 8 (12.7)                  | 12 (17.9)                 | 6 (15.0)                  |
|                      | 3    | 14 (22.2)                 | 11 (16.4)                 | 8 (20.0)                  |
|                      | 4    | 12 (19.0)                 | 12 (17.9)                 | 4 (10.0)                  |
|                      | 5    | 8 (12.7)                  | 11 (16.4)                 | 11 (27.5)                 |
| Convalescent-phase   | 6    | 14 (22.2)                 | 7 (11.1)                  | 4 (10.0)                  |
|                      | 7    | 13 (20.6)                 | 7 (11.1)                  | 4 (10.0)                  |
|                      | 8    | 13 (20.6)                 | 7 (11.1)                  | 4 (10.0)                  |
|                      | 9    | 7 (11.1)                  | 4 (10.0)                  |                           |
|                      | 10   | 12 (19.0)                 |                           |                           |
|                      | 11   | 11 (16.4)                 |                           |                           |
|                      | 12   | 1 (1.6)                   |                           |                           |
|                      | 13   | 1 (1.6)                   |                           |                           |
|                      | 14   | 1 (1.6)                   |                           |                           |
|                      | 15   | 1 (1.6)                   |                           |                           |
|                      | 36   | 1 (1.6)                   |                           |                           |
| Total                | 63   | 67                        | 40                        |                           |

* Panel A samples are PCR-positive acute-phase samples with paired serum samples from the convalescent phase, panel B includes acute-phase samples with false-negative PCR results and corresponding convalescent-phase samples showing seroconversion, and panel C samples are PCR-positive acute-phase specimens with paired convalescent-phase IgM-negative specimens.

MATERIALS AND METHODS

Clinical samples. All sera used in this study are part of the archived serum collection at the Dengue Branch (DB) of the CDC Division of Vector-Borne Infectious Diseases (DVBID). All sera were collected through routine surveillance conducted in Puerto Rico from 1998 to the present. The following groups of samples were selected for the creation of three separate serum panels to be tested by the Platelia DENV NS1Ag test: (i) 63 anti-DENV-IgM-positive convalescent-phase samples (defined as samples collected ≥5 DPO) for which there was a paired acute-phase sample (collected <5 DPO) that tested positive by RT-PCR for RNA from DENV (4 positive for DENV serotype 1 [DENV1], 38 for DENV2, 20 for DENV3, and 1 for DENV4) (serum panel A); (ii) 67 acute-phase samples that tested negative for DENV by RT-PCR for which there was a paired anti-DENV-IgM-positive sample (indicating seroconversion) (serum panel B); and (iii) 40 acute-phase samples that tested positive by RT-PCR for RNA from DENV (2 for DENV1, 20 for DENV2, 16 for DENV3, and 2 for DENV4) for which there was a paired convalescent-phase sample that tested negative for anti-DENV IgM but showed a 4-fold or greater increase in the anti-DENV IgG titer compared to that in the acute-phase sample (indicating a secondary infection) (serum panel C) (16, 21). Samples were characterized by previously described laboratory analyses, including MAC-ELISA and anti-DENV IgG ELISA (3, 16), viral isolation from C6/36 cells (8), and multiplex real-time RT-PCR (4). Sera were taken from the archived specimens, thawed, and aliquoted into anonymous panels. Characteristics of these panels are shown in Table 1.

DENV NS1 antigen capture test. NS1 was measured using the Platelia DENV NS1 antigen capture test (Bio-Rad Laboratories, Marnes-La-Coquette, France), which we previously determined to have an overall sensitivity of 83.2% (95% confidence interval [CI]95, 77.5 to 87.7%) and a specificity of 100% (CI95, 92.1 to 100%) for acute-phase samples positive for DENV1, DENV2, DENV3, or DENV4 taken 1 to 5 DPO (1). All testing was performed in duplicate in strict adherence to the manufacturer’s instructions provided in the kit insert. All controls and standards were provided by the manufacturer. Samples and reagents were equilibrated to room temperature prior to testing. Briefly, diluted serum was complexed with a horseradish peroxidase (HRP)-conjugated anti-NS1 monoclonal antibody (MAB). The MAB-MAB complex was added to 96-well microtiter plates precoated with a capture anti-NS1 MAB. Plates were incubated at 37°C for 90 min. Complexes were then detected by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, and the reaction was stopped with 1 N H2SO4 after 30 min. Optical densities (OD) were determined by reading the plates at 450 nm with a 630-nm reference filter. A sample ratio was determined by dividing the sample OD by the average for the cutoff control.

Statistical analysis. For serum panels A and B, a generalized linear model (glm) was fit to the data with DPO as an explanatory variable and the NS1 test outcome (positive or negative) as the response. Because the response is binary, a binomial distribution for the number of positive NS1 tests was assumed and we used a logit link. Standard diagnostic analyses were performed, including tests for over- or underdispersion. In cases of over- or underdispersion, quasilihood methods were used for parameter estimates and standard errors. Analysis of deviance was used to determine whether or not DPO is a statistically significant predictor for a positive NS1 test.

For serum panel C, an exponential decay model was fit to the data by using weighted least squares; this model was a better fit than the glm. DPO was again the explanatory variable, and the proportion of positive NS1 tests was used as the response. Standard diagnostic tests were used to confirm the fit and assumptions for this model. A t test with the model coefficient was used to determine whether or not DPO is a statistically significant predictor of the proportion of positive NS1 tests.

RESULTS

Sensitivity of the Platelia DENV NS1Ag test for convalescent-phase samples. Figure 1A shows the proportion of samples that tested positive for NS1 antigen for each time point (DPO). In the analysis of the sensitivity of the NS1 assay, only convalescent-phase samples (from ≥6 DPO) that had a paired acute-phase NS1-positive sample were used (Fig. 1B). Thus, for the purpose of assessing the sensitivity of the test, it was assumed that NS1 was present in each of the convalescent-phase samples used. For the data in Fig. 1B, DPO was not a statistically significant predictor of sensitivity for NS1 detection (P = 0.35). Additionally, no significant correlation between NS1 sensitivity and the number of days elapsed between the collection of the acute- and convalescent-phase samples was found (data not shown). The sensitivity of the Platelia DENV NS1Ag test for NS1 in convalescent-phase samples from panel A was 22% (CI95, 13 to 36%; n = 63).

NS1 antigen can be detected in PCR-negative acute-phase samples. Figure 2 shows the proportion of samples with false-negative RT-PCR results that tested positive for the NS1 antigen for each DPO. It was found that DPO was not a statistically significant predictor of sensitivity for NS1 detection (P = 0.28). The Platelia DENV NS1Ag test detected NS1 in 37% (CI95, 27 to 49%; n = 67) of samples that tested negative for DENV by RT-PCR but for which the corresponding convalescent-phase sample exhibited IgM seroconversion (RT-PCR false negatives; serum panel B).

NS1 antigen is detected in IgM-negative samples from patients with secondary DENV infection. The Platelia DENV NS1Ag test detected NS1 antigen in 20% (CI95, 8 to 42%; n = 20) of samples that tested negative for IgM but were determined to represent secondary DENV infections based on an RT-PCR-positive acute-phase serum sample and a 4-fold or greater rise in the IgG titer in the convalescent-phase sample compared to that in the acute-phase sample (serum panel C). Figure 3 shows the proportion of IgM-negative samples from patients with secondary DENV infections that tested positive for DENV by detection of NS1 antigen for each DPO. DPO was not found to be a significant predictor of sensitivity for these samples (P = 0.11).

The sensitivity to NS1 for serum panel A, in which IgM was
present in the convalescent-phase samples, was observed to be 0.22. The sensitivity to NS1 for serum panel C, in which IgM was absent in the convalescent-phase samples, was observed to be 0.20. The P value for the difference in sensitivity between the two panels is less than 0.01. The 95% confidence interval for the difference in sensitivity between the two panels is 0.19 to 0.24. These results suggest that the presence of IgM has no effect on the detection of NS1.

DISCUSSION

Commercially developed NS1 antigen capture assays have the potential to improve dengue diagnosis. However, understanding the clinical circumstances under which they are most useful is important in developing a diagnostic algorithm that maximizes the likelihood of a correct diagnosis using these tests. Routine diagnostics for dengue include using RT-PCR or viral isolation during the acute phase of the infection (0 to 5 DPO) and serology tests including IgM and IgG antibody detection in the convalescent phase of the infection (6 to 14 DPO). The NS1 assay has not been routinely incorporated into the current diagnostic scheme, and the manufacturer of the test kit suggests using the assay during the first 8 DPO; however, this suggestion requires validation. Therefore, the product’s primary use has been for the acute phase of dengue infection (0 to 5 DPO), for which the highest and most consistent sensitivities have been observed in independent studies. These independent studies determined that the sensitivity of the NS1 assay by Bio-Rad ranged from 63 to 97% and that the specificity ranged from 98 to 100% (2, 5, 9, 12, 13, 15, 23). In addition, previous studies performed in our laboratory noted differences in sensitivity among DENV serotypes (for DENV1, 93%; for DENV2, 82%; for DENV3, 87%; and for DENV4, 71%) (1).

The study presented here revealed that the Bio-Rad kit was able to detect NS1 in 37% of the acute-phase samples positive for DENV (with positivity confirmed by seroconversion) that were incorrectly classified by PCR (false negatives). Because the sensitivity of PCR for acute-phase specimens has been shown to be as low as 80%, depending on the primer/probe sets and the assay used for detection, an additional assay capable of improving the sensitivity of the diagnostic algorithm for a single acute-phase sample would be extremely valuable (4, 14). With our current surveillance strategy, final classification of approximately 50 to 60% of samples remains unresolved as a result of failure to obtain a convalescent-phase sample for serological confirmation in cases in which the acute-phase sample is negative by PCR testing (unpublished CDC data). The ability to resolve the identity of the etiological agent from a single acute-phase sample is important for clinical triage for patients, as well as epidemiological investigations. In addition to being used for acute-phase samples, the NS1 assay may resolve difficult cases, including those of secondary DENV infection convalescent-phase samples that do not express detectable levels of IgM as well as late-convalescent-phase samples in which IgM titers have already declined (11). This study was designed to determine the utility of this assay for these difficult sample types.

FIG. 1. (A) Proportion of all samples (acute and convalescent phase) that tested positive for NS1 at each DPO. (B) Levels of sensitivity as indicated by proportions of convalescent-phase samples for which the paired acute-phase sample was positive for NS1. Although the sensitivity appears to decrease with DPO, this trend was not statistically significant.

FIG. 2. Proportion of NS1-positive panel B serum samples by DPO. Panel B includes acute-phase specimens with false-negative PCR results for which the corresponding convalescent-phase samples showed IgM seroconversion.
IgM response is considered to indicate a recent flavivirus infection and not an acute infection. In other words, a positive NS1 response in this type of sample redefines the clinical diagnosis from a recent dengue infection to an acute dengue infection. Furthermore, a positive NS1 response further identifies the infection as a DENV infection versus a flavivirus infection because the NS1 assay is 98 to 100% specific for DENVs, in contrast to an IgM ELISA, which is between 70 and 90% specific depending on the ELISA format used (10). Therefore, NS1 can be utilized for differential diagnosis in cases in which the antigen for the IgM ELISA cross-reacts with other anti-flavivirus antibodies and the infecting virus cannot be identified by conventional RT-PCR.

The Platelia DENV NS1Ag test allowed us to significantly improve upon the sensitivity of RT-PCR alone for the detection of DENV in acute-phase serum samples. Additionally, the improved sensitivity afforded by the Bio-Rad assay for the subset of secondary-infection-associated samples that do not demonstrate IgM seroconversion makes it a valuable addition to a DENV testing algorithm aimed at diagnosis of infection with a single clinical sample. The use of an algorithm based on a single clinical sample has the potential to reduce the number of indeterminate diagnoses due to failure to obtain a convalescent-phase sample. Moreover, since secondary dengue infections are likely to cause a more severe form of disease than primary infections, this alternative testing may prove valuable in the clinical setting, when early diagnosis and treatment can decrease mortality to <1% (17). The excellent sensitivity and specificity of the assay, combined with its relatively low technological and resource requirements, make it an attractive alternative to RT-PCR for diagnosis of acute DENV infection in resource-poor areas with a potential for definitive point-of-care diagnosis in a single clinic visit.

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