Short Report: Relationship between Nonstructural Protein 1 Detection and Plasma Virus Load in Dengue Patients

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Abstract. We report data from a prospective observational study performed in Martinique during a co-epidemic of dengue virus serotype 2 (DENV-2) and serotype 4 (DENV-4). Among 70 serum samples from patients with DENV-2 (n = 21) or DENV-4 (n = 49) infections, 47 (67.1%) were positive for dengue nonstructural protein 1 (NS1). Antigenemia correlated with plasma virus load and was independent of immune status and the time of sampling. Increased viremia 4–6 days after onset of illness was associated with NS1 positivity, secondary infection, and severe disease. Testing for NS1 could help identify the potentially most severely ill patients during the critical phase of dengue.

Dengue nonstructural 1 protein (NS1) is secreted by cells infected with dengue virus (DENV). This glycoprotein is highly conserved for all DENV serotypes and is strongly immunogenic. The NS1 antigen and NS1-specific antibodies may play a central role in the pathogenesis of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). It has been suggested that high plasma levels of NS1 could help identify patients at risk for plasma leakage. Commercial enzyme-linked immunosorbent assays (ELISAs) for detection of DENV NS1 in human serum samples have been proposed for dengue diagnosis, but the value of NS1 detection in predicting clinical severity remains to be evaluated.

In a previous study conducted during a co-epidemic of DENV serotype 2 (DENV-2) and serotype 4 (DENV-4), blood samples were obtained form patients with acute dengue infection and stored in a serum bank. These serum samples provided an opportunity to screen serum samples from dengue patients for NS1 and to explore the relationships between NS1 antigenemia, plasma virus loads, dengue serotypes, immune status, and outcomes of illnesses.

The data reported were derived from a prospective observational study. All patients who came to an emergency department in Martinique with a history of acute febrile illness were invited to participate in the study after providing written informed consent. Clinical data were recorded at the bedside in a computerized medical record system by means of a standardized questionnaire. The final severity of illness was diagnosed on the basis of data recorded at the first visit and during follow-up. The distinction between uncomplicated dengue fever, DHF/DSS, and other severe clinical forms of dengue illnesses was adapted from the 1997 World Health Organization classification system.

Blood was obtained by venous puncture at admission time, and serum aliquots were stored at −70°C for virologic studies. Laboratory methods for the diagnosis of dengue infections and plasma virus load measurements have been described. Briefly, a hemi-nested reverse transcription–polymerase chain reaction (RT-PCR) was carried out with DENV generic and serotype specific primers, as described by Lanciotti and others. Quantitative real-time PCR was performed using generic primers, the iQ SYBR Green Supermix Kit, and the iCycler iQ Real Time PCR Detection System (Bio-Rad, Marne la Coquette, France). Plasma virus load was derived from standard curves obtained by serial dilution of titrated DENV-2 and DENV-4 supernatants and were expressed as plaque-forming unit (PFU) equivalents per milliliter. In addition, an ELISA for detection of NS1 was performed using PLATELIA™ Dengue NS1Ag Kits (Bio-Rad, Marne la Coquette, France) according to the manufacturer’s recommendations. Dengue-specific antibodies were detected using IgM capture, IgG capture, and IgG indirect ELISA kits (Panbio, Brisbane, Queensland, Australia). A positive IgG capture test result for a serum sample obtained within six days of the onset of fever indicated a secondary infection. Serum samples with negative results by IgG capture and IgG ELISA indicated a primary infection.

Where indicated, values are expressed as the median and 25–75% interquartile range (IQR). Data were compared across groups by using non-parametric tests. Logistic regression analysis was used to identify independent association between NS1, plasma virus load, dengue serotype, immune status, and other variables of clinical interest. Variables were entered into multivariate analysis by using a backward selection algorithm. All analyses were done in Statview version 4.5 software (Abacus Concepts, Berkeley, CA).

In the initial study, 110 (28.6%) of 384 patients with acute febrile illness had confirmed dengue infection by RT-PCR. The hemi-nested procedure identified 64 DENV-4 infections, 39 DENV-2 infections, 6 DENV-3 infections, and 1 DENV-1 infection. Testing for NS1 and measurement of virus load were performed in 70 (68%) of 103 DENV-2 and DENV-4 cases, for which sufficient serum was available. Although serum samples tested were not randomly allocated, the distribution of the clinical variables between the DENV-2 patients and the DENV-4 patients was similar to that observed in the original population.

The median age of the patients was 30 years (25–75% IQR = 23 years), and the male-to-female ratio was 0.6:1. The median time from the onset of fever to admission to the emergency department (time of presentation and sampling) was 2 days (25–75% IQR = 2 days) in DENV-4 patients and 3 days (25–75% IQR = 3 days) in DENV-2 patients (P = 0.024, by Mantel-Cox log-rank test). Serologic testing indicated a primary dengue infection in 9 (42.9%) of 21 DENV-2 patients and 43 (82.7%) of 49 DENV-4 patients (P < 0.001, by Fisher’s exact test). Regarding the final severity of illness, uncomplicated dengue fever was diagnosed in 39 (79.6%) of 49 DENV-4 patients and 11 (47.6%) of 21 DENV-2 patients (P = 0.041, by Fisher’s exact test). However, the trend towards more DHF/DSS

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in DENV-2 patients than in DENV-4 patients was not statistically significant (7 [33.3%] of 21 versus 9 [18.4%] of 49; \( P = 0.218 \), by Fisher’s exact test).

The median plasma viral load was 5,790 PFU equivalents/mL (25–75% IQR = 45,248 PFU equivalents/mL), and values ranged from 5.6 to 4,940,000 PFU equivalents/mL (Figure 1, top panel). Viremia levels were significantly higher in patients with DENV-2 infections than in those with DENV-4 infections (Figure 1, middle panel). Plasma virus loads decreased significantly over the time of sampling in patients with primary infections (\( P = 0.001 \), by Mantel-Cox log-rank test), but remained increased until the sixth day of illness in those with secondary infections (\( P = 0.915 \), by Mantel-Cox log-rank test). During the first three days of the disease, the distribution of virus loads was not different between patients with primary infections and those with secondary infections (Figure 2A).

**Figure 1.** Percentile distribution of plasma virus loads for 70 patients with symptomatic dengue infections on admission to an emergency department in Martinique. PFU = plaque-forming unit; NS1 = non-structural protein 1. \( P \) values were calculated by using the Mantel-Cox log-rank test.

**Figure 2.** Distribution of plasma virus loads in 70 patients with symptomatic dengue infections on admission to an emergency department, Martinique. A and B. Patients admitted within 1–3 days after onset of fever. C and D. Patients admitted 4–6 days after onset of illness. Box plots show median values (horizontal line in the box), 25–75% interquartile range (lower–upper limits of the box), and 90% range of data (additional bars). PFU = plaque-forming unit; NS = not significant; \( P \) values were calculated using the Mantel-Cox log-rank test.
and between those with uncomplicated illness and those with severe illness (Figure 2B). During days 4–6, patients with secondary infections or with severe illnesses had higher virus loads than those with primary infections or uncomplicated illnesses (Figure 2C and D).

Dengue virus NS1 was detected in 47 (67.1%) of 70 patients positive for dengue by RT-PCR. There was no difference in epidemiologic, clinical, and biological variables between NS1-positive and NS1-negative patients, with the exception of higher plasma virus loads in NS1-positive patients (Table 1 and Figure 1C, bottom panel). The sensitivity of NS1 testing increased with virus load. Nonstructural protein 1 was detected in 9 (45%) of 20 serum samples that had virus loads <1,000 PFU equivalents/mL, 17 (63%) of 27 serum samples that had virus loads between 1,000 and 30,000 PFU equivalents/mL, and 21 (91.3%) of 23 serum samples that had virus loads >30,000 PFU equivalents/mL. (P = 0.003, by chi-square test).

To control for confounding, multivariate analysis was performed and confirmed that the detection of NS1 was independently associated with plasma virus load at any time of sampling, dengue serotype, age, sex, and immune status of the patients, and final severity of illness (odds ratio per log10 increase in virus loads = 1.74, 95% confidence interval = 1.13–2.67, P = 0.012, by Wald test). Interestingly, the distribution of NS1-positive serum samples among primary and secondary infections was significantly correlated with the time elapsed since onset of fever. When tested during days 1–3 of illness, 31 (91.2%) of 34 NS1-positive patients were diagnosed with primary dengue infections and 3 (8.7%) of 34 were diagnosed with secondary infections. When tested during days 4–6, the proportions of NS1-positive serum samples from patients with primary and secondary infections were 5 (38.5%) of 13 and 8 (61.5%) of 13, respectively (P < 0.001, by Fisher’s exact test).

The pathogenesis of severe dengue disease is multifactorial and involves interactions between virus characteristics and immune response. Secondary infection is considered the most significant risk factor for DHF/DSS, and it has been suggested that more efficient DENV-2 replication in primed hosts confers enhanced pathogenicity. As reported in our previous study, most severe cases, including DHF, were observed in patients with secondary DENV-2 infections, whereas patients with primary DENV-4 infection showed mainly uneventful outcomes. Viremia levels are postulated to be involved in severe diseases. Higher virus loads were associated with severe secondary DENV-2 infections, particularly after three days of illness. This observation is consistent with the hypothesis of slower virus clearance in DHF patients.

Assays for detecting NS1 have been shown to be more sensitive for patients with primary than for those with secondary dengue infection. Nonstructural protein 1–specific IgG is known to be present during the first few days of secondary dengue infection and may trap a substantial amount of the NS1 within the immune complex. As expected, we found a higher sensitivity of NS1 detection for primary than secondary infections during the first three days of illness. However, the rate of NS1 positivity during days 4–6 was higher in those with secondary infections. According to the duration of dengue illness, a nadir of NS1 detection rates has been reported around the time of defervescence, followed by an increase. Secondary infections with DENV-2 may result in prolonged viral replication and NS1 release from specific cells, e.g., immune, reticuloendothelial, or hepatic cells.

The proportion of NS1-positive patients correlated with plasma virus loads, and both parameters were prolonged in secondary and severe infections. This finding suggests that NS1 testing could help in identifying the patients likely to develop severe outcomes during the so-called critical phase of dengue. This phase occurs in some patients at approximately 3–5 days of illness and is characterized by development of a vascular permeability syndrome that leads to various degrees of plasma volume loss and hypotension. In addition to warning signs such as a rapid decrease in body temperature, persistent intractable vomiting, increasing abdominal pain, increased hemocrit, and a decrease in thrombocyte counts, a positive NS1 test result would be of special interest for quickly confirming the diagnosis of dengue and suggesting a high probability of persistent viremia.

Future evaluations of the prognostic value of NS1 detection should focus on the time of sampling and clinical phase of the disease. Quantitative assays are also needed because the kinetics of NS1 antigenemia may be of interest for predicting the risk of DHF or other severe complications.
Received March 3, 2010. Accepted for publication May 11, 2010.

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