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

Laurent Thomas,* Fatiha Najioullah, Olivier Verlaeten, Jenny Martial, Ségolène Brichler, Stéphane Kaidomar, Victor Moravie, André Cabié, and Raymond Césaire

Centre Hospitalier Universitaire, Fort-de-France, Martinique; Centre d'Investigation Clinique et d'Epidémiologie Clinique Antilles Guyane (CIC-EC INSERM U802), Fort-de-France, Martinique

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).¹ 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).² It has been suggested that high plasma levels of NS1 could help identify patients at risk for plasma leakage.^{3–5} Commercial enzyme-linked immunosorbent assays (ELISAs) for detection of DENV NS1 in human serum samples have been proposed for dengue diagnosis,^{6,7} 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 from patients with acute dengue infection and stored in a serum bank.⁸ 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.⁸ 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.^{8,9}

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.⁸ 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.¹⁰ 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.¹¹ 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 infections.⁸ 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.⁸

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

*Address correspondence to Laurent Thomas, Centre Hospitalier Universitaire, Fort-de-France, Martinique. E-mail: laurent.thomas@chu-fortdefrance.fr

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)

A Plasma viral loads (PFU equivalents /mL)

FIGURE 1. Percentile distribution of plasma virus loads for 70 patients with symptomatic dengue (DENV) 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.

Plasma viral loads (PFU equivalents /mL)

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.

TABLE 1

Clinical data for 70 symptomatic dengue patients on admission in Martinique*

Clinical data	NS1 positive (n = 47)	NS1 negative (n = 23)	P†
Plasma virus load × 10 ³ plaque-forming unit equivalent/mL	13.1 [71.8]	1.06 [8]	0.012
Epidemiology			
Age, years	30 [28]	32 [25]	0.837
Male:female	17:30	9:14	1
DENV-2:DENV-4 infection	13:34	8:15	0.590
Primary:secondary infection	36:11	16:7	0.569
Sampled days 1–3:4–6	34:13	15:8	0.586
Vital signs			
Body temperature, °C	38.7 [1.4]	38.7 [1.7]	0.762
Pulse pressure, mm Hg	48 [24]	49 [20]	0.626
Heart rate, beats/minute	93 [30]	89 [26]	0.684
Signs and symptoms, no. (%)			
Headache	42 (89)	20 (87)	1
Retroorbital pain	18 (41)	9 (39)	1
Muscle and/or joint pain	36 (77)	15 (68)	0.559
Backache	38 (81)	17 (74)	0.545
Epigastralgia	19 (41)	9 (39)	1
Gastrointestinal signs	24 (51)	12 (52)	1
Fatigue	34 (74)	17 (74)	1
Cough	12 (26)	6 (26)	1
Rash	5 (11)	0 (0)	0.161
Bleeding	5 (11)	3 (13)	1
Postural hypotension	5 (11)	1 (4)	0.656
Presyncope/syncope	12 (26)	5 (22)	1
Biology			
Hematocrit, %	39 [6]	40 [5]	0.609
Hemoglobinemia, g/L	134 [26]	135 [24]	0.435
Thrombocyte count × 10 ³ /mL	154 [94]	171 [105]	0.753
Lymphocyte count/mL	570 [420]	595 [340]	0.355
Neutrophile count/mL	3,705 [2,530]	4,580 [2,050]	0.167
ASAT (U/L)	30 [43]	43 [44]	0.270
ALAT (U/L)	20 [39]	27 [30]	0.161
Creatine kinase (U/L)	135 [235]	186 [202]	0.204
Total proteinemia (g/L)	74 [6]	72 [5]	0.565
C-reactive protein (mg/L)	6.3 [28]	10.6 [38]	0.274
Uncomplicated dengue, no. (%)	34 (69.4)	16 (69.6)	1
Hospitalized, no. (%)	20 (43)	8 (35)	0.609

* NS1 = nonstructural protein 1; DENV = dengue virus; AST = aspartate aminotransferase; ALT = alanine aminotransferase. Continuous data are expressed as median [25–75% interquartile range].

† By Fisher's exact test (categorical data) or Mann-Whitney test (continuous data).

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,¹² and it has been suggested that more efficient DENV-2 replication in primed hosts confers enhanced pathogenicity.¹³ As reported in our previous study,⁸ 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.^{4,13} 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.¹⁴

Assays for detecting NS1 have been showed to be more sensitive for patients with primary than for those with secondary dengue infection.^{6,7} Nonstructural protein 1-specific IgG is known to be present during the first few days of secondary dengue infection¹⁵ and may trap a substantial amount of the NS1 within the immune complex.^{16,17} 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.⁶ Secondary infections with DENV-2 may result in prolonged viral replication and NS1 release from specific cells, e.g., immune, reticulo-endothelial, or hepatic cells.¹⁸

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.¹⁹ In addition to warning signs such as a rapid decrease in body temperature, persistent intractable vomiting, increasing abdominal pain, increased hematocrit, 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.

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Authors' addresses: Laurent Thomas, Stéphane Kaidomar, and Victor Moravie, Service des Urgences, Centre Hospitalier Universitaire, Fort-de-France, Martinique. Fatiha Najioullah, Olivier Verlaeten, Jenny Martial, Ségolène Brichler, and Raymond Césaire, Laboratoire de Virologie-Immunologie, Centre Hospitalier Universitaire, Fort-de-France, Martinique. André Cabié, Service des Maladies Infectieuses et Tropicales, Centre Hospitalier Universitaire, Fort-de-France, Martinique.

REFERENCES

1. Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V, 1999. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. *J Virol* 73: 6104–6110.
2. Falconar AK, 1997. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. *Arch Virol* 142: 897–916.
3. Young R, Hilditch PA, Bletchly C, Halloran W, 2000. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. *J Clin Microbiol* 38: 1053–1057.
4. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, Green S, Vaughn DW, Nisalak A, Ennis FA, Rothman AL, 2002. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *J Infect Dis* 186: 165–168.
5. Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmea S, Auethavornanan K, Jairungsri A, Kanlaya R, Tangthawornchaikul N, Puttikhunt C, Pattanakitsakul SN, Yenchitsomanus PT, Mongkolsapaya J, Kasinrerak W, Sittisombut N, Husmann M, Blettner M, Vasanawathana S, Bhakdi S, Malasit P, 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. *J Infect Dis* 193: 1078–1088.
6. Dussart P, Petit L, Labeau B, Breman L, Leduc A, Moua D, Matheus S, Baril L, 2008. Evaluation of two new commercial tests for the diagnosis of acute dengue virus infection using NS1 antigen detection in human serum. *PLoS Negl Trop Dis* 2: e280.
7. Hang VT, Nguyen NM, Trung DT, Tricou V, Yoksan S, Dung NM, Van Ngoc T, Hien TT, Farrar J, Wills B, Simmons CP, 2009. Diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses. *PLoS Negl Trop Dis* 3: e360.
8. Thomas L, Verlaeten O, Cabié A, Kaidomar S, Moravie V, Martial J, Najioullah F, Plumelle Y, Fonteau C, Dussart P, Césaire R, 2008. Influence of the dengue serotype, previous dengue infection, and plasma viral load on clinical presentation and outcome during a dengue-2 and dengue-4 co-epidemic. *Am J Trop Med Hyg* 78: 990–998.
9. World Health Organization, 1997. *Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control*. Second edition. Geneva: World Health Organization.
10. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV, 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 30: 545–551.
11. Vazquez S, Hafner G, Ruiz D, Calzada N, Guzman MG, 2007. Evaluation of immunoglobulin M and G capture enzyme-linked immunosorbent assay Panbio kits for diagnostic dengue infections. *J Clin Virol* 39: 194–198.
12. Halstead SB, 1988. Pathogenesis of dengue: challenges to molecular biology. *Science* 239: 476–481.
13. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA, Nisalak A, 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis* 181: 2–9.
14. Wang WK, Chen HL, Yang CF, Hsieh SC, Juan CC, Chang SM, Yu CC, Lin LH, Huang JH, King CC, 2006. Slower rates of clearance of viral load and virus-containing immune complexes in patients with dengue hemorrhagic fever. *Clin Infect Dis* 43: 1023–1030.
15. Shu PY, Chen LK, Chang SF, Yueh YY, Chow L, Chien LJ, Chin C, Lin TH, Huang JH, 2003. Comparison of capture immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) and nonstructural protein NS1 serotype-specific IgG ELISA for differentiation of primary and secondary dengue virus infections. *Clin Diagn Lab Immunol* 10: 622–630.
16. Koraka P, Burghoorn-Maas CP, Falconar A, Setiati TE, Djamiatun K, Groen J, Osterhaus AD, 2003. Detection of immune-complex-dissociated nonstructural-1 antigen in patients with acute dengue virus infections. *J Clin Microbiol* 41: 4154–4159.
17. Lapphra K, Sangcharaswichai A, Choekphaibulkit K, Tiengrim S, Piriakarnsakul W, Chakorn T, Yoksan S, Wattanamongkolsil L, Thamlikitkul V, 2008. Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness. *Diagn Microbiol Infect Dis* 60: 387–391.
18. Martina BE, Koraka P, Osterhaus AD, 2009. Dengue virus pathogenesis: an integrated view. *Clin Microbiol Rev* 22: 564–581.
19. World Health Organization, 2009. *Dengue. Guidelines for Diagnosis, Treatment, Prevention and Control*. Third edition. Geneva: World Health Organization.