Determination of Viremia and Concentration of Circulating Nonstructural Protein 1 in Patients Infected with Dengue Virus in Mexico

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Abstract. Higher levels of viremia and circulating nonstructural protein 1 (NS1) have been associated with dengue disease severity. In this study, viremia and circulating NS1 levels were determined in 225 serum samples collected from patients in Mexico infected with dengue virus serotypes 1 and 2 (DENV-1 and DENV-2). Patients with dengue hemorrhagic fever (DHF) who were infected with DENV-1 showed higher levels of circulating NS1 than patients with dengue fever (DF) ($P = 0.0175$). Moreover, NS1 levels were higher in patients with primary infections with DENV-1 than in patient infected with DENV-2 ($P < 0.0001$) and in patients with primary infections with DENV-2 than in patients with secondary infections with DENV-2 ($P = 0.0051$). Unexpectedly, viremia levels were higher in patients with DF than in those with DHF infected with either DENV-1 or DENV-2 ($P = 0.0019$ and $P = 0.001$, respectively) and in patients with primary infections than those with secondary DENV-2 infections ($P < 0.0001$). Results indicate that levels of circulating NS1 vary according to the infecting serotype, immunologic status (primary or secondary infection), and dengue disease severity.

INTRODUCTION

Dengue is the most prevalent human viral disease transmitted by mosquitoes. Each year, more than 50 million cases occur, which are associated with more than 25,000 deaths, especially in children less than five years of age.¹ Dengue is endemic to more than 100 countries and nearly one-third on the world's population lives in risk areas, which are tropical and subtropical regions.² Because of the great disease burden associated with dengue, the World Health Organization considers dengue as a major public health problem and has issued a mandate to develop strategies to prevent and treat this disease. However, there is currently no licensed vaccine or a specific treatment for dengue.³

Dengue virus (DENV) is a member of the family *Flaviviridae*, genus *Flavivirus*, and is transmitted to humans by the bite of *Aedes* mosquitoes, mainly *Aedes aegypti*.^{4–6} The infection with any of the four DENV serotypes can either be asymptomatic or manifest in three clinical forms of increasing severity: dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome.^{4–7} Dengue fever is characterized by fever, headache, myalgia, and arthralgia, and in some cases by leukopenia and increased levels of aminotransferases.^{6,7} Dengue hemorrhagic fever is characterized by thrombocytopenia, increase in vascular permeability, and plasma leakage. In severe cases, circulatory failure, shock (dengue shock syndrome), and death can occur.^{6,7} The reasons for such disparate clinical outcomes upon infection are largely unknown, although both viral and host factors have been implicated.⁸

Nonstructural protein 1 (NS1) is secreted from infected cells and circulates at high levels in serum of infected patients.^{9–11} Detection of NS1 in serum or plasma is an early marker for the diagnosis of dengue and commercial enzyme-linked immu-

nosorbent assays (ELISAs) or immunochromatographic rapid tests for NS1 detection are widely used for early diagnosis of dengue.^{2,12} Prospective studies conducted in Asia suggest a relationship between levels of circulating NS1 and dengue disease severity. In a pioneer study, Libraty and others¹³ followed-up 32 children with DENV-2 infections in a prospective hospital-based study and concluded that increased levels of free circulating NS1 may be a marker for identifying patients at risk for DHF. In another prospective study conducted in Thailand, Avirutnan and others¹⁴ also observed that the mean level of circulating NS1 in patients with DHF was higher than that in patients with DF. However, a recent study conducted in the AG129 mouse model failed to find an association between circulating NS1 levels and disease severity.¹⁵ This evidence, although compelling, is limited by the fact that it was generated in a mouse model that lacks a proper interferon response.

A relationship between NS1 positivity rates and viremia has also been reported. Antigenemia was found to correlate with viremia in a prospective observational study that included 24 serum samples from patients infected with DENV-2 and 49 serum samples from patients infected with DENV-4 in Martinique.¹⁶ The same conclusion was reached by Duong and others¹⁷ after the analysis of more than 200 serum samples collected from hospitalized patients in Cambodia. However, in a recent prospective study that analyzed 167 sequential samples collected from children in Vietnam, the circulating concentrations of NS1 were observed to vary according to the infecting serotype and the immune status of the patient.¹⁸ Regarding a possible association between viremia levels and disease severity, conflicting results have been reported. Although several studies^{12,19,20} reported a positive association between viremia levels and disease severity, other studies have not.^{14,17} Because identification of markers for disease severity or increase risk of progression to the more severe forms of the disease is of major interest in dengue research, the aim of this study was to evaluate viremia and circulating NS1 levels in serum samples sera collected from patients in Mexico with dengue and to determine if the results obtained in the studies conducted in Asia may be reproduced in different epidemiologic and genetic settings.

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MATERIALS AND METHODS

Serum samples. A total of 225 human serum samples collected during September–December 2009 were used for this transverse retrospective study. Human serum specimens were provided by the Instituto de Diagnóstico y Referencia Epidemiológicos (Mexico City, Mexico), and all were collected as part of the national program for dengue surveillance. Because dengue is a disease of obligatory report in Mexico, no informed consent from the patients is required. However, the anonymity of patients was fully preserved. Accompanying epidemiologic data were obtained from the clinical history included with each sample. Clinical cases were classified as DF or DHF following the 1997 World Health Organization criteria.²¹ Efforts were made to have equivalent numbers of DF and DHF cases, thus 120 patients with DF and 105 patients with DHF were included. Samples were taken from patients during the first five days post-onset of fever, which corresponds to the acute period of infection. Day zero was defined as the calendar day during which fever began in a patient.

RNA extraction and reverse transcription–polymerase chain reaction. RNA was extracted from 200 μ L of serum (or virus-infected cells, used as controls) by using an automatic device (MagNa Pure LC 2.0 System, Roche, Indianapolis, IN)

TABLE 1

Characteristics of the patient population (n = 225) that contributed to the sample panel used in this study*

Characteristic	DENV-1		DENV-2	
	DF	DHF	DF	DHF
No. patients	64	62	56	43
No. females	31	35	20	16
No. males	33	27	36	27
No. primary infections	61	57	43	24
No. secondary infections	3	5	13	19

*DENV = dengue virus; DF = dengue fever; DHF = dengue hemorrhagic fever.

and the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche) according to the manufacturer's instructions. Extracted RNA was eluted in 100 μ L of nuclease-free water.

To determine the infecting virus serotype, serum samples were tested for viral RNA by real-time reverse transcription–polymerase chain reaction (RT-PCR) using the CFX96 System (Bio-Rad Laboratories, Hercules, CA) and the multiplex TaqMan fluorogenic assay described by Johnson and others.²² In brief, 5 μ L of RNA were mixed with 50 pmol of serotype-specific primers and 9 pmol probes in a total reaction mixture of 25 μ L using the Super Script III Platinum, One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA).

FIGURE 1. Viremia levels in dengue infected patients according to clinical condition. Viremia (PFU eq/mL) in serum samples from patients with dengue fever (DF) or dengue hemorrhagic fever (DHF) infected with dengue virus 1 (DENV-1) or dengue 2 (DENV-2) were determined by using a singleplex real time reverse transcription–polymerase chain reaction. Box-and-whisker plots show median values (horizontal line in the box), 25–75% interquartile range (upper–lower limits of the box), 95% range of data (error bars), and outliers (black circles). *P* values were calculated by using the Mann-Whitney U test. Statistical significance was $P \leq 0.05$. n = number of patients in each condition.

FIGURE 2. Viremia levels in dengue-infected patients by days after fever onset. Viremia (PFU eq/mL) in serum samples from patients infected with dengue virus 1 (DENV-1) or dengue 2 (DENV-2) collected 0–2 days or 3–5 days after fever onset were determined by using a singleplex real time reverse transcription–polymerase chain reaction. Box-and-whisker plots show median values (horizontal line in the box), 25–75% interquartile range (upper–lower limits of the box), 95% range of data (error bars), and outliers (black circles). Statistical significance was $P \leq 0.05$.

Four serotype-specific probes were labeled at their 5' ends with a reporter and at their 3' ends with a thermoquencher as follows: FAM-BHQ1, Texas Red-BHQ2, HEX-BHQ1, and Quasar 670-BHQ2 for DENV-1, DENV-2, DENV-3, and DENV-4, respectively. Amplification and real-time detection consisted of the following cycle profile: reverse transcription at 50°C for 30 minutes; followed by 95°C for 10 minutes; and 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. Fluorescence data were continuously collected. Viremia was calculated by using a singleplex qRT-PCR and standard curves obtained by serial dilutions of DENV-1 and DENV-2 stocks titrated by plaque assays in BHK-21 cells²³ and expressed as plaque-forming units per milliliter equivalents (PFUeq/mL).^{16,22}

Serologic assays. To define the immune status of the patients, serum samples were tested for dengue-specific IgM and IgG by using a capture ELISA (PanBio, Brisbane, Queensland, Australia) system. On the basis of the manufacturer's instructions, the sample value (PanBio units) was determined for each serum by dividing the absorbance of the test sample by the calculated cut-off value and then multiplying by 10. A sample was defined as positive for IgM or IgG when the PanBio units were ≥ 11 and ≥ 22 , respectively. Because all serum samples were collected early after fever onset (between 0 and 5 days), a sample was defined as primary infection

when negative for IgG and as secondary infection when positive for IgG.^{24,25}

The presence of NS1 was qualitatively determined using the Platelia™ NS1 Ag enzyme immunoassay (Bio-Rad Laboratories, Marnes-la-Coquette, France) as indicated by the manufacturer. For quantification of NS1 levels in serum samples with the same assay, standard curves were constructed by using two-fold serial dilutions of affinity-purified recombinant NS1 from DENV-2 expressed in *Escherichia coli* (kindly provided by Dr. Ronaldo Mohana-Borges, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil). The recombinant protein was obtained by using a refolding protocol that enabled generation of dimeric NS1 in a conformation similar to that of the protein expressed in eukaryotic cells.²⁶ Serum samples obtained from DENV-1 patients were diluted 1:1,000 and those obtained from DENV-2 patients were diluted 1:200 to generate optical readings within the linear range of the curve, which ranged from 0.073 ng/mL to 0.60 ng/mL (Supplemental Figure 1). The dilution factor required for analysis of serum samples corresponding to DENV-1 and DENV-2 patients was determined empirically by testing samples at multiple dilutions. Reproducibility between plates was controlled by including negative, positive, and cut-off control serum samples in each plate.

Statistical analysis. The Mann-Whitney U test was used to compare median values of viremia and NS1 levels between groups. Graphs were generated with the GraphPad Prism 5 software (GraphPad, San Diego, CA).

RESULTS

Study population. A total of 225 serum samples were collected in six states (Colima, Jalisco, Morelos, Nayarit, Chiapas, and Yucatán) of Mexico during 2009. All serum samples were positive for NS1. The median age of the patients was 21 years, and the male:female ratio was 1.2. Analysis by multiplex real-time PCR indicated that 56% (126) of the patients were infected with DENV-1 and 44% (99) were infected with DENV-2. Infections with DENV-3 or DENV-4 were not identified. On the basis of negative results for IgG obtained in the PanBio capture ELISA, 93.7% (118) of the infections caused by DENV-1 and 67.7% (67) of the infections caused by DENV-2 were considered primary infections (Table 1).

Viremia levels. Although the best method to measure viremia levels would be viral titration by plaque assay, the low viral titers in some serum samples plus the number of serum samples analyzed make this method cumbersome. Thus, to avoid lack of sensibility and to achieve high reproducibility, viremia was measured by qRT-PCR and results were expressed as PFUeq/mL.

Median viremia for patients infected with DENV-1 was 257.86 PFUeq/mL (25–75% interquartile range [IQR] = 17.85–9,652 PFUeq/mL, range = 0.32–267,516 PFUeq/mL), and the median viremia for patients infected with DENV-2 was 1,560.02 PFUeq/mL (25–75% IQR = 83.59–10,563 PFUeq/mL, range = 0.30–124,097 PFUeq/mL). However, differences in viremia levels between DENV-1 and DENV-2 patients did not reach statistical significance ($P = 0.0785$) (Figure 1). When viremia levels were compared between patients with DF and patients with DHF, significant differences were found between the two clinical conditions regardless of the infecting serotype. For DENV-1 patients, median viremia levels in patients with DF (median = 1,152 PFUeq/mL; 25–75% IQR = 63.79–26,251 PFUeq/mL, range = 0.32–235,958 PFUeq/mL) were significantly higher ($P = 0.0019$) than in patients with DHF (median = 48 PFUeq/mL, 25–75% IQR = 8.83–4,025 PFUeq/mL, range = 0.42–267,516 PFUeq/mL). Similarly, for those patients infected with DENV-2, median viremia levels were significantly higher ($P = 0.001$) in patients with DF (median = 2,898 PFUeq/mL, 25–75% IQR = 270.4–17,259 PFUeq/mL, range = 0.35–124,097 PFUeq/mL) than in patients with DHF (median = 218.5 PFUeq/mL, 25–75% IQR = 14.52–4,576 PFUeq/mL, range = 0.30–86,558 PFUeq/mL) (Figure 1).

Viremia levels clearly decreased as infections progressed (Figure 2). For DENV-1 infections, median viremia decreased from 4,632 PFUeq/mL (25–75% IQR = 61.97–34,424 PFUeq/mL,

FIGURE 3. Viremia levels in dengue virus serotype 2 (DENV-2) infected patients with primary or secondary infections. Viremia (plaque-forming units [PFU eq/mL]) in serum samples were determined by using a singleplex real time reverse transcription–polymerase chain reaction. Box-and-whisker plots show median values (horizontal line in the box), 25–75% interquartile range (upper–lower limits of the box), 95% range of data (error bars), and outliers (black circles). Statistical significance was $P \leq 0.05$.

FIGURE 4. Levels of circulating nonstructural protein 1 (NS1) in serum samples of dengue-infected patients according to clinical condition. Concentrations (ng/mL) of circulating NS1 in serum samples from patients with dengue fever (DF) or dengue hemorrhagic fever (DHF) infected with dengue virus 1 (DENV-1) or DENV-2 were determined by using a commercial enzyme-linked immunosorbent assay with modifications. Box-and-whisker plots show median values (**horizontal line in the box**), 25–75% interquartile range (**upper–lower limits of the box**), 95% range of data (**error bars**), and outliers (**black circles**). Statistical significance was $P \leq 0.05$.

range = 0.65–267,516 PFUeq/mL) for serum samples collected 0–2 days after fever onset to 82.42 PFUeq/mL (25–75% IQR = 9.75–1,910 PFUeq/mL, range = 0.32–166,580 PFUeq/mL) for serum samples collected 3–5 days after fever onset ($P = 0.0002$). Likewise, for serum samples collected from DENV-2 patients, median viremia decreased from 7,200 PFUeq/mL (25–75% IQR = 701.4–22,658 PFUeq/mL, range = 32.47–124,098 PFUeq/mL) to 399 PFUeq/mL (25–75% IQR = 11.3–3,026 PFUeq/mL, range = 0.29–49,488 PFUeq/mL), for serum samples 0–2 days and 3–5 days after fever onset, respectively ($P < 0.0001$). The reduction in viremia as infection progresses was observed irrespective of the clinical condition.

For patients infected with DENV-2, viremia levels were significantly higher ($P < 0.0001$) in primary infections than in secondary infections (median = 4,966 PFUeq/mL, 25–75% IQR = 265–19,896 PFUeq/mL, range = 0.29–124,098 PFUeq/mL versus median = 161 PFUeq/mL, 25–75% IQR = 17.77–1,369 PFUeq/mL, range = 2.05–7,081 PFUeq/mL) (Figure 3). Viremia levels were reported to decay faster during secondary infections.^{18,20} However, this difference was observed regardless if the day of serum sample collection after fever onset was taken into consideration. Because of the small number of secondary infections (6.3%) detected, similar comparisons with DENV-1 patients were not possible.

Levels of circulating NS1. Levels of circulating NS1 were determined for all 225 serum samples collected. Levels of circulating NS1 for patients infected with DENV-1 ranged between 13.51 ng/mL and 1886 ng/mL (median = 126 ng/mL, 25–75% IQR = 54.95–304.9 ng/mL). Levels of circulating NS1 in DENV-2 patients were much lower and ranged from 3.64 ng/mL to 265.7 ng/mL (median = 6.42 ng/mL, 25–75% IQR = 4.6–28 ng/mL) (Figure 4). Thus, difference in levels of circulating NS1 between patients infected with DENV1 and DENV-2 were highly significant ($P < 0.0001$).

When median levels of circulating NS1 were compared between patients with DF and DHF, clear significant differences were found for patients infected with DENV-1 but not for those infected with DENV-2. For DENV-1 patients, median levels of NS1 in serum samples were 104 ng/mL (25–75% IQR = 48.32–181.3 ng/mL, range = 14.82–877.8 ng/mL) and 156 ng/mL (25–75% IQR = 56–397 ng/mL, range = 13.51–1,886 ng/mL) for DF and DHF, respectively ($P = 0.0175$) (Figure 4). In contrast, no significant differences in levels of circulating NS1 were observed between patients with DF or DHF caused by DENV-2 (median = 7.84 ng/mL, 25–75% IQR = 4.6–26.4 ng/mL, range = 3.8–194.7 ng/mL versus 6.02 ng/mL, 25–75% IQR = 4.52–53.28 ng/mL, range = 3.64–265.7 ng/mL ($P = 0.3660$)) (Figure 4).

For patients infected with DENV-1, levels of circulating NS1 increased from 88.04 ng/mL (25–75% IQR = 45–155.7 ng/mL, range = 13.51–865.4 ng/mL) for serum samples collected 0–2 days after fever onset to 156.30 ng/mL (25–75% IQR = 72.93–372.8 ng/mL, range = 14.82–1,886 ng/mL) for those collected 3–5 days after fever onset (Figure 5). In contrast, for patients infected with DENV-2, NS1 levels remained fairly constant during this period. Median levels were 8.26 ng/mL (25–75% IQR = 4.7–43.55 ng/mL, range = 3.68–194.7 ng/mL) and 5.95 ng/mL (25–75% IQR = 4.52–20.13 ng/mL, range = 3.64–265.7 ng/mL) for serum samples collected 0–2 days and 3–5 days after fever onset, respectively (Figure 5).

Finally, for patients infected with DENV-2, significantly higher median values in levels of circulating NS1 levels were observed in persons with primary infections than those with secondary infections (8.04 ng/mL, 25–75% IQR = 4.68–60.28 ng/mL, range = 3.68–265.7 ng/mL versus 5.5 ng/mL, 25–75% IQR = 4.4–8.59 ng/mL, range = 3.64–81.22 ng/mL) ($P = 0.0051$) (Figure 6).

DISCUSSION

Identification of host or viral makers that will enable recognition of patients with increased risk of progression towards

the more severe forms of dengue would be of great help not only for improving clinical management of the patients but also to reduce costs in unnecessary hospitalizations. Thus, several studies have evaluated the usefulness of viral factors (viremia and circulating NS1 levels) or host factors, such as cytokines levels, platelet counts, or complement activation products, to identify early during the disease, patients with increased risks for developing severe dengue.^{27,28} In the current study, we evaluated viremia and circulating NS1 levels in a group of patients in Mexico with dengue in an attempt to corroborate and expand earlier results obtained mostly with patients from Asia, which suggest that NS1 levels and/or viremia are higher in patients with DHF than in patients with DF, and thus may be useful markers of disease progression.

In this study, higher in viremia levels were observed in patients with DF than in patients with DHF. Levels of viremia have been associated with disease severity in several studies^{13,16,19} but not in others.^{14,29} Our results, consistent with previous results, indicate that viremia levels decay fast after fever onset^{13,14} and that primary infections have higher viremias than secondary infections.^{18,29} The reasons for these discrepant results are unknown, but indicate that viremia levels seem to be the results of a complex interplay between viral and host factors.^{4–8,15} The observation that infections

FIGURE 5. Levels of circulating nonstructural protein 1 (NS1) in serum samples of dengue-infected patients by days after fever onset. Concentrations (ng/mL) of circulating NS1 in serum samples from patients infected with dengue 1 virus (DENV-1) or dengue 2 (DENV-2) collected 0–2 days or 3–5 days after fever onset were determined by using a commercial enzyme-linked immunosorbent assay with modifications. Box-and-whisker plots show median values (horizontal line in the box), 25–75% interquartile range (upper–lower limits of the box), 95% range of data (error bars), and outliers (black circles). Statistical significance was $P \leq 0.05$.

FIGURE 6. Levels of circulating nonstructural protein 1 (NS1) in serum samples of dengue virus serotype 2-infected patients with primary or secondary infections. Concentrations of circulating NS1 in serum samples were determined by using a commercial enzyme-linked immunosorbent assay with modifications. Box-and-whisker plots show median values (horizontal line in the box), 25–75% interquartile range (upper–lower limits of the box), 95% range of data (error bars), and outliers (black circles). Statistical significance was $P \leq 0.05$.

with different serotypes occur with different levels of viremia^{16,18,20} but not clear differences in clinical outcomes suggest that viremia level and disease severity are not correlated.

Our results are consistent with those of studies that indicate that levels of circulating NS1 are higher in patients with DHF than in patients with DF.^{13,14,30} However, in this study, differences in circulating NS1 levels were observed only during primary infections with DENV-1 because DHF and DF patients with DENV-2 virus infections showed little differences in circulating NS1 levels and the few serum samples collected precluded any comparison for DENV-1 secondary infections. Low levels of circulating NS1 detected in serum samples from patients with DENV-2 secondary infections may have prevented detection of any differences between different clinical conditions. In that regard, our data differs from those reported by Libraty and others,¹³ whose findings of higher NS1 levels in DHF patients were obtained for a cohort of children with secondary DENV-2 infections. Similarly, almost 91% of the infections studied by Avirutnan and others¹⁴ were DENV-1 and DENV-2 secondary infections. Differences in the detection system used to measure NS1 levels may be responsible for these discrepancies. Commercial assays, such as the one used in this study, have been shown to be more sensitive for NS1 detection during primary infections than during secondary infections.^{17,31,32}

Our results also suggest that median concentrations of circulating NS1 are higher for infections caused by DENV-1 than by DENV-2. For this study, only primary infections could be compared. However, similar observations were recently reported by Duyen and others¹⁸ for primary and secondary infections. It has been reported that the sensitivity of the PLATELIA™ commercial assay for detection of NS1 is higher for DENV-1 than for DENV-2.^{17,33} Our standard curve was created by using a recombinant NS1 protein obtained from DENV-2. Thus, the possibility that NS1 levels in serum sample from DENV-1 patients are overestimated cannot be excluded. However, differences in levels of circulating NS1 found between serum samples collected from DENV-1 and DENV-2 patients were very significant ($P < 0.0001$).

In addition, for those infections with DENV-2, much higher levels of NS1 were observed for primary infections than for secondary infections. Viremia and plasma concentration of circulating NS1 have been shown to decay faster during secondary infections than during primary infections.^{18,20} These authors also reported that seven of nine patients with undetectable NS1 responses were DENV-2 patients with secondary infections. Formation of antibodies against NS1 immune complexes has also been related to less efficient detection of NS1 during secondary infections.^{31,34} Thus, the more vigorous immune response reported after secondary

infection may be related to inefficient detection of NS1 during secondary infections.^{35–38}

In summary, these results suggest that during primary infections, levels of circulating NS1 are higher in patients with DHF than in patients with DF. Nonetheless, our results and other results^{18,20} also suggest that concentrations of circulating NS1 are affected by the infecting serotype and the immunologic status (i.e., primary or secondary infection) of the patient. Given the high beneficial impact for patient care and health costs that a marker for disease severity may provide, prospective studies should be conducted in different epidemiologic settings to evaluate the value of circulating NS1 levels.

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