Dengue Virus 4 (DENV-4) Re-Emerges after 30 Years in Brazil: Cocirculation of DENV-2, DENV-3, and DENV-4 in Bahia

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SUMMARY: Dengue fever (DF) is a mosquito-borne viral disease of great concern in tropical and subtropical regions of the world. One important cause of the increase in DF is rapid development and urbanization that has led to proliferation of the Aedes aegypti mosquito, the vector responsible for transmission of the illness. Surveillance of dengue virus (DENV) infection in Brazil shows the predominance of DENV-1, DENV-2, and DENV-3 until 2010. This study reports the reappearance of DENV-4 in Brazil for the first time in 30 years. Serum samples were collected from individuals (n = 214) exhibiting fever and muscular pain in Bahia, Brazil, during 2011–2012. These samples were subjected to reverse transcription-polymerase chain reaction (RT-PCR)/nested PCR, which revealed that 82% of samples were positive for DENV-4; most were older age groups and exhibited a serological pattern consistent with a primary infection. The cocirculation of multiple DENV serotypes within the same city places the population at risk for a fatal form of the disease. Therefore, with the increasing incidence of severe DF cases, early diagnosis will be a priority for public health efforts in Brazil.

INTRODUCTION

Dengue fever (DF) is a mosquito-borne viral disease of great concern in tropical and subtropical regions of the world. The World Health Organization estimates that 50–100 million cases of DF occur annually. Morbidity and mortality due to dengue virus (DENV) infections have increased dramatically in recent decades worldwide (1). One important cause of the increase in DENV infections is rapid development and urbanization, which has led to proliferation of the Aedes aegypti mosquito, the vector responsible for DENV transmission (2). DF is clinically characterized as a nonspecific febrile disease with myalgia and arthralgia. The more severe and life-threatening form of DF also involves bleeding, thrombocytopenia, and increased vascular permeability that can result in death (3).

DENV belongs to the family Flaviviridae and has 4 serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. Its positive single-stranded RNA genome encodes 10 proteins: 3 structural (C, prM, and E) and 7 nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (4,5). Among these proteins are highlighted protein E and NS1. The E glycoprotein is highly immunogenic, and NS1 protein is expressed on the surface of infected cells and can be detected in the serum (6).

DF is an important health problem in Brazil. Every year, the fight against proliferation of the mosquito is intensified, with community workers visiting homes to detect possible reservoirs that facilitate the proliferation of the mosquito. Since 1987, DENV infection has been an important health problem in Bahia (7). In March 2011, the public health authorities in Salvador, Bahia, registered the reappearance of DENV-4 in the country for the first time in 30 years (8). Interestingly, DENV-4 isolates in Salvador are of genotype I, which is derived from Asiatic lines (GenBank JX523699, JX523700, JX523701, and JX5023702) that had not been detected previously in Brazil.

With the increasing incidence of DENV infection, early diagnosis principally during the acute phase of the illness will be a public health priority in Brazil (9,10). Routine diagnostic test for DENV are based on the detection of NS1 viral protein and serum anti-DENV IgM/IgG (11,12). NS1 is a highly conserved protein present in high concentrations in serum from days 1–9 after the onset of fever in infected or reinfected patients (13). During primary viral infection, IgM becomes detectable on days 3–5 after the onset of illness and persists for up to 3 months. IgG appears during the second week of infection and persists for life (14–16). The detection of viral nucleic acid, the gold standard test, can be used to identify circulating serotypes (17). However, this test is not used in routine diagnoses. The aim of this study is to report the appearance of the new DENV-4 serotype circulating in the city of Salvador during 2011–2012 and to describe the serological profile of infected patients based on the detection of NS1 and anti-DENV IgM/IgG.

MATERIALS AND METHODS

Patient serum samples: During 2011–2012, clinicians from Aliança Hospital (Salvador, Bahia) collected sera from individuals (n = 214) with suspected cases of DF.
(exhibiting fever and 2 of the following symptoms: frontal headache, retro-orbital pain, myalgias, arthralgias, hemorrhagic manifestations, or rash) (1). The collected samples were stored at ~70°C until laboratory processing. This research study was approved by the Ethics Committee for Human Research of Salvador University under protocol number n° 04.10.49 FR: 270807.

RNA extraction: The RNA was extracted from 140 μL of serum using the QIAamp viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

DENV RNA detection by reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR: RNA (8 μL) was reverse transcribed (RT) using the SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA), and viral serotyping was performed as reported by Lanciotti et al. (17). Briefly, cDNA obtained by RT was amplified using the D1 forward (5'-TCAATATGCTGAAACGGCAGAAAC CG-3') and D2 reverse (5'-TGACCAACAGCTCAAT GTCTTCAGGTTC-3') primers in PCR Super Mix (Invitrogen). The cycling conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. cDNA obtained (5 μL) was used for an additional round (nested PCR) of 95°C for 2 min and 20 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min of amplification using the D1 primer and type-specific internal primers for the C-PrM genomic junction region as follows: TS1-DENV-1 reverse, 5'-CGTCA TCGTGGTCCGGGGG-3'; TS2-DENV-2 reverse, 5' CGGCACAAGGCCATGAACAG-3'; TS3-DENV-3 reverse, 5'-TAACATCATCATGAGACAGACG-3'; TS4-DENV-4 reverse, 5'-CTCTTGTTCTCTTAAACAA GAGA-3'. Amplified products of 482 nt (DENV-1), 119 nt (DENV-2), 290 nt (DENV-3), and 392 nt (DENV-4) were analyzed on ethidium bromide-stained 2% agarose gels.

Detection of NS1 antigen, IgM, and IgG: The sera were tested for NS1 protein and anti-DENV IgM/IgG according to the manufacturer’s instructions using Dengue Duo Test Bioeasy (Bioeasy Diagnostica, Belo Horizonte, MG, Brazil).

Statistical analysis: Sensitivity and specificity values were calculated using RT-PCR as the reference method. The agreement between the tests was calculated according to the Kappa index (18).

RESULTS

Cohort age distribution: The age distribution of included patients (n = 214) is presented in Fig. 1A. This graph shows that 61% of patients were 25–65 years of age. Children and adolescents represented 29% of patients. Unfortunately, the age of a small number of patients was not registered (8%, 17/214).

Detection of anti-DENV IgM antibodies, anti-DENV IgG antibodies, and viral protein NS1: All 214 samples collected between May 2011 and May 2012 were subject- ed to the immuno-chromatographic Dengue Duo Test. Of these samples, 183 (85.5%) were positive and 31 (14.5%) were negative. Fig. 1B shows the distribution of the positive samples according to the 3 serological markers of the test: 147 were positive for viral protein NS1, 54 were positive for anti-DENV IgM, and 32 were positive for anti-DENV IgG. The intersections of the graph represent the simultaneous detection of the serological markers and demonstrate that 11 samples tested positive for all 3 serological markers. Fig. 1B also highlights the high number of samples positive for anti-DENV IgM antibodies (n = 54). However, the presence of anti-DENV IgG antibodies (n = 32) was low compared with that of anti-DENV IgM antibodies and was always combined with one or both of the other markers.

Detection and identification of DENV serotypes: Serum samples were analyzed for the viral detection using RT-PCR and nested PCR. Of 214 samples analyzed, 43.4% (n = 93) were DENV-positive and 56.6% (n = 121) were DENV-negative. Of positive samples, serotyping revealed that 82.8% (n = 77) were DENV-4, 14% (n = 13) were DENV-2, and 3.2% (n = 3) were DENV-3.

Comparison of viral detection between assays based on NS1 protein and viral genome: Of 214 samples analyzed, 78 samples were positive for both tests, and 52 samples were negative for both tests. However, RT-PCR did not detect DENV in 69 samples that were positive for NS1. Considering that RT-PCR is the gold standard diagnostic test, statistical analysis demonstrated that the detection of NS1 by the Dengue Duo Test had a sensitivity of 53%, a specificity of 77%, and a positive predictive value of 83.3% compared with viral identification by RT-PCR. The Kappa index revealed a reasonable agreement (κ, 0.254) between the 2 tests.

Simultaneous detection of viral protein NS1, anti-DENV IgM/IgG antibodies, and viral genome by RT-PCR: Table 1 compares the results for virus detection using the 3 serological markers (viral protein NS1, anti-DENV IgM/IgG antibodies) and viral genome detection by RT-PCR. Of 214 samples, 193 were positive for either a serologic maker or viral genome by RT-PCR. Twenty-one samples were negative for all tests. It is noteworthy that 118 samples (118/214) were positive for viral protein NS1 but negative for anti-DENV antibodies. Ninety-nine of these 118 samples (83.8%) were from patients older than 25 years of age (see also Table 2). Another finding reported in Table 1 is the high number of individuals (n = 33) who tested positive only for anti-DENV IgM (predominantly children aged 0–14 [14/33]) (Table 2) or who tested positive for anti-DENV IgM and IgG (n = 20). The presence of anti-DENV IgG alone was detected in a small number of patients (n = 12). Table 2 shows these test results in relation to the patients’ ages. Of patients aged 41–65 years, 42% had...
positive results by RT-PCR (39/93), of which DENV-4 was the most frequently detected serotype (89.7%, 35/39 positive cases). Patients aged 25–40 years had the second highest rate of positive results by RT-PCR. This age group had 19.4% of positive cases (18/93), of which DENV-4 was the only serotype detected (100%; 18/18 positive cases). In these 2 age groups, the detection of anti-DENV IgM or IgG antibodies was low. DENV-2 was the most frequently detected serotype in children and adolescents (77.7%; 7/9 positive cases).

**Serological profiles of individuals with only anti-DENV IgG antibodies:** A significant percentage of patients with only anti-DENV IgG antibodies were between the ages of 46 and 63 years (66.66%; 8/12) (Table 3). Many of these patients were also NS1-positive (75%; 9/12). This profile is compatible with the acute phase of a secondary infection or reinfection. We also noted that 3 patients were positive for both DENV-4 and NS1 pro-
The sensitivity of the test used in the hospital was fundamental to the early diagnosis of DENV infection in these patients. Our data confirm some researchers have demonstrated regarding this immunochromatographic test that it has greater sensitivity for the detection of antigen NS1 than do other tests based on enzyme linked immunosorbent assay (ELISA) methods (11,12).

The immune response of patients infected with DENV is easily monitored with the commercially available serological tests. Anti-DENV IgM appears on days 3-5 after the onset of fever, immediately followed by the presence of anti-DENV IgG (14,15). The detection of viral protein NS1 is inversely associated with the presence of antibodies because the formation of immunocomplexes lowers the circulation NS1 in the blood (21,22). Therefore, the presence of antibodies makes it difficult to detect NS1. This phenomenon was clearly observed in our study patients. We observed an inverse relationship between the detection of NS1 protein and anti-DENV antibodies.

The immunochromatographic test used in this study showed that the presence of anti-DENV IgM was greater than the presence of anti-DENV IgG, the latter being an immunoglobulin with little relevance in patients studied, as it is an indicator of recurrent infection/convalescence. This profile (high occurrence of DENV-4, increased anti-DENV IgM, low anti-DENV IgG) reinforced the hypothesis that individuals in the acute phase of the disease were undergoing their first exposure to the virus (19,23,24). This response profile was detected in the vast majority of patients in this study.

Secondary infections or reinfections are characterized by a transient IgM and IgG response and a rapid decrease in the detection of the circulating virus or NS1 (13,15). This response profile was observed in a group of patients with the presence of anti-DENV IgG alone. In these individuals, the detection of NS1 antigen and/or the virus in the serum suggested a secondary infection during the acute phase. These results confirm findings from secondary DENV infections demonstrating that anti-DENV IgG is higher than that in primary cases, particularly in the early stages of the illness (16,25-28). We also show that RT-PCR is a reliable test for detecting secondary DENV infections when serum samples are collected in the febrile period during the early, acute phase of infection. The probability of reinfection in a city with the endemic virus increases with the age of the individual. Interestingly, 75% of patients in the group with secondary DENV infections were between 40 and 63 years of age. These data contrast with the observation that 50% of patients with anti-DENV IgM antibodies alone were children and adolescents aged 14 years and below. This finding demonstrates that in an endemic population, exposure to the virus begins in early childhood (19,24).

Our findings show that DENV infection is a great challenge for the Brazilian public health authorities despite a well-established Aedes mosquito control program. Other factors must be involved in the emergence or re-emergence of this illness, such as population growth, increased tourism, migration, and international commerce, particularly in a heavily visited city such as Salvador. The introduction of the new serotype

<table>
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<th>Sample IgG + (Age)</th>
<th>DENV&lt;sup&gt;1&lt;/sup&gt; serotype</th>
<th>NS1&lt;sup&gt;2&lt;/sup&gt; detection</th>
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</thead>
<tbody>
<tr>
<td>1 (59)</td>
<td>DENV-4</td>
<td>Positive</td>
</tr>
<tr>
<td>2 (18)</td>
<td>DENV-4</td>
<td>Positive</td>
</tr>
<tr>
<td>3 (58)</td>
<td>DENV-4</td>
<td>Positive</td>
</tr>
<tr>
<td>4 (20)</td>
<td>DENV-2</td>
<td>Negative</td>
</tr>
<tr>
<td>5 (56)</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>6 (52)</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
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<tr>
<td>8 (48)</td>
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<tr>
<td>9 (63)</td>
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<td>10 (52)</td>
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<tr>
<td>11 (13)</td>
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<td>12 (46)</td>
<td>Negative</td>
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<sup>1</sup>: Viral detection by RT-PCR.
<sup>2</sup>: Dengue Duo Test Bioeasy.

Table 3. Detailed analysis of patients with only anti-DENV IgG antibodies
DENGUE-4 in the endemic population of Salvador distinguishes this city from other areas in Brazil, as all 4 serotypes are circulating. With the spread of all 4 serotypes, the need for rapid, early diagnosis increases, as severe DF cases requiring early clinical treatment will be seen more frequently. Our study demonstrates that a rapid test with high sensitivity (such as the immunochromatographic Dengue Duo Test) that simultaneously detects the serologic markers NS1 and anti-DENV IgM/IgG would be of great utility for early detection and rapid treatment of DENV infection.

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Conflict of interest None to declare.

REFERENCES