Characterization of a Dengue Patient Cohort in Recife, Brazil

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Abstract. From 2004 to 2006, 658 patients with suspected dengue virus infections were enrolled in a clinical dengue cohort established in Recife, Pernambuco, located at the northeastern region of Brazil. A total of 2,364 blood samples were collected, and serum, plasma, and cells were cryopreserved. Among the suspected cases, 354 (54%) were confirmed as acute DENV-3 infection based on reverse transcription-polymerase chain reaction, virus isolation, and ELISA-IgM. According to WHO criteria, 29.4% of the positive acute cases were classified as dengue fever (DF) and 8.2% of the cases as dengue hemorrhagic fever (DHF), grade 1 or 2. The DHF cases represent 100% of those confirmed in Recife during the period of the study. The dengue cases that did not fulfill the definition of either DHF or DF were classified as DF complicated and accounted for 44.0% of the cases. All the acute cases were classified as either primary or secondary acute dengue virus infections. Secondary infection was predominant in patients with DF; however, there was no predominance of either primary or secondary infections in patients with DHF.

INTRODUCTION

Dengue virus (DENV) infection in humans causes a wide spectrum of illness, ranging from mild sub-clinical disease to a severe and occasionally fatal hemorrhagic form. In general, the initial dengue virus infection is moderately severe in adults and milder in children. Severe complications of the disease, such as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), are mainly associated with sequential dengue infection. However, several other studies have suggested that host factors and viral strain virulence should be considered as potential risk factors.

Thanks to the efforts to combat yellow fever during the first half of the 20th century, Brazil was free of the dengue mosquito vector Aedes aegypti in urban areas until 1976. However, a rapid and disorganized urban growth, associated with a lack of maintenance of the mosquito combat program, has allowed the resurgence of mosquito vectors in urban areas. Thus, after DENV entry in 1986 (serotype 1) and in 1990 (serotype 2), dengue fever outbreaks that occurred in Brazil were caused by epidemic waves of DENV-1 and DENV-2 serotypes (1986–1999) in specific regions. After DENV-3 entry (2000–2006), dengue became endemic throughout the country.

According to the Brazilian Ministry of Health (2007), from 1986 to 2006, > 4 million dengue cases had been reported in the country; 1.8 million of them were reported in the northeastern region. The first cases of DHF in Brazil were confirmed in 1990 after DENV-2 entry and, since then, both the number of DHF cases and the mortality caused by this disease have increased. Factors that have contributed to the current situation in Brazil include an increased proportion of lower-income residents in urban areas, a high household density, and deficiencies in the water supply and garbage collection. The northeastern region of Brazil is the second-most populous region, with 28% of the total Brazilian population, and accounted for almost 50% of the reported dengue cases in the country. The state of Pernambuco, the second most populous state in this region (16.5% of all inhabitants), was responsible for 21% of the dengue cases reported in the northeast region.

In 2002, the introduction of DENV-3 led to the largest dengue epidemic in Pernambuco, with 120,000 cases reported (an incidence rate of 1,457 cases/100,000 inhabitants). In 2003, the number of dengue cases dropped dramatically, likely as a result of herd immunity, signifying a brief inter-epidemic period. DENV serotypes 1, 2, and 3 have been circulating in Pernambuco since 2002, and from 2004 to 2006, ~36,000 cases were reported. In Recife, the capital of Pernambuco and one of the largest urban areas in the northeast, 1,690 dengue fever cases and 29 DHF cases were confirmed in the past 3 years. Here we describe a dengue cohort in Recife, Pernambuco, Brazil, that has been in active development since 2004. This cohort has been the subject of clinical studies that have been carried out to examine several aspects of dengue pathogenesis and support the rational development of prophylactic strategies and tools for improving the diagnosis/prognosis of the disease.

MATERIALS AND METHODS

Cohort design and study population. Patients, ≥ 5 years of age, with suspected dengue fever who were admitted to one of three hospitals in the city of Recife, in Pernambuco, Brazil (the Hospital Esperança, Hospital Santa Joana and Instituto Materno Infantil [IMIP]), were invited to participate in this study. In this ongoing study, clinical and laboratory evaluations were performed on the first and fourth days of patient enrollment, with additional examinations when necessary. Laboratory examinations include hemoglobin, hematocrit, white blood cell count, differential leukocytes count, platelet count, serum albumin, serum aspartate transaminase, and serum alanine transaminase. Clinical and laboratory data and blood samples from patients were collected at the time of the first medical appointment and they were followed, with blood sample collection, for a period of 5 years.

Ethical considerations. Written consent to participate in the study was obtained from each subject (or the guardian of the patient) enrolled in the dengue cohort, after a full expla-
nation of the study proposed. All data were handled confidentially and anonymously. This study was reviewed and approved by ethics committee of the Brazilian Ministry of Health, number, CONEP: 4909, Process 25000.119007/2002-03; CEP: 68/02. It was also reviewed by the Johns Hopkins Institutional Review Board as protocol JHIM-IRB-3: 03-08-27-01.

**Blood sample collection.** Sequential blood samples were obtained from 658 patients enrolled in the dengue cohort, totaling 2,364 samples. From each patient, up to four blood samples were collected within 30 days from the start of the symptoms. Normally, at Days 1, 3, 7, and 15 after enrollment into the study; two additional samples were obtained 6 and 12 months later. From then on, blood samples were collected every year; this annual collection of samples will be continued for the remainder of the 5-year study. Disease Day 1 was the day of onset of symptoms (usually fever), as reported by the patient. Blood samples were collected into 10-mL Vacutainer tubes. Serum samples were separated and stored into two cryovials (1 mL per tube) at −80°C and −20°C for virus isolation and serology, respectively. Plasma and peripheral blood mononuclear cells (PBMCs) were also separated and cryopreserved for further studies.

**Database.** The study data were integrated into a customized digital database that includes complete clinical data, research results, and the respective inventories of cryopreserved samples of PBMCs, plasma, and serum.19

**Virus isolation and identification.** A total of 658 serum samples obtained during the acute phase of the disease were processed for virus isolation. Dengue virus was isolated by inoculating 20 μL of serum samples onto monolayer of C6/36 cells,20 maintained in Leibovitz L-15 medium (GIBCO, Invitrogen, Grand Island, NY) containing 2% fetal calf serum. Virus was identified by immunofluorescence test,21 with serotype-specific anti-dengue monoclonal antibodies (Bio-Manguinhos, Fundação Oswaldo Cruz, Brazil).

**Reverse transcriptase-polymerase chain reaction.** Viral RNA was extracted from 658 serum samples using QIAquick polymerase chain reaction (PCR) purification kits (Qiagen, Valencia, CA). A two-step nested reverse transcriptase (RT)-PCR was carried out on all first serum samples.22 In brief, cDNA copies of a portion of the viral genome (capsid/prM) were synthesized and amplified using two consensus primers designed to anneal to the four dengue virus serotypes. A second round of amplification was carried out using dengue serotype-specific primers, and the amplification products produced DNA with molecular sizes specific to each of the four DENV serotypes.22 PCR products were resolved by 2% (wt/vol) agarose gel electrophoresis and stained with ethidium bromide. Negative and positive controls were included in all steps. An internal PCR control containing 10^5 cDNA copies/mL of each of the serotypes was also included to determine reaction efficiency and sensitivity.

**Serology.** A total of 2,364 serum samples were analyzed to detect DENV-specific IgM and IgG antibodies. Anti-dengue IgM-capture ELISA (Bio-Manguinhos or PanBio, Brisbane, Australia) was performed according to manufacturer’s instructions. Results were interpreted as negative or positive according to the assay manual. Anti-dengue IgG indirect ELISA (PanBio) was performed following the recommended guidelines. Results were calculated and interpreted as negative or positive according to the manufacturer’s instructions. The hemagglutination inhibition (HI) assay23 modified to a microtiter plate format was performed on samples of all the 29 DHF cases recruited. HI antibodies against DENV serotypes 1–4 and yellow fever virus were measured in paired serum samples collected in the acute and convalescent phase of disease. Antigens used in the HI assay were provided by the Evandro Chagas Institute, Belém (Pará), Brazil. A 4-fold increase or greater in antibody titer was considered positive for acute infection. The dengue-immunological history of the DHF patients were also classified using the HI assays according to WHO criteria as follows: cases with no HI antibodies titers (< 1:20) in acute phase serum, obtained before the fourth day of illness, and convalescent phase serum samples with an HI titer < 1:1280, were classified as primary infections; and when the HI antibodies titers were > 1:20 in the acute phase serum samples and the convalescent HI antibody titer > 1:2,560 infections were considered secondary dengue infections.24

**Classification of primary and secondary serologic response.** Dengue cases were laboratory-confirmed by DENV isolation and/or viral RNA detection by RT-PCR and/or by a positive anti-dengue IgM ELISA. Primary infection was characterized by the absence of dengue-specific IgG antibodies in the acute serum sample and presence of anti-dengue IgM, virus isolation, and/or viral RNA detection, followed by the presence of anti-dengue IgG in convalescent serum samples. Secondary infection was characterized by detection of specific anti-dengue IgG in the acute sample and the absence of anti-dengue IgM, associated with a positive RT-PCR and/or virus isolation; followed by the presence of anti-dengue IgM in convalescent serum samples. With the exception of a few secondary cases, confirmed by virus isolation and/or RT-PCR, anti-dengue IgM was present in all convalescent serum samples. All dengue cases were classified according to these criteria. The HI assay results were also used to confirm the classification of the 29 DHF cases. There was a good agreement in both methods used for immune response characterization.

**Clinical definitions of laboratory-confirmed DENV infection.** Dengue cases were classified, following the WHO Guidelines24 on dengue fever, characterized by a high fever, accompanied by at least two of the associated symptoms: severe headache, retro-orbital pain, myalgia, arthralgia, and rash, and DHF, which were defined by the same clinical manifestations as for DF, but with hemorrhagic manifestations, including positive tourniquet test, thrombocytopenia (platelet count < 100,000/mm^3), hemoconcentration, or other sign of plasma leakage.24 An additional classification was designated “DF complicated” (DFC), when classic dengue fever presented with hemorrhagic manifestations, and thrombocytopenia (< 100,000/mm^3), but the clinical/laboratorial parameters did not fulfill the WHO criteria for DHF. This third group classification has importance in other ongoing dengue cohort studies.

**Statistical analysis.** Statistical analysis was performed with Stat Pac Software for Windows (version 10.2; Stat Pac, Bloomington, MN) and Microsoft Office Excel, version 3.0 (Microsoft, Redmond, WA). χ^2 tests were used for proportions, with statistical significance set at P < 0.05.

**RESULTS**

**Volunteer recruitment.** This dengue cohort was first established in February 2004. The recruitment of volunteers for
this study was dependent on the number of suspected dengue cases identified at the three hospitals participating in this study. In 2004, the main period in which confirmed cases occurred was from March to June; in 2005, from May to August; and in 2006, April and May. During the first 3 years of the project (2004–2006), 658 individuals suspected of dengue virus infection were enrolled, and enrollment is expected to continue until 2008. A FileMaker-based database has been programmed and hosted at the Laboratory of Virology and Experimental Therapy/CPqAM and has been used for cohort management and patient/sample tracking. A limited version of the cohort database, compliant with the ethical requirements, is accessible through the Internet by request of a password (http://augustlab.bs.jhmi.edu/index.html).

A total of 2,364 blood samples from the 658 volunteers have been collected thus far; serum, plasma, and PBMC samples are stored. The number of PBMC samples (4 × 10^6 cells per vial) cryopreserved is ~9,000 vials. The age of the volunteers ranged from 5 to 86 years, with 348 (52.9%) female and 310 (47.1%) male individuals (Table 1). On average, the patients of our cohort sought medical assistance 5.3 days after the onset of the symptoms. The first samples were collected at the first medical visit, which was the day of enrollment. All first serum samples collected from the 658 volunteers were analyzed by RT-PCR, virus isolation, and serology (IgM and IgG). Anti-dengue IgM and anti-dengue IgG tests were performed for all the subsequent samples. Of the 658 volunteers, 354 subjects were judged to have acute dengue virus infection were enrolled, and enrollment is expected to continue until 2008. A FileMaker-based database has been programmed and hosted at the Laboratory of Virology and Experimental Therapy/CPqAM and has been used for cohort management and patient/sample tracking. A limited version of the cohort database, compliant with the ethical requirements, is accessible through the Internet by request of a password (http://augustlab.bs.jhmi.edu/index.html).

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**Serology.** Anti-dengue IgM and IgG detection was performed for all 2,364 serum samples collected. Among 658 serum samples obtained on the admission day (first samples), anti-dengue IgM was detected in 177 samples (26.9%). The average day of IgM detection was Day 5.3. In subsequent samples, IgM was detected in another 125 cases, resulting in a total of 302 IgM-positive cases. Among the 354 laboratory-positive cases, 46.6% (165/354) were confirmed exclusively by anti-dengue IgM detection, and in 52 cases (14.7%), anti-dengue IgM was not detected.

Anti-dengue IgG assay performed on the first serum samples indicated that 374 cases (56.8%) were positive for IgG, whereas 284 (43.2%) were negative. Of the 374 suspected dengue cases with an IgG-positive assay, 179 cases (47.9%) were further confirmed as acutely infected with dengue on the basis of anti-dengue IgM and/or RT-PCR positivity in subsequent samples. Thus, these cases were characterized as secondary (sequential) infections. Of the 284 cases with an IgG-negative first sample, 175 cases (61.6%) became IgG-positive in a subsequent sample and thus were characterized as primary dengue infections. The average time at which IgG became positive was 5.4 days (approximately 11 days after the onset of the symptoms) after the first blood sample collected, which was at enrollment day.

**RT-PCR and virus isolation.** RT-PCR and virus isolation assays were performed on all 658 samples obtained on admission; all subsequent samples were analyzed for IgM and IgG ELISA. Of the 354 laboratory-positive samples, 53.4% (189/354) were confirmed by RT-PCR: 81.5% (154/189) of these RT-PCR-positive samples were collected by Day 5 from the onset of symptoms, and only 18.5% (35/189) were collected from Day 6 to Day 9. DENV-3 was the serotype identified in all the confirmed acute dengue infections during this period. This virus was isolated from 74/354 serum samples (21%) before Day 6 and 9.5% from Day 6 to Day 8. No other dengue virus serotype was isolated or appeared in the PCR assays. It was not possible to isolate virus from 115 samples of the cases that were positive by RT-PCR; however, all of these samples were either IgM-positive or have seroconverted to IgG. Thus, in this analysis, RT-PCR was 2.5 times more sens-

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Confirmed/suspected (N = 354/658) (% positive)</th>
<th>Type response (% positive)</th>
<th>Clinical form (N = 354)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>P</td>
<td>S</td>
<td>DF (N = 104)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>DFC (N = 156)</td>
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<td></td>
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<td>DHF (N = 29)</td>
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<td></td>
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<td>D (N = 65)</td>
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<tr>
<td>5–9</td>
<td>56/106 (53)</td>
<td>38 (68)</td>
<td>29</td>
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<tr>
<td>10–14</td>
<td>38/69 (55)</td>
<td>22 (58)</td>
<td>13</td>
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<tr>
<td>15–24</td>
<td>41/91 (45)</td>
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<td>9</td>
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<tr>
<td>25–34</td>
<td>71/135 (52)</td>
<td>32 (45)</td>
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<td>35–44</td>
<td>69/113 (61)</td>
<td>35 (51)</td>
<td>15</td>
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<tr>
<td>45–54</td>
<td>43/82 (52)</td>
<td>13 (30)</td>
<td>16</td>
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<tr>
<td>≥ 55</td>
<td>36/62 (58)</td>
<td>8 (22)</td>
<td>6</td>
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<tr>
<td>Total</td>
<td>354/658 (54)</td>
<td>175 (49)</td>
<td>104</td>
</tr>
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| P, primary infection; S, secondary infection; DF, dengue fever; DFC, dengue fever complicated (platelet count < 100,000 mm^3); DHF, dengue hemorrhagic fever; D, dengue case (not clinically classified).

<table>
<thead>
<tr>
<th>Assays</th>
<th>Negative (%) (N = 177)</th>
<th>Positive (%) (N = 177)</th>
<th>Total (%) (N = 354)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolation</td>
<td>Negative 116 (65.5)</td>
<td>164 (92.7)</td>
<td>280 (79.1)</td>
</tr>
<tr>
<td></td>
<td>Positive 61 (34.5)</td>
<td>13 (7.3)</td>
<td>74 (20.9)</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Negative 42 (23.7)</td>
<td>123 (69.5)</td>
<td>165 (46.6)</td>
</tr>
<tr>
<td></td>
<td>Positive 135 (76.3)</td>
<td>54 (30.5)</td>
<td>189 (53.4)</td>
</tr>
<tr>
<td>IgM (ELISA)</td>
<td>Negative 80 (45.2)</td>
<td>81 (45.8)</td>
<td>161 (45.5)</td>
</tr>
<tr>
<td></td>
<td>Positive 97 (54.8)</td>
<td>96 (54.2)</td>
<td>193 (54.5)</td>
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<tr>
<th>IgM detection after second or later samples</th>
<th>Total cases (N = 354)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM (ELISA)</td>
<td>52 (14.7)</td>
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</table>
sitive than virus isolation and is the ideal diagnostic method for the first 5 days of symptoms.

**Laboratory-positive dengue cases.** A total of 1,353 blood samples were collected from the cases with laboratory-positive acute dengue infection. Among the 354 positive subjects, 183 (51.7%) were women and 171 (48.3%) were men. The age of the study subjects ranged from 5 to 86 years, with a median age of 30.7 years (SD, 17.9 years); 26.6% of them were <15 years old and 73.4% were ≥15 years of age. During the study period, primary and secondary dengue infection rates were 49.4% (175/354) and 50.6% (179/354), respectively (Table 1). For the cases with primary dengue infection, the average age was 25.6 ± 16 years, ranging from 5 to 78 years. For those with secondary infection, the average age was 35.6 ± 18 years, ranging from 5 to 84 years. The modal ages in primary and secondary infection cases were 6 and 48 years of age, respectively. Among 5- to 9-year-old children, 68% (38/56) of dengue infections were primary, and only 32% (18/56) were secondary (Table 1); this difference was statistically significant (OR, 4.46; 95% CI, 2.05–9.67; P < 0.001 by χ² test). Among subjects <15 years of age, the majority (63.8%) of the acute dengue infections had a serologic primary response (OR, 3.11; 95% CI, 1.73–5.66; P < 0.001); 36.2% had a secondary response in this age group. However, among volunteers ≥15 years old, there was no significant difference in the frequencies of the two types of serologic responses: 44.2% (115/260) were primary infections and 55.8% (145/260) were secondary infections. Among all the positive volunteers, there was no significant difference between the number of men and women for either primary or secondary infections.

**Clinical forms of disease: DF, DHF, and DFC.** Based on clinical presentation of symptoms and laboratory records, 29.4% (104/354) of positive cases were classified as DF and 8.2% (29/354) as DHF, according to the WHO guidelines, with 16 being grade 1 and 13 grade 2 (Table 1). (A table with the serologic exams of the DHF patients is provided online in the supplemental material including the HI test). The other 156 cases (44.0%) did not fulfill the DHF criteria established based on classification adopted by the Brazilian Ministry of Health. The average age of DHF cases was 38 ± 19 years and ranged from 10 to 84 years; the modal age was 21 years. Sixty-five laboratory-positive dengue cases (18.4%) have not yet been clinically classified because complementary tests are still underway. Among those with DF, there were significantly more cases (62 cases, 59.6%) with secondary infections than with primary infections (42 cases, 40.4%; OR, 2.00; 95% CI, 1.14–3.51; P = 0.016). For DHF, there were 16 cases (55.2%) of primary infection and 13 cases (44.8%) of secondary infection; this difference was not statistically significant (OR, 1.28; 95% CI, 0.6–2.7; P = 0.519). For DFC, there were 72 cases (46.2%) of primary infection and 84 cases (53.8%) of secondary infection; again, this difference was not statistically significant (OR, 1.26; 95% CI, 0.83–1.92; P = 0.273). With regard to DF and DFC, the sex distribution was essentially equal: of those with DF, 54 (52%) were women and 50 (48%) were men; for DFC, 73 (46.8%) were women and 83 (53.2%) men. However, for DHF, there was a significantly higher number of women (22 cases, 75.9%) than men (7 cases, 24.1%; OR, 3.20; 95% CI, 1.38–7.40; P = 0.0007).

**DISCUSSION**

For the last 20 years, dengue fever has been considered a serious public health, environmental, and economic problem in Brazil as has been recognized in many other South American countries. The area of Brazil that is represented by our cohort is one of the most severely affected regions of the country. The northeastern region of Brazil accounted for almost one half of the reported cases between 2004 and 2006, with the state of Pernambuco being the second most affected.

The city of Recife, Pernambuco’s capital, was the most severely affected area during the dengue epidemics that have occurred in the state of Pernambuco, with an average annual incidence rate of 411.6 ± 179.6 cases/100,000 inhabitants from 1995 to 2001. In 2002, DENV-3 was introduced into the city and the annual incidence rate reached 2,782/100,000 (a total of 39,981 cases), the largest dengue epidemic to date in Pernambuco; in 2003, the number of cases reported in Recife dropped dramatically to 1,208 cases, an incidence rate of 81.2/100,000 inhabitants. From 2004 to 2006, Recife experienced a low endemic period, with an average of 37 cases/100,000 inhabitants. This decline was probably caused by either intensification in the vector control measures or a high prevalence of protective immunity in the population. During this period (2004–2006), 1,690 DF cases and 29 DHF cases were confirmed in Recife.

Thus, the incidence of dengue reported thus far and the presence of three DENV serotypes (DENV-1, DENV-2, and DENV-3) has made Recife a particularly interesting and important place in which to study this disease during an inter-epidemic period. During this study, we observed that the peak of number of confirmed dengue cases has occurred during the rainy season in this region (between April and August). This seasonality has also been observed in other studies in Brazil and Trinidad and is linked to the growth of the mosquito vector population during this season.

Thus far, 658 individuals have enrolled in this study, with a total of 2,364 blood samples collected. Taken together, serology and RT-PCR allowed us to identify 354 dengue-positive cases. Thus, it seems to be critical to use these diagnostic tools together to confirm acute infection with DENV. RT-PCR was extremely important during the first 7 days of symptoms, whereas IgM became a more important diagnostic tool after the first 7 days.

When all 354 positive cases were considered, there was only a small difference in the numbers for the two sexes (51.7% women versus 48.3% men); however, women were more susceptible to DHF. The predominance of female patients suffering the most severe clinical manifestations suggests that women are at relatively higher risk of developing DHF than men (relative risk, 1.53; OR, 3.2). However, these data should be interpreted with caution, given the relatively low number of DHF cases in our cohort. Other epidemiologic studies performed in Brazil, based on the public health care system database, have shown an overall higher incidence of dengue in women, when all clinical forms were considered. The reason for this sex bias is not clear. Lower income has been associated with a higher risk for dengue transmission. Thus, the balanced sex ratio identified in the cohort presented here might reflect the fact that two of the three hospitals from which the dengue patient volunteers have been recruited have
a relatively higher-income client population; therefore, the socio-economic profile of the population in our study differs from that in the studies cited above. It is therefore possible that sex inequalities in medical care are the source of the bias toward increased diagnosis of dengue in women of the lower-income population. The reason for the higher incidence of DHF in women in this cohort needs to be further studied.

Only 26.6% of our cohort population was < 15 years of age. This low apparent rate of infection in patients < 15 years of age has also been reported in other studies in Brazil\textsuperscript{29-31} and likely mirrors the underestimation of dengue cases in children, in whom it is frequently milder and confused with other exanthemtic diseases, according to our preliminary sero-prevalence data obtained in this age range (unpublished data). Similarly, Campagna and others\textsuperscript{27} reported that exanthema was one of the most commons dengue symptoms in a cohort of 71 children 5–12 years old in the state of Mato Grosso do Sul, in the central-western region of Brazil. In contrast, some studies performed in Asia have indicated that children there generally develop the full range of symptoms.\textsuperscript{33–37} One possible reason for this difference is that the co-circulation of the four DENV serotypes at the same time in Asia results in a short period between dengue re-infections in this endemic area,\textsuperscript{38} whereas in Recife during the past 3 years, predominantly only one serotype has been circulating and re-infections have been much more sparse.

ELISA IgG detection during the first days of symptoms was used here to determine whether the acute dengue infections represented a first time exposure to the virus or whether the patients had been exposed to dengue virus before. It was observed that 56.8% of volunteers had antibodies to dengue virus at the time of enrollment. Among the 354 laboratory-confirmed cases, 175 (49.4%) were characterized as primary infections and 179 (50.6%) as secondary infections. Among 5- to 9-year-old children, primary infections accounted for 68% (38/56) of cases, and only 32% (18/56) were secondary infections (OR, 4.46; 95% CI, 2.05–9.67; \(P < 0.001\)). This difference was still significant even for the group of < 15-year-olds: 63.8% with primary infection versus 36.2% with secondary infection (OR, 3.11; 95% CI, 1.73–5.66; \(P < 0.001\)). However, among subjects \(\geq 15\) years old, there was no significant difference between the number of cases with primary (44.2%) and secondary infections (55.8%). Moreover, the sex distribution was close to equal for both primary and secondary infections.

Based on the clinical presentation of symptoms and laboratory data, we were able to classify 104 (29.4%) cases as DF and 29 (8.2%) as DHF, but the other 156 cases (44.0%) were classified as DFC because they did not fulfill the DHF definition according to WHO guidelines,\textsuperscript{24} despite the presence of some of the relevant clinical manifestations (e.g., thrombocytopenia and hemorrhagic manifestations). Duarte and França\textsuperscript{39} have noted that DFC cases represent an important proportion of the confirmed dengue cases reported by the Public Health System in Brazil. Therefore, the emergence of more severe dengue cases that do not fulfill the DHF definition underscores the need for a re-definition of DHF cases by WHO to improve clinical management, as well as support the analysis of the underlying immunopathogenic mechanisms of dengue virus.\textsuperscript{39}

In our cohort, the frequency of primary versus secondary infection was not statistically different for either DHF or DFC. This finding is in contrast to what has been described on epidemiology studies of DHF/DSS, where heterotypic secondary infection and dengue severity have been found to be highly correlated.\textsuperscript{3-5,40} Interestingly, a study performed in Rio de Janeiro, Brazil, during the dengue epidemic in 1990, found an association between secondary infections and DHF in six fatal cases.\textsuperscript{41} However, another study performed on 37 serum specimens from patients who died during the DENV-3 epidemic in Rio de Janeiro classified 20 (54.1%) cases as primary infection, 9 (24.3%) cases as secondary infection, and 8 (21.6%) cases as inconclusive. In addition, among nonfatal DHF cases (\(N = 88\)), 55.7% were classified as primary infection and 44.7% as secondary infection, which corroborate with our findings.\textsuperscript{42} Moreover, another study performed in Latin America, undertaken by Harris and others\textsuperscript{34} in Nicaragua, found that secondary infection was not significantly associated with either DHF/DSS. Interestingly, other studies have shown that DENV-3 causes DHF in primary infections to a greater extent than DENV-2 or DENV-4.\textsuperscript{44} Therefore, the equilibrated rate between primary and secondary infection found in this cohort among patients with DHF suggests that, in this group, some other mechanism, other than antibody-dependent enhancement or sequential infection, might play a role modulating the immune responses in some patients and lead to the development of the most severe form of the disease. Among other reasons, those differences may be caused by the distinctive epidemiologic characteristics of dengue infections in Brazil versus Asia. In Asia, the sequential dengue infections occur in a much shorter period because all four serotypes co-circulate, whereas in Brazil, there were long intervals between the outbreaks of different serotypes.

In Brazil thus far, dengue, and especially DHF, affects most commonly the adult population, whereas in Southeast Asian countries, children are the most frequently affected by the most severe form of the disease.\textsuperscript{13,34} The reasons for this difference are still unknown but may be linked to likely a combination of multiple hosts and epidemiologic and virologic factors. The reason for the presence of low rates of DHF/DSS cases in Brazil still needs to be further studied. We have undertaken several studies with our cohort considering the possibility of a high prevalence of DHF dengue resistance genes in the Brazilian population. Evidences supporting the existence of host DHF resistance genes has also been observed in studies on the Cuban population,\textsuperscript{45} and hypothesis regarding these findings have also been postulated by Halstead.\textsuperscript{46}

Recently, an increased rate of severe cases among children in the Amazon region of Brazil has been reported.\textsuperscript{13} Although it is still early to affirm that the epidemiologic pattern is changing in Brazil, if this intense circulation of DENV persists, Brazil may face a disastrous increase in the number of DHF/DSS cases in children in the near future and start to see a similar pattern of dengue as in Asia. Since 2002, DENV-3 has been the predominant serotype isolated in the city of Recife, as in other cities in Brazil.\textsuperscript{42,47,48} Thus, the current epidemiologic situation in Recife is unique, because the population under study has been exposed to only the DENV-3 serotype and one genotype at this moment. However, if DENV-1, DENV-2, or DENV-4 starts to circulate into this environment, we may observe a great increase in DHF cases in children.

Phylogenetic analysis of isolates from dengue epidemics that occurred in the state of Pernambuco from 1995 to 2003 has been performed. The genomic sequence corresponding to
the DENV-1 and DENV-2 E/NS1 gene regions and DENV-3 prM/E gene region were performed. The genotypes found corresponded to genotype V (America/Africa), genotype III (Southeast Asia/America), and genotype III (Sri Lanka/India) for DENV-1, DENV-2, and DENV-3, respectively (unpublished data). The Brazilian DENV-3 genotype III is similar to the strains currently circulating in the American continent and is related to the strains that caused DHF epidemics in Sri Lanka and India (1989–1991). The DENV-3 genotype III has been identified also in Venezuela and Cuba in the last years. It seems that the same genotypes have been circulating all over the country during the last two decades, with little genetic variation in the virus population. Thus, the variance related to differences in the virulence of circulating viral strains are likely to be minimal, and we can infer that the clinical differences seen in this cohort are likely related to host and epidemiologic factors.

According to the Brazilian Ministry of Health, 1,690 DF cases and 29 DHF cases were confirmed during the past 3 years in Recife. The cohort described here has enrolled 100% of the DF cases and 92.2% of the total DF cases reported in this city, thanks to cooperation between Recife’s Health Secretary and LaVITE/CpqAM/Fiocruz. Thus, the sample bank established from this cohort effectively represents the current status of dengue in Recife and provides a critical resource for the development and validation of diagnostics/prognostic tools for studies of dengue immunopathogenesis and for the ex vivo evaluation of the immunogenicity of candidate vaccines.

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Note: Supplementary table 1: “Laboratory results of dengue hemorrhagic fever cases” and Supplementary table 2: “Hemaglutination inhibition antibodies results from the dengue hemorrhagic fever cases” appear online at www.ajtmh.org.

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