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Increasing Clinical Severity during a Dengue Virus Type 3 Cuban Epidemic: Deep Sequencing of Evolving Viral Populations

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ABSTRACT

During the dengue virus type 3 (DENV-3) epidemic that occurred in Havana in 2001 to 2002, severe disease was associated with the infection sequence DENV-1 followed by DENV-3 (DENV-1/DENV-3), while the sequence DENV-2/DENV-3 was associated with mild/asymptomatic infections. To determine the role of the virus in the increasing severity demonstrated during the epidemic, serum samples collected at different time points were studied. A total of 22 full-length sequences were obtained using a deep-sequencing approach. Bayesian phylogenetic analysis of consensus sequences revealed that two DENV-3 lineages were circulating in Havana at that time, both grouped within genotype III. The predominant lineage is closely related to Peruvian and Ecuadorian strains, while the minor lineage is related to Venezuelan strains. According to consensus sequences, relatively few nonsynonymous mutations were observed; only one was fixed during the epidemic at position 4380 in the NS2B gene. Intrahost genetic analysis indicated that a significant minor population was selected and became predominant toward the end of the epidemic. In conclusion, greater variability was detected during the epidemic’s progression in terms of significant minority variants, particularly in the nonstructural genes. An increasing trend of genetic diversity toward the end of the epidemic was observed only for synonymous variant allele rates, with higher variability in secondary cases. Remarkably, significant intrahost genetic variation was demonstrated within the same patient during the course of secondary infection with DENV-1/DENV-3, including changes in the structural proteins premembrane (PrM) and envelope (E). Therefore, the dynamic of evolving viral populations in the context of heterotypic antibodies could be related to the increasing clinical severity observed during the epidemic.

IMPORTANCE

Based on the evidence that DENV fitness is context dependent, our research has focused on the study of viral factors associated with intraepidemic increasing severity in a unique epidemiological setting. Here, we investigated the intrahost genetic diversity in acute human samples collected at different time points during the DENV-3 epidemic that occurred in Cuba in 2001 to 2002 using a deep-sequencing approach. We concluded that greater variability in significant minor populations occurred as the epidemic progressed, particularly in the nonstructural genes, with higher variability observed in secondary infection cases. Remarkably, for the first time significant intrahost genetic variation was demonstrated within the same patient during the course of secondary infection with DENV-1/DENV-3, including changes in structural proteins. These findings indicate that high-resolution approaches are needed to unravel molecular mechanisms involved in dengue pathogenesis.

Dengue viruses (DENVs) cause the most important arthropod-borne viral disease in humans, with latest estimates of 390 million dengue infections per year, of which 96 million manifest some level of disease severity (1). This figure is more than three times the dengue burden estimate of the World Health Organization (2). Latin America has progressively evolved a region with low dengue endemicity to a region of hyperendemicity, with local transmission of the four dengue virus serotypes (DENV 1 to 4) in practically all countries (3). DENVs are assigned to the genus Flavivirus in the family Flaviviridae. The genomes of flaviviruses comprise a single-stranded RNA molecule encoding three structural proteins, the capsid (C), premembrane/membrane (PrM/M), and envelope (E), and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (4). Infection with any DENV serotype may present as an asymptomatic infection or as a symptomatic infection ranging from mild to severe illness: dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (5) or dengue and severe dengue, according to the new dengue case classification (2).

The etiology of DHF/DSS has been the subject of research for...
over 40 years (6, 7). In Cuba, secondary infection has been implicated as the most relevant risk factor to developing severe disease, combined with the introduction of DENV strains of Asian origin. Likewise, other risk factors, including age, genetic background, and chronic diseases, like bronchial asthma, sickle cell anemia, and diabetes, have also been implicated in dengue severity (8, 9). Notably, during two severe epidemics (1981 and 1997) characterized by the circulation of only one serotype (DENV-2), 4 and 20 years, respectively, after the massive DENV-1 epidemic, a marked month-to-month increase in clinical severity was observed in secondary infection cases (8, 10, 11).

In June 2001, the nationwide dengue case surveillance system identified a DENV-3 outbreak in Havana City, which eventually involved 12,889 confirmed cases, including 78 DHF/DSS and identified a DENV-3 outbreak in Havana City, which eventually involved 12,889 confirmed cases, including 78 DHF/DSS and three fatalities (12). Here, we report that significant monthly increases in the proportion of DHF/DSS cases also occurred during this epidemic, 24 years after the DENV-1 epidemic and 20 years after one caused by DENV-2. Since the epidemic was circumscribed to Havana City, which has an admixed population with homogeneous ethnic composition, and since all severe cases were in adults, the hypothesis related to the outbreak moving into different regions with different population demographics to explain the increasing severity was refuted. Likewise, host factors did not appear to explain this increase because it is not logical to assume that the most susceptible individuals would all be infected toward the end of the epidemic.

However, the theory that cross-immunity could play a significant role in shaping viral population diversity, selecting for more fit viruses toward the end of the epidemics that produce severe dengue, is plausible. Previous studies in this particular context have suggested that the phenomenon of increasing clinical severity with the epidemic’s progression could be related to temporary changes that occur in the virus causing the epidemic (10, 13, 14). In this regard, little is known about the extent of intrahost genetic diversity of DENVs in relation to immune status and their implications for dengue pathogenesis and disease severity. Initial studies using cloning techniques demonstrated higher levels of DENV-3 intrahost genetic diversity in patients than in mosquitoes (15). In addition, the extent and pattern of DENV-1 diversity during acute infection were related to disease outcome (16), while no relationship was found for the same serotype in a different scenario (17). Later on, studies using deep-sequencing approaches, although in different epidemiological contexts, have shown low genetic diversity in humans (18, 19). Most recent papers on dengue intrahost diversity have been focused on viral population variations during human/mosquito host switching (20–22).

In the present study, we explored intrahost DENV-3 genetic diversity in serum samples collected at different time points during the 2001-2002 Cuban epidemic, using whole-genome amplification and next-generation sequencing to determine the role of the virus in increasing disease severity. Our results demonstrate that changes in the viral population occurring with the epidemic’s progression could have an impact on viral fitness.

MATERIALS AND METHODS

Epidemic characterization in terms of dengue severity. The proportion of DHF/DSS per dengue case was compared every four epidemiological weeks as an expression of increasing clinical severity, based on confirmed dengue case figures that were notified during the epidemic period through the laboratory surveillance system (12). Analysis for a linear trend in proportion was done by a chi-square test using Epinfo, version 3.2. Odds ratios (OR) were calculated taking the weeks 33 to 36 as a reference because no DHF/DSS cases were observed in preceding weeks.

Samples. Twenty-two DENV-3 acute positive serum samples corresponding to 21 patients collected at different time points during the 2001-2002 Cuban epidemic were utilized for molecular characterization (Table 1). Two samples (Cuba_553_2001 and Cuba_558_2001) correspond to the same patient. All samples had been processed during the epidemic period for viral isolation on C6/36 HT cells and identification by indirect immune fluorescence with monoclonal antibodies (23) and then stored at −80°C in the strain bank of the National Reference Laboratory of Virology at Pedro Kouri Institute of Tropical Medicine. Informed consent was obtained from all patients at the moment of sample collection. All cases were classified clinically at that time as DF or DHF/DSS according to the Guidelines for Control and Prevention of Dengue and Dengue Hemorrhagic Fever in the Americas (24). In addition, the most recent clinical classification (2) was obtained after analysis of data available in the clinical records of the patients. Ten patients included in the study, classified as DF because they did not fulfill the strict WHO criteria to classify them as DHF according to the 1997 guidelines, presented warning signs and required hospitalization. Indeed, they were at risk of severe dengue. However, early hospitalization policies combined with proper clinical management prevented complications. Patients without warning signs treated at home were visited daily by the family doctor to accurately define the final disease outcome. The Institutional Ethical Review Committee of the Pedro Kouri Institute of Tropical Medicine approved the present study (IRB number CEI-IPK-13-11).

IgG detection. All samples were processed by an enzyme-linked immunosorbent assay (ELISA) inhibition test to determine anti-dengue virus IgG titer. Samples with titers less than 1/20 were considered primary infections, and samples with titers higher than 1/1,280, were considered secondary infections (25).

Sequence of infection. Sera from patients in the convalescent phase of the infection were analyzed for neutralizing antibodies to all DENV serotypes using the 50% endpoint plaque reduction neutralization test described by Morens et al. (26), with some modifications (27). According to criteria previously established (28), patients with neutralizing antibody titers of ≥1:30 to only one DENV serotype were considered to have experienced a primary dengue virus infection. Patients with neutralizing antibody titers of ≥1:30 against two or more serotypes were considered to have experienced a second or third infection.

Full-length viral genome amplification. Briefly, viral RNA was extracted from 140 µl of serum sample using a QIAamp viral RNA minikit (Qiagen, Germany). cDNA was synthesized using a Transcriptor High Fidelity cDNA Synthesis kit (Roche Applied Science, Germany) using 600 µM hexamer random primer according to the manufacturer’s instructions. An aliquot of 3 µl of cDNA was subjected to PCR using an Expand High Fidelity Plus PCR system (Roche Applied Science, Germany) according to the manufacturer’s instructions. Two independent DNA libraries (a and b) were constructed using two different sets of primers for each sample. Five pairs of primers were utilized to obtain five overlapping fragments of 2 to 3 kb (fragments F1a to F5a), covering the complete genome of the DENV-3 virus as previously published (29). In addition, a second set of primers was designed (available from the authors on request); these fragments were named F1b to F5b.

Population diversity determined by deep sequencing. To estimate the population diversity of variants by deep sequencing, PCR fragments were purified via a QIAQuick PCR purification kit (Qiagen, Germany), and total DNA was quantified by Pico Green fluorescence (Invitrogen, USA). Amplicons were then fragmented using Fragmentase and linked to Illumina multiplex adapters; they were subjected to clustering and sequencing with Illumina cBot and GAIIx technology and analyzed with established deep-sequencing data analysis tools. Illumina technology was selected since it is capable of producing enough sequencing data to enable
identification of rare variants present in as low as a 1:1,000 ratio in samples of modest reference size. Improvements in accuracy were made with the ViVAN (Viral Variant ANalysis) pipeline (30), a robust algorithm based on the reference size. Improvements in accuracy were made with the identification of rare variants present in as low as a 1:1,000 ratio in samples of modest reference size. Improvements in accuracy were made with the ViVAN (Viral Variant ANalysis) pipeline (30), a robust algorithm based on the reference size. Improvements in accuracy were made with the identification of rare variants present in as low as a 1:1,000 ratio in samples of modest reference size. Improvements in accuracy were made with the identification of rare variants present in as low as a 1:1,000 ratio in samples of modest reference size. Improvements in accuracy were made with the identification of rare variants present in as low as a 1:1,000 ratio in samples of modest reference size. 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Consensus sequence analysis. Consensus full polyprotein nucleotide sequences of each DENV-3 isolate obtained in the present study were aligned using ClustalX (34), together with relevant sequences retrieved from GenBank (available from the authors on request) such that representative sequences from all the known DENV-3 genotypes were present. From the initial data set, identical sequences and known recombinant sequences were removed from the alignments. This produced a total data set of 104 sequences of 10,170 nucleotides in length. Phylogenetic analyses were performed using Bayesian analysis in MrBayes, version 3.1.2 (35), with a minimum of 20 million generations and a burn-in of 10%. Stationarity was assessed at effective sample size (ESS; >400) using Tracer, version 1.4.1 (part of the BEAST package) (36).
RESULTS
Increasing clinical severity during the epidemic. The proportions of DHF/DSS cases per dengue case were compared every four epidemiological weeks as an expression of increasing clinical severity. According to epidemiological data the first dengue case was reported by epidemiological week 22 (beginning of June). However, the first DHF/DSS case occurred by epidemiological week 36 (beginning of September). From weeks 45 to 48 the number of confirmed dengue cases had a tendency to decrease while the proportion of DHF/DSS cases increased notably \((P < 0.05)\). The last seven DHF/DSS cases appeared during January 2002, with five of them occurring during the first week. The risk of severe dengue increased noticeably from September 2001 to January 2002 by 7.25-fold (Table 2). These findings confirmed that increasing clinical severity occurred toward the end of the epidemic.

Phylogenetic analysis. The Bayesian phylogenetic tree constructed with complete polyprotein sequences indicated that all Cuban isolates collected during the 2001-2002 epidemic grouped within genotype III, introduced in Latin America since 1994 (Fig. 1). Therefore, as expected, the Nicaraguan strain (NI_BID_V2420_1994) isolated around this time was located at the base of the Latin American group. All major nodes were statistically reliable according to the estimates of posterior probability. The phylogenetic tree further suggested that two lineages were circulating in Havana. It was noticeable that most Cuban isolates (20 isolates) representing the main lineage formed an independent monophyletic subgroup, closely related to Peruvian and Ecuadorian isolates from 2000 to 2002, but two isolates from the beginning of the epidemic (Cuba_118_2001 and Cuba_167_2001) appeared slightly distant, closely related to Venezuelan isolates from 2001.

Genetic variability at the consensus sequence level. Analysis of nucleotide sequences of the DENV-3 Cuban isolates, excluding samples Cuba_118_2001 and Cuba_167_2001, corresponding to a different lineage, revealed that relatively few nonsynonymous mutations occurred (Table 3). Notably, these mutations were generally unique for particular isolates, suggesting that they were not fixed within the population during the time of study, except for the mutation at nucleotide position 4380, which became fixed. This nonsynonymous mutation led to a conservative amino acid change of serine to asparagine, namely, S93N that is located in the NS2B protein. Puzzlingly, only the first isolate (Cuba_15_2001) had the S residue at this position, which was uncommon for DENV-3 strains of any genotype. Being a fairly indifferent amino acid, serine can reside both within the interior of a protein and on the protein surface. Its small size means that it is relatively common within tight turns on the protein surface, where it is possible for the serine side chain hydroxyl oxygen to form a hydrogen bond with the protein backbone, effectively mimicking proline. However, asparagine prefers generally to be on the surface of proteins, exposed to an aqueous environment (37).

Finally, samples Cuba_553_2001 and Cuba_558_2001 co-

| Sample no. | Sample code | C  | E  | NS1 | NS2A | NS2B | NS3 | NS4A | NS4B | NS5 |
|------------|-------------|----|----|-----|------|------|-----|------|------|-----|
| 1          | Cuba_15_01  | K  | T  | V   | H    | K    | A   | V    | I    | T   | S   | H   | V   | R   | E   | I   | H   | T   | R   | H   | V   |
| 2          | Cuba_26_01  |    |    |     |      |      |     |      |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3          | Cuba_73_01  |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 4          | Cuba_118_01 | N  | Y  | E   | T   | V    | N   | Y   | I    | K   | V   |     |     |     | R   | I   |     |     |     |     |     |     |
| 5          | Cuba_167_01 | N  | Y  | E   | T   | V    | N   | Y   | I    | K   | V   |     |     |     | R   | I   |     |     |     |     |     |     |
| 6          | Cuba_463_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 7          | Cuba_492_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 8          | Cuba_504_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 9          | Cuba_513_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 10         | Cuba_523_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 11         | Cuba_546_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 12         | Cuba_547_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 13         | Cuba_553_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 14         | Cuba_557_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 15         | Cuba_558_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 16         | Cuba_568_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 17         | Cuba_580_01 |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 18         | Cuba_16_02  |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 19         | Cuba_11_02  |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 20         | Cuba_17_02  |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 21         | Cuba_20_02  |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 22         | Cuba_21_02  |    |    |     |      |      |     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |

* The reference sequence of Cuba_15_2001 is shown in boldface. Only substitutions are shown. Samples 4 and 5 correspond to a different lineage.
lected 2 days apart from the same patient showed identical consensus nucleotide sequences.

**Viral population analysis.** Synonymous and nonsynonymous variant allele rates per 10,000 bases at the complete-genome level were calculated according to time of isolation. An increasing trend toward the end of the epidemic was observed only for synonymous variant allele rates (Fig. 2). Interestingly, in terms of nonsynonymous variant alleles, the viral population analysis indicated that at position 4380 in the NS2B gene, a significant minor population (A, 0.875%; T, 0.0%; C, 0.0%; G, 99.111%) present in the first isolate collected during the epidemic (Cuba_15_2001) was selected and became predominant (A, 99.943%; T, 0.0%; C, 0.0%; G, 0.664%) at the end of the epidemic. Taking into account this pattern, variants at low frequency (<1%) were considered relevant; therefore, unique significant minority variants (>0.1%) that appeared with the epidemic’s progression were analyzed using the first isolate (Cuba_15_2001) as a reference.

Greater variability was observed in the nonstructural genes than in the structural genes in terms of significant minority variants, involving mainly NS5, NS3, and NS4B genes, and particularly toward the end of the epidemic. Across the genome, the number of positions with significant minority variants (>0.1%) ranged from 12 to as high as 48 in both primary and secondary infections without significant differences between these groups (Fig. 3).

However, different results were obtained when higher-frequency minority variants (>1%) were analyzed (Fig. 4). Still greater variability was observed in the nonstructural genes than in the structural genes, but it was noteworthy that patients with secondary infections showed greater variability than patients with primary infections. In addition, patients with secondary infections presented minority variants in the structural genes (PrM and E), some of which were nonsynonymous. In contrast, patients suffering primary infections had only mostly synonymous minority variants (>1%) in nonstructural genes (Tables 4 and 5).

Intersample cluster analyses using RMSD values based on MDS showed greater variability with the epidemic’s progression. Samples with similar characteristics were reflected in the plot by their close spatial proximity to each other. Isolates collected at the end of the epidemic were located on the periphery of the plotting area, indicating higher variability (Fig. 5). Dendrograms using unique significant minority variants (>0.1%, >0.5%, and >1%) showed similar results; isolates collected at the very beginning were closely related and had less genetic variability than late isolates, based on RMSD values (Fig. 6).

Finally, significant minority variants present in samples Cuba_553_2001 and Cuba_558_2001 collected from the same patient at days 2 and 4 after fever onset were compared (Table 6). The analysis revealed changes in the viral population structure during the course of a secondary infection that were well supported by our data since high-quality sequences were obtained (Table 6), and similar results were obtained in two independent DNA libraries (a and b). Two silent nucleotide substitutions, C/T at position 5371 in NS3 and C/T at position 9142 in NS5, were found in both samples as the predominant population. Notably, these variants were present as significant minority variants in the

![FIG 2](http://jvi.asm.org/)

Synonymous variant allele rate per 10,000 bases at the complete-genome level according to time of sample collection during the 2001-2002 epidemic. Data sets a and b correspond to two different DNA libraries processed for each acute-phase sample through deep sequencing. For the synonymous variant allele rate in data set a (syna), $R^2 = 0.547$, and for data set b (synb), $R^2 = 0.693$; for the linear tendency for data set a, $P = 1.94e-4$, and for data set b, $P = 5.19e-6$. 

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first isolate, Cuba_15_2001. In addition, seven unique significant minority variants (>1%) were found in sample Cuba_553_2001 that were absent in sample Cuba_558_2001 (six of which were synonymous). Likewise, six unique significant minority variants (>1%) were found in sample Cuba_558_2001 that were absent in sample Cuba_553_2001 (four of which were non-synonymous). Interestingly, significant minority variants in the sample collected at day 2 corresponded exclusively to non-structural genes, while significant minority variants present in the sample collected at day 4 included nonsynonymous changes in the PrM, E, and NS5 genes. The change E9D in PrM protein was located in the N terminus of Pr near motif 6, one of the prominent

![FIG 3](http://jvi.asm.org/)

**FIG 3** Number of positions with unique significant minority variants common for sets a and b (>0.1%), taking as a reference the first isolate of the DENV-3 Cuban epidemic of 2001 to 2002. Samples are grouped according to type of infection in primary and secondary infections by date of sample collection (Table 1). UTR, untranslated region.

![FIG 4](http://jvi.asm.org/)

**FIG 4** Number of positions with unique significant minority variants common for sets a and b (>1%), taking as a reference the first isolate of the DENV-3 Cuban epidemic of 2001 to 2002. Samples are grouped according to type of infection in primary and secondary infections by date of sample collection (Table 1).
TABLE 4 Unique significant variants (>1%) common for set a and b for primary infection cases taking as a reference the first isolate obtained during the Cuban epidemic, 2001 to 2002

| Sample no. | Feature | Position (nt) | Reference allele | Set a variant | Amino acid no. | Change | Frequency (%) | Allele | Amino acid no. | Change | Frequency (%) |
|------------|---------|---------------|-----------------|--------------|----------------|--------|--------------|--------|----------------|--------|--------------|
| 9          | —       | —             | —               | —            | —              | —      | —            | —      | —              | —      | —            |
| 10 NS1     | 3424    | C             | T               | 344          | Synonymous ↔ N | 1.145  | 1.107        | T      | 344           | Synonymous ↔ N | 0.966        |
| 12 NS5     | 9803    | C             | T               | 754          | Synonymous ↔ L | 4.539  | 4.338        | T      | 754           | Synonymous ↔ L | 4.371        |
| 14 NS1     | 2924    | C             | T               | 178          | Synonymous ↔ L | 5.312  | 5.186        | T      | 178           | Synonymous ↔ L | 6.398        |
| 13 NS3     | 3571    | C             | T               | 293          | Synonymous ↔ A | 1.086  | 1.049        | T      | 293           | Synonymous ↔ A | 0.134        |

| Sample no. | Feature | Position (nt) | Reference allele | Set b variant | Amino acid no. | Change | Frequency (%) | Allele | Amino acid no. | Change | Frequency (%) |
|------------|---------|---------------|-----------------|--------------|----------------|--------|--------------|--------|----------------|--------|--------------|
| 18 3′ UTR  | 10369   | G             | T               | *            | *              | 1.641  | —            | —      | —              | —      | —            |
| 22         | —       | —             | —               | —            | —              | —      | —            | —      | —              | —      | —            |

a Indicates minority variants represent <1%.
b Indicates position in noncoding region of the 3′ untranslated region (UTR).
c nt, nucleotide.
TABLE 5 Unique significant variants (>1%) common for set a and b for secondary infection cases taking as a reference the first isolate obtained during the Cuban epidemic, 2001 to 2002a

| Sample no. | Feature | Position (nt)b | Reference allele | Set a variant | Set b variant |
|------------|---------|----------------|-----------------|--------------|--------------|
|            |         |                | Allele | Amino acid no. | Change | Frequency (%) | Allele | Amino acid no. | Change | Frequency (%) |
| 2          | 3’UTR   | 10404          | G      | A              | *      | 11.859        | A      | *              | *      | 12.027        |
| 3          | NS4B    | 6853           | G      | C              | 18     | E → D         | C      | 18             | E → D  | 62.998        |
|            | NS5     | 8212           | C      | T              | 223    | Synonymous → G | T      | 223            | Synonymous → G | 0.905       |
|            | 2K peptide)d | 6751          | C      | T              | 7      | Synonymous → L | T      | 7              | Synonymous → L | 26.578      |
|            | NS3     | 5956           | C      | T              | 488    | Synonymous → H | T      | 488            | Synonymous → H | 24.625      |
| 6          | PrM     | 519            | T      | C              | T      | 35            | T      | 35             | I      | 4.605         |
|            | NS5     | 8080           | T      | C              | 179    | Synonymous → I | C      | 179            | Synonymous → I | 1.961       |
|            | NS5     | 9743           | C      | T              | 734    | Synonymous → L | T      | 734            | Synonymous → L | 1.11         |
| 7          | NS5     | 10000          | G      | A              | 819    | Synonymous → E | A      | 819            | Synonymous → E | 52.01        |
|            | 3UTR    | 10481          | C      | T              | *      | 27.601        | T      | *              | *      | 27.616        |
|            | NS5     | 5098           | G      | A              | 202    | Synonymous → R | A      | 202            | Synonymous → R | 7.952        |
|            | NS2A    | 3531           | G      | T              | 28     | G → V         | T      | 28             | G → V      | 2.237        |
|            | 3UTR    | 10359          | C      | T              | *      | 1.881         | T      | *              | *      | 1.805         |
|            | NS5     | 9265           | C      | T              | 574    | Synonymous → N | T      | 574            | Synonymous → N | 1.423        |
|            | NS4B    | 7366           | C      | A              | 189    | Synonymous → A | A      | 189            | Synonymous → A | 1.417        |
| 8          | NS2B    | 4294           | G      | A              | 64     | Synonymous → E | A      | 64             | Synonymous → E | 7.562        |
| 11         | E       | 1834           | G      | A              | 307    | Synonymous → K | A      | 307            | Synonymous → K | 99.93        |
|            | E       | 991            | G      | A              | 26     | Synonymous → E | A      | 26             | Synonymous → E | 99.862       |
|            | NS5     | 10049          | G      | A              | 836    | V → I         | A      | 836            | V → I      | 7.418         |
|            | NS5     | 10223          | G      | A              | 894    | E → Q         | C      | 894            | E → Q      | 6.061         |
| 13         | NS5     | 9142           | C      | T              | 533    | Synonymous → D | T      | 533            | Synonymous → D | 98.715       |
|            | NS5     | 5371           | C      | T              | 293    | Synonymous → A | T      | 293            | Synonymous → A | 99.878       |
|            | NS5     | 9985           | C      | T              | 814    | Synonymous → N | T      | 814            | Synonymous → N | 3.15         |
|            | NS4B    | 7012           | G      | A              | 71     | Synonymous → Q | A      | 71             | Synonymous → Q | 1.678        |
|            | NS5     | 7975           | G      | A              | 144    | Synonymous → L | A      | 144            | Synonymous → L | 1.686        |
|            | NS5     | 7744           | G      | A              | 67     | Synonymous → E | A      | 67             | Synonymous → E | 1.484        |
|            | NS3     | 5326           | C      | T              | 278    | Synonymous → N | T      | 278            | Synonymous → N | 1.003        |
|            | NS4B    | 7366           | C      | T              | 189    | Synonymous → A | T      | 189            | Synonymous → A | 0.921        |
|            | NS5     | 3500           | C      | T              | 18     | L → F         | T      | 18             | L → F      | 1.474         |
| 15         | NS5     | 9142           | C      | T              | 533    | Synonymous → D | T      | 533            | Synonymous → D | 97.096       |
|            | NS5     | 5371           | C      | T              | 293    | Synonymous → A | T      | 293            | Synonymous → A | 99.666       |
|            | NS2B    | 4315           | C      | T              | 71     | Synonymous → S | T      | 71             | Synonymous → S | 5.037        |
|            | E       | 1510           | G      | A              | 199    | M → I         | A      | 199            | M → I      | 2.212        |
|            | NS5     | 9092           | A      | G              | 517    | I → V         | G      | 517            | I → V      | 1.941        |
|            | PrM     | 442            | G      | T              | 9      | E → D         | T      | 9              | E → D      | 1.283        |
|            | NS1     | 3373           | C      | T              | 327    | Synonymous → D | T      | 327            | Synonymous → D | 1.032        |
|            | E       | 2235           | C      | T              | 441    | A → V         | T      | 441            | A → V      | 0.902        |
| 16         | NS5     | 8655           | G      | A              | 371    | R → K         | A      | 371            | R → K      | 98.923       |
|            | NS5     | 6184           | C      | T              | 564    | Synonymous → C | T      | 564            | Synonymous → C | 6.419        |
|            | NS4B    | 7444           | C      | A              | 215    | Synonymous → T | A      | 215            | Synonymous → T | 2.41         |
| 17         | NS1     | 3310           | G      | A              | 306    | Synonymous → K | A      | 306            | Synonymous → K | 99.847       |
|            | NS5     | 8428           | C      | T              | 295    | Synonymous → D | T      | 295            | Synonymous → D | 96.876       |
|            | NS5     | 8875           | C      | T              | 444    | Synonymous → G | T      | 444            | Synonymous → G | 0.075        |
|            | NS5     | 9127           | C      | T              | 528    | Synonymous → A | T      | 528            | Synonymous → A | 0.102        |
|            | 3UTR    | 10283          | C      | T              | *      | *             | T      | *              | *      | 0.067         |
|            | NS5     | 8659           | C      | T              | 372    | Synonymous → V | T      | 372            | Synonymous → V | 0.086        |
|            | NS5     | 8743           | G      | A              | 400    | Synonymous → K | A      | 400            | Synonymous → K | 0.09         |
|            | NS5     | 7594           | A      | G              | 17     | Synonymous → L | G      | 17             | Synonymous → L | 1.16         |
| 19         | NS4A    | 6574           | T      | C              | 75     | Synonymous → G | C      | 75             | Synonymous → G | 1.116        |
|            | 3UTR    | 10661          | A      | G              | *      | *             | 0.6    | G              | 0.838       |

(Continued on following page)
interval [CI], 1.94 to 2.01) whereas Boyeros (48), one of the last municipalities affected by the epidemic, had an $R_0$ of 61.06 (95% CI, 60.44 to 61.68).

Considering the main lineage as the one involved in the phenomenon of increasing clinical severity, temporary changes that appear at the consensus level in the 20 DENV-3 samples conforming to this lineage were analyzed. Contrary to expectations, the clear pattern of evolution observed during the 1997 Cuban epidemic (14) was not demonstrated here. A unique amino acid change, S93N in NS2B, differentiates all the studied isolates from the first isolate of the epidemic. Interestingly, only the first isolate had serine at this position, which is uncommon for DENV of any serotype. However, according to deep sequencing, the genetic variant coding for the common motif asparagine was also present in the first isolate as a minor variant (1%). The fact that this motif remains invariant for DENV-3 at deeper phylogenetic levels suggests that it is favored in nature. Importantly, the replication of DENV requires the correct processing of the polyprotein by the viral NS3 protease (NS3pro). For full enzymatic activity NS3pro requires the hydrophilic part of the integral membrane protein NS2B as a cofactor (residues 49 to 95) (49–51).

TABLE 5 (Continued)

| Sample no. | Feature | Position (nt)$^b$ | Reference allele | Amino acid no. | Change | Frequency (%) | Set a variant | Reference allele | Amino acid no. | Change | Frequency (%) | Set b variant |
|------------|---------|------------------|------------------|----------------|--------|----------------|---------------|------------------|----------------|--------|----------------|---------------|
| 20         | NS3     | 5791             | G                | A              | 433    | Synonymous $\rightarrow$ V | 96.402        | A                | 433            | Synonymous $\rightarrow$ V | 99.509 |
| PrM        | 652     | G                | T                | 79             | Synonymous $\rightarrow$ T | 4.65         | A                | 79             | Synonymous $\rightarrow$ T | 0.073 |
| 3UTR       | 10514   | G                | C                | *              | *      | Synonymous $\rightarrow$ L | 3.956         | C                | *              | *      | Synonymous $\rightarrow$ L | 4.416 |
| NS3        | 4685    | C                | T                | 65             | Synonymous $\rightarrow$ L | 3.66         | T                | 65             | Synonymous $\rightarrow$ L | 0.286 |
| 3UTR       | 10577   | A                | C                | *              | *      | Synonymous $\rightarrow$ G | 2.459         | C                | *              | *      | Synonymous $\rightarrow$ G | 2.533 |
| 2K peptide | 6769    | C                | A                | 13             | Synonymous $\rightarrow$ G | 2.048        | A                | 13             | Synonymous $\rightarrow$ G | 2.463 |
| 21         | NS5     | 8212             | C                | T              | 223    | Synonymous $\rightarrow$ G | 39.457        | T                | 223            | Synonymous $\rightarrow$ G | 97.343 |
| NS4A       | 6700    | G                | T                | 117            | Synonymous $\rightarrow$ V | 8.487        | T                | 117            | Synonymous $\rightarrow$ V | 8.528 |
| NS4B       | 6853    | G                | C                | 18             | E $\rightarrow$ D | 1.086        | C                | 18             | E $\rightarrow$ D | 0.797 |

$^a$ Boldface indicate that these variants became predominant in the particular patient.

$^b$ nt, nucleotide.

$^c$ Indicates positions in noncoding region of the 3’ untranslated region (UTR).

$^d$ 2K peptide, the DENV 2K-signal sequence is a 17-amino-acid peptide linking NS4A with NS4B.

FIG 5 Multidimensional scaling using root mean square deviation (RMSD) values calculated using significant minority variants (>1%) for data set a (red dots) and b (blue dots). Numbers represent the 20 studied samples ordered by collection time, as indicated in Table 1. Samples 4 and 5 that correspond to a different lineage were excluded.

FIG 6 Dendrogram clustering of Cuban isolates collected at different time points during the 2001-2002 epidemic using an RMSD-based distance matrix including data sets a and b. Samples that correspond to a different lineage were excluded.
In the present study, viral population analysis also showed portions of sequence space in which to increase its fitness. According to previous seroepidemiological studies, all severe cases from the acute phase in this patient. Changes in the antibody response profile were demonstrated during transmission from mosquito back to human were not examined. The viral population changed in 2 days; remarkably, new variants that arose on day 4 presented nonsynonymous changes at the structural genes PrM and E. Moreover, changes in the antibody response profile were demonstrated during the acute phase in this patient.

Could this variability depend on the immunity of the patient? According to previous seroepidemiological studies, all severe cases observed during the 2001-2002 Cuban epidemic had the sequence of DENV-1 infection followed by DENV-3 (DENV-1/DENV-3) or experienced tertiary infection (DENV-1/DENV-2/DENV-3) (56). However, the infection sequence DENV-2/DENV-3 was associated with asymptomatic infection or mild disease (57). Similar results were observed in a different epidemiological setting (58), and it is suggested that antibodies against DENV-2 could have the ability to neutralize and downregulate DENV-3 infections. In accordance, a recent study that characterized the antigenic diversity in the DENV types by antigenic maps constructed from neutralizing antibody titers has shown that whereas DENV isolates are usually located closer to other viruses of the same type, some viruses, both modern and historical, have greater antigenic resemblance to viruses of a different type than to some viruses of the same type (59). Therefore, the viral population transmitted to a healthy individual from an infected mosquito may vary depending on the immunological background of the previous individual on which the mosquito fed. Likewise, the immunological background of the new human host could have an effect on the viral population during the course of infection. Recently, Sim et al. used whole-genome amplification and next-generation sequencing to characterize DENV intrahost genetic diversity in both patient-derived and matched-mosquito-derived virus populations. Mosquitoes were infected by direct feeding on patients, enabling the authors to track changes in viral populations during human-to-mosquito transmission. However, changes in viral populations during transmission from mosquito back to human were not examined, and the immunological background of the patients enrolled in the study was not determined (20).

In the present situation, the DENV-3 mutant spectrum could be replicating in total absence of DENV heterologous antibodies during many cycles of primary infections since a large part of the population was naive (at least all individuals born after 1981). Alternatively, during secondary infections in the sequence DENV-

| TABLE 6 Unique significant variants (>1%) common for set a and b present in two samples corresponding to the same patient at days 2 and 4 after fever onset, taking as a reference the first isolate obtained during the Cuban epidemic, 2001 to 2002 |
| --- |
| Sample and feature | Position (nt) | Read coverage | Reference allele | Amino acid no. | Change | Frequency (%) | Allele | Amino acid no. | Change | Frequency (%) |
| **Cuba_553_2001** |  |  |  |  |  |  |  |  |  |  |
| NS5 | 9142 | 30,246 | C | T | 533 | Synonymous → D | 99.288 | T | 533 | Synonymous → D | 98.715 |
| NS3 | 5371 | 8,024 | C | T | 293 | Synonymous → A | 96.832 | T | 293 | Synonymous → A | 99.878 |
| NS4B | 7012 | 16,819 | G | A | 71 | Synonymous → Q | 1.723 | A | 71 | Synonymous → Q | 1.678 |
| NS5 | 7975 | 19,551 | G | A | 144 | Synonymous → L | 1.511 | A | 144 | Synonymous → L | 1.686 |
| NS5 | 7744 | 18,047 | G | A | 67 | Synonymous → E | 1.472 | A | 67 | Synonymous → E | 1.484 |
| NS3 | 5326 | 8,654 | C | T | 278 | Synonymous → N | 1.386 | T | 278 | Synonymous → N | 1.003 |
| NS4B | 7366 | 15,691 | C | T | 18 | Synonymous → A | 1.259 | T | 18 | Synonymous → A | 0.921 |
| NS2A | 3500 | 12,713 | C | T | 18 | L → F | 0.983 | T | 18 | L → F | 1.474 |
| **Cuba_558_2001** |  |  |  |  |  |  |  |  |  |  |
| NS5 | 9142 | 13,986 | C | T | 533 | Synonymous → D | 98.963 | T | 533 | Synonymous → D | 97.096 |
| NS3 | 5371 | 18,626 | C | T | 293 | Synonymous → A | 97.515 | T | 293 | Synonymous → A | 99.666 |
| NS2B | 4315 | 27,529 | C | T | 71 | Synonymous → S | 3.495 | T | 71 | Synonymous → S | 5.037 |
| E | 1510 | 24,416 | G | A | 199 | M → I | 2.366 | A | 199 | M → I | 2.212 |
| NS5 | 9092 | 12,493 | A | G | 517 | I → V | 1.579 | G | 517 | I → V | 1.941 |
| PrM | 442 | 19,513 | G | T | 9 | E → D | 1.114 | T | 9 | E → D | 1.283 |
| NS1 | 3373 | 18,492 | C | T | 327 | Synonymous → D | 1.096 | T | 327 | Synonymous → D | 1.032 |
| E | 2235 | 30,874 | C | T | 441 | A → V | 0.914 | T | 441 | A → V | 0.902 |

a Boldface indicate that this variant became predominant.

b nt, nucleotide.

c Variant A (synonymous → E) at position PrM 442 was detected in sample Cuba_553_2001 at a frequency of 0.1% (day 2).

d Read coverage, the average number of reads that align to each base of the reference sequence.
1/DENV-3, higher viral load could be expected via antibody-dependent enhancement. Viral load, together with population genetic heterogeneity, permits exploration of sequence space for a fitness increase. As viral fitness is environment and population size dependent, it can change according to the immunological landscape.

The emergence of significant minority variants with changes in the structural proteins PrM and E late during the acute phase of the disease in a secondary infection case (DENV-1/DENV-3) is described for the first time in dengue. However, its implications concerning pathogenesis should be examined with a higher number of well-characterized serial samples, with data including the immunological background of the patients. The high-resolution analysis of intrahost genetic diversity published by Thai et al. in serial plasma samples taken from 17 patients infected with DENV-1 revealed that nucleotide sequence diversities of viral populations were very low, ranging from 0 to 0.0013 among different samples collected from the same patient (17). While this study explored only the E gene (exclusively the fragment coding domain III) and used cloning techniques rather than deep sequencing, its observations fit within the results of the present study, which observed the lowest diversity in the structural genes. Moreover, the significant minority variants with changes in the E gene that emerged in the same patient late during the course of infection did not involve domain III. Lack of diversity in the E gene has also been attributed to strong purifying selection (60).

In conclusion, our results suggest that changes in the viral population swarm occurred with the epidemic’s progression and that these changes could have had an impact on viral fitness. Therefore, the dynamics of evolving viral populations in the context of heterotypic antibodies could be related to the increasing clinical severity observed during dengue epidemics. Definitely, new experiments are required to prove in vivo and/or ex vivo the role of particular mutations on the increased viral fitness toward the end of the epidemic. In this regard, the impact of synonymous alleles deserves further study, focused on the biological basis of the selective advantage of silent mutations in DENVs. More importantly, studies addressing the extent and pattern of intrahost genetic diversity during the course of dengue secondary infections using deep-sequencing approaches should be tackled to unravel molecular mechanisms involved in dengue virus pathogenesis.

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