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Molecular epidemiology of type 1 and 2 dengue viruses in Brazil from 1988 to 2001

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Abstract

Dengue is a mosquito-borne viral infection that in recent decades has become a major international public health concern. Epidemic dengue fever reemerged in Brazil in 1981. Since 1990 more than one dengue virus serotype has been circulating in this tropical country and increasing rates of dengue hemorrhagic fever and dengue shock syndrome have been detected every year. Some evidence supports the association between the introduction of a new serotype and/or genotype in a region and the appearance of dengue hemorrhagic fever. In order to study the evolutionary relationships and possible detection of the introduction of new dengue virus genotypes in Brazil in the last years, we analyzed partial nucleotide sequences of 52 Brazilian samples of both dengue type 1 and dengue type 2 isolated from 1988 to 2001 from highly endemic regions. A 240-nucleotide-long sequence from the envelope/nonstructural protein 1 gene junction was used for phylogenetic analysis. After comparing the nucleotide sequences originally obtained in this study to those previously studied by others, and analyzing the phylogenetic trees, we conclude that, after the initial introduction of the currently circulating dengue-1 and dengue-2 genotypes in Brazil, there has been no evidence of introduction of new genotypes since 1988. The increasing number of dengue hemorrhagic fever cases seen in Brazil in the last years is probably associated with secondary infections or with the introduction of new serotypes but not with the introduction of new genotypes.

Key words
- Dengue virus
- Nucleotide sequence
- E/NS1 region
- Molecular epidemiology
- Dengue fever
- Dengue hemorrhagic fever

Introduction

Dengue viruses belong to the Flaviviridae family and are transmitted to humans through the bite of female *Aedes* mosquitoes. As the most important arthropod-borne viral infection of humans, dengue represents an important public health problem for urban population in the tropical and subtropical areas of the world. About 2.5 billion people in 100 countries are at risk for infection, and over 100 million cases of human infections and about 20,000 deaths occur each year. Symptomatic human infections may range from a mild, flu-like syndrome, sometimes associated with a rash (dengue fever, DF) to a more severe form

of disease associated with plasma leakage, thrombocytopenia, hemorrhage (dengue hemorrhagic fever, DHF) and/or shock (dengue shock syndrome, DSS) (1-3).

The dengue virus genome is an ~11-kb single-strand positive sense RNA with a single-open reading frame which encodes a polyprotein precursor of about 3,400 amino acid residues. Proteolytic cleavages generate 10 proteins that are detected in infected cells (C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (4,5). On the basis of antigenic variability, dengue viruses are classified into 4 serotypes (DEN-1 to 4). In addition to serotype classification, significant variation in genomic composition among viruses of each serotype permits genotype classification (5). Unlike other RNA viruses, some segments of the dengue virus genome have a high degree of stability where fixed mutations are common. Partial sequencing of some genomic regions has been successfully employed to determine the genetic variation of dengue viruses and to characterize genotypes within serotypes (6-11).

The genomic regions encoding the envelope protein (E) and the nonstructural protein 1 (NS1) seem to be the most appropriate to characterize genotypes within serotypes, especially a 240-nucleotide long sequence spanning the E/NS1 junction (6,8,12). Studies of the evolutionary relationships of dengue viruses have revealed several major genotypes among each of the four serotypes. For DEN-1 viruses five genotypes have been described: one group representing strains from the Americas, Africa and Southeast Asia (I), a Sri Lankan group (II), a Japanese group (III), a fourth group including strains from Southeast Asia, the South Pacific, Mexico, and Australia (IV), and a fifth group composed of Taiwanese and Thai strains (V). For DEN-2 viruses, phylogenetic analysis initially identified five genotypes: strains from the Caribbean and South America (I); strains from the South Pacific, i.e., Taiwan, Philippines and New Guinea C prototype viruses and an older Thai strain (II); Vietnamese, Jamaican and Thai strains (III); isolates from Indonesia, the Seychelles, Burkina Fasso and Sri Lanka (IV), and finally, isolates from rural Africa (V) (6,8). Further studies incorporating additional DEN-2 strains resulted in the merging of genotypes II and III into one genotype (Asian/American-Asian genotype) (13). Infection by one serotype does not protect against infection by a second serotype, and epidemiologic and laboratory studies have shown that cross-reactive immune responses, including infection-enhancing antibodies, contribute to the higher frequency of DHF/DSS in persons with sequential infections (14,15). The occurrence of DHF/DSS in some regions has been associated with the introduction of new serotypes and/or genotypes of dengue virus (6,8,13,16).

As in many other countries in Latin America, Brazilian people have been seriously affected by dengue infections. About 80% of notified dengue cases in the Americas occurred in Brazil, and in the last years more than 1 million cases have been reported in all five Brazilian geographic regions (17). In spite of the importance of dengue as a serious public health problem in Brazil, only a few dengue viruses isolated from endemic regions have been analyzed with respect to genomic variability (6,7,9,16,18-20).

In the present study, we analyzed partial nucleotide sequences of a significant number of DEN-1 and DEN-2 strains isolated in Brazil since 1988 from regions of high endemicity in order to determine the evolutionary relationships among them and the introduction and circulation of new genotypes that could be associated with the more severe cases seen in the last years.

Material and Methods

Viruses

All 25 strains of dengue viruses originally analyzed in this study were isolated from acute-phase sera of patients with DF

who had been infected in different states of Brazil from 1995 to 2001. Twenty-two of these strains were randomly selected from the collection of the Arbovirus Section, Adolfo Lutz Institute, São Paulo, São Paulo State, Brazil. Two other DEN-2 strains were obtained from the collection of Evandro Chagas Institute, Belém, Pará State, Brazil, and the most recent strain (D1-BRA/SP/01) was isolated by the investigators from a patient with DF living in São Paulo State. Acute-phase sera were used to infect monolayers of a mosquito cell line (C6/36 - *Aedes albopictus*) and serotypes were identified by indirect fluorescent antibody tests using type-specific monoclonal antibodies (15F3-1 and 3H5-1) kindly donated to Adolfo Lutz Institute by the Centers for Disease Control and Prevention, Atlanta, GA, USA (21,22). All strains were submitted to a single passage in cell culture. After growth for 7 days at 28ºC, virus-infected supernatants were collected, clarified by centrifugation and stored at -70ºC until the time for use. The identification of viral strains and their distribution in Brazilian regions are listed in Table 1.

Viral RNA extraction and RT-PCR amplification

Viral RNA was extracted from 140 µl supernatant medium of virus-infected cells using the QIAamp® Viral RNA system according to the manufacturer's protocol (Qiagen®, Chatsworth, CA, USA). Viral RNA was reverse transcribed to cDNA in a 20-µl reaction volume with he Superscript II reverse transcriptase system (Invitrogen, Carlsbad, CA, USA) and pd$(N)_6$ random primers (Amersham-Pharmacia, Piscataway, NJ, USA). Reverse transcription was allowed to proceed at 42ºC for 50 min followed by reverse transcriptase inactivation at 70ºC for 15 min. cDNA amplification was performed with synthetic primers as previously described (6). Both sense and antisense primers were used to amplify a 408-bp fragment of the E/NS1 junction region of the viral RNA; the 240-nucleotide segment for genetic comparison is comprised within this fragment (nucleotides 2.282 to 2.521 for DEN-1 and 2.311 to 2.550 for DEN-2). PCR amplifications consisted of 35 cycles of denaturation (94ºC for 1 min), annealing (55ºC for 1 min) and extension (72ºC for 2 min) for both DEN-1 and DEN-2 strains in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, Foster City, CA, USA). A final extension step was carried out at 72ºC for 10 min. Each PCR was run with positive and negative controls and the fragments were separated by 2% agarose gel electrophoresis, stained with 1 µg/ml ethidium bromide, and detected under ultraviolet light.

Sequencing

Direct nucleotide sequencing of both strands was performed with an automated Sequence Detector System (ABI Prism 310 sequencer; Applied Biosystems) using a commercially available kit (BigDye Terminator Cycle Sequencing Ready Reaction®, Applied Biosystems) according to the manufacturer's protocol. Briefly, for each sequencing reaction, 50 to 100 ng DNA was mixed with 3.2 pmol of a sense or antisense primer, 5 µl water and a reaction mixture containing the four dye-labeled dideoxynucleotide terminators. Sequencing reactions were performed in 25 cycles of denaturation (96ºC for 30 s), annealing (50ºC for 1 min) and extension (60ºC for 4 min) on a GeneAmp PCR System 2400 (Applied Biosystems). The final reaction mixture was purified with 75% isopropanol and the cycle-sequenced DNA was then dried in a vacuum centrifuge for 20 min. The pellet was resuspended in 20 µl of template suppression reagent (Applied Biosystems) and loaded onto the sequencer. Sequences were base-called using the DNA Sequencing Analyses software (Applied Biosystems).

Table 1. Dengue virus strains compared by sequence analysis.

Code	Strain	Year	Location	Reference	GenBank accession No.
Serotype 1 (DEN-1)					
d1-BRA/PR/95.1	IAL H 158615	1995	Brazil/Paraná (S)	¶	AY159268
d1-BRA/PR/95.2	IAL H 158646	1995	Brazil/Paraná (S)	¶	AY159269
d1-BRA/MT/96.1	IAL H 162923	1996	Brazil/Mato Grosso (MW)	¶	AY159262
d1-BRA/MT/96.2	IAL H 163022	1996	Brazil/Mato Grosso (MW)	¶	AY159263
d1-BRA/MT/96.3	IAL H 163361	1996	Brazil/Mato Grosso (MW)	¶	AY159264
d1-BRA/RJ/96	IAL H 163743	1996	Brazil/Rio de Janeiro (SE)	¶	AY159270
d1-BRA/BA/96.1	IAL H 165358	1996	Brazil/Bahia (NE)	¶	AY159257
d1-BRA/BA/96.2	IAL H 166877	1996	Brazil/Bahia (NE)	¶	AY159258
d1-BRA/PE/97.1	IAL H 167200	1997	Brazil/Pernambuco (NE)	¶	AY159267
d1-BRA/BA/97	IAL H 167307	1997	Brazil/Bahia (NE)	¶	AY159259
d1-BRA/PA/98.1	IAL H 172708	1998	Brazil/Pará (N)	¶	AY159265
d1-BRA/PA/98.2	IAL H 173963	1998	Brazil/Pará (N)	¶	AY159266
d1-BRA/MG/99	IAL H 182774	1999	Brazil/Minas Gerais (SE)	¶	AY159260
d1-BRA/RJ/99	IAL H 183745	1999	Brazil/Rio de Janeiro (SE)	¶	AY159271
d1-BRA/MS/99	IAL H 184508	1999	Brazil/Mato Grosso do Sul (MW)	¶	AY159261
d1-BRA/SP/01	SMRP 01/1	2001	Brazil/São Paulo (SE)	¶	AY159272
d1-BRA/PE/97.2	BR/97 233	1997	Brazil/Pernambuco (NE)	30	AF311958
d1-BRA/PE/97.3	BR/97 409	1997	Brazil/Pernambuco (NE)	30	AF311957
d1-BRA/PE/97.4	BR/97 111	1997	Brazil/Pernambuco (NE)	30	AF311956
d1-BRA/PR/01	BR/01 MR	2001	Brazil/Pernambuco (NE)	30	AF513110
d1-BRA/RJ/90	BR 90	1990	Brazil/Rio de Janeiro (SE)	30	AF226685
d1-BRA/RJ/88	28973/HS	1988	Brazil/Rio de Janeiro (SE)	6	M32908
d1-Nauru Island	16299	1974	Nauru Island	6	M32904
d1-Philippines	027	1988	Philippines/Manila	6	M32892
d1-Japan	Mochizuki	1943	Japan	6	M32929
d1-Colombia	351094	1987	Colombia	6	M32911
d1-Haiti	1413	1983	Haiti	6	M32903
d1-Surinam	816879	1981	Surinam	6	M32918
d1-Mexico	1412	1983	Mexico	6	M32902
d1-Australia	TI4	1981	Australia/Thursday Island	6	M32931
d1-El Salvador	1916	1987	El Salvador	6	M32905
d1-Taiwan	779172	1988	Taiwan/Kaohsiung	6	M32917
Serotype 2 (DEN-2)					
d2-BRA/MA/96	IAL H 164954	1996	Brazil/Maranhão (NE)	¶	AY159274
d2-BRA/MT/97	IAL H 167178	1997	Brazil/Mato Grosso (MW)	¶	AY159276
d2-BRA/PE/97	IAL H 168330	1997	Brazil/Pernambuco (NE)	¶	AY159278
d2-BRA/MA/98.1	IAL H 172702	1998	Brazil/Maranhão (NE)	¶	AY159275
d2-BRA/BA/99	IAL H 182803	1999	Brazil/Bahia (NE)	¶	AY159273
d2-BRA/PB/99	IAL H 182896	1999	Brazil/Paraíba (NE)	¶	AY159277
d2-BRA/SE/99	IAL H 182931	1999	Brazil/Sergipe (NE)	¶	AY159279
d2-BRA/PA/98.1	IEC H 635380	1998	Brazil/Pará (N)	¶	AY277245
d2-BRA/PA/98.2	IEC H 635020	1998	Brazil/Pará (N)	¶	AY277246
d2-BRA/RJ/90.1	39056	1990	Brazil/Rio de Janeiro (SE)	16	U91859
d2-BRA/RJ/90.2	38998	1990	Brazil/Rio de Janeiro (SE)	16	U91860
d2-BRA/RJ/90.3	40247	1990	Brazil/Rio de Janeiro (SE)	16	U91861
d2-BRA/RJ/90.4	41464	1990	Brazil/Rio de Janeiro (SE)	16	U91862
d2-BRA/RJ/90.5	41576	1990	Brazil/Rio de Janeiro (SE)	16	U91863
d2-BRA/TO/91	H506525	1991	Brazil/Tocantins (N)	16	U91867
d2-BRA/RJ/90.6	39122	1990	Brazil/Rio de Janeiro (SE)	20	AF529064
d2-BRA/RJ/90.7	39325	1990	Brazil/Rio de Janeiro (SE)	20	AF529065
d2-BRA/BA/95.1	51502	1995	Brazil/Bahia (NE)	20	AF529066
d2-BRA/BA/95.2	51504	1995	Brazil/Bahia (NE)	20	AF529067
d2-BRA/ES/95	52582	1995	Brazil/Espírito Santo (SE)	20	AF529068
d2-BRA/BA/96	55968	1996	Brazil/Bahia (NE)	20	AF529069

Continued on next page

Table 1 continued.

Code	Strain	Year	Location	Reference	GenBank accession No.
d2-BRA/RJ/98.1	61321	1998	Brazil/Rio de Janeiro (SE)	20	AF529070
d2-BRA/RN/98.1	61654	1998	Brazil/Rio Grande do Norte (NE)	20	AF529071
d2-BRA/RJ/98.2	62515	1998	Brazil/Rio de Janeiro (SE)	20	AF529072
d2-BRA/RN/98.2	64020	1998	Brazil/Rio Grande do Norte (NE)	20	AF529073
d2-BRA/RJ/99.1	64627	1999	Brazil/Rio de Janeiro (SE)	20	AF529074
d2-BRA/RJ/99.2	64905	1999	Brazil/Rio de Janeiro (SE)	20	AF529075
d2-BRA/ES/00.1	66703	2000	Brazil/Espírito Santo (SE)	20	AF529076
d2-BRA/ES/00.2	66718	2000	Brazil/Espírito Santo (SE)	20	AF529077
d2-BRA/RJ/00	66985	2000	Brazil/Rio de Janeiro (SE)	20	AF529078
d2-Puerto Rico	PR159S1	1969	Puerto Rico	6	M32968
d2-Jamaica	JAH	1982	Jamaica	6	M32960
d2-Vietnam	57S	1987	Vietnam/Hanoi	6	M32948
d2-Philippines	028	1988	Philippines/Manila	6	M32932
d2-Indonesia	8720	1973	Indonésia	6	M32951
d2-Sri Lanka	1334	1981	Sri Lanka	6	M32938
d2-Taiwan	766635	1987	Taiwan/Kaohsiung	6	M32949
d2-Thailand	D82-137	1982	Thailand/Bangkok	13	U87341
d2-Senegal	ArD20761	1974	Senegal/Kedougou	6	M32957
d2-Ivory Coast	DakA578	1980	Ivory Coast	6	M32958

IAL = viruses donated by Adolfo Lutz Institute and isolated on C6/36 *Aedes albopictus* cell line. IEC = viruses donated by Evandro Chagas Institute and isolated in the C6/36 *Aedes albopictus* cell line. SMRP = strain isolated at the School of Medicine of Ribeirão Preto. Notation for Brazilian strains: Brazil (BRA)/state (region); S = South; SE = Southeast; N = North; NE = Northeast; MW = Midwest. ¶Sequence data obtained in the present study.

Phylogenetic analysis

A data set of 72 E/NS1 junction sequences was used for comparison and phylogenetic analyses (Table 1). These data included the 25 sequences of Brazilian dengue viruses first described in the present study (16 DEN-1; 9 DEN-2) combined with 27 sequences of Brazilian dengue viruses previously reported (6 DEN-1; 21 DEN-2), and 20 global reference sequences of different genotypes of both serotypes (10 DEN-1; 10 DEN-2) deposited in GenBank. None of the viruses compared had additions or deletions in the genomic region studied. Alignments were done manually using nucleotide sequences. Phylogenetic analyses and construction of phylogenetic trees for both DEN-1 and DEN-2 strains were done using the neighbor-joining method and p-distance (MEGA software, version 2.1, Temple, AZ, USA) (23). Sequences from representative strains of dengue serotypes 3 and 4 (strain H87, Philippines, 1956) and 4 (strain 814669, Dominican Republic, 1981) obtained from GenBank (accession numbers M93130 and M14931, respectively) were used as an outgroup to root the trees. The bootstrap method, with 500 replicates, was used to estimate the reliability of the predicted trees.

Results

In the present study, we obtained and analyzed original partial nucleotide sequences from 16 strains of DEN-1 and nine strains of DEN-2 selected to represent the viruses circulating in all five Brazilian geographical regions. All viruses had undergone only one passage in C6/36 cells and nucleotide sequences were deposited in GenBank (accession numbers AY159257-AY159279, AY277245 and AY277246). Analysis of these nucleotide sequences in comparison to reference virus sequences revealed new mutations for both DEN-1 and

DEN-2, probably representing evolutionary mutations. The majority occurred in the third base of codons resulting in silent mutations. All mutations in the DEN-1 sequences were silent, whereas for DEN-2, mutations in three codons resulted in amino acid changes between residues of the same non-polar hydrophobic class.

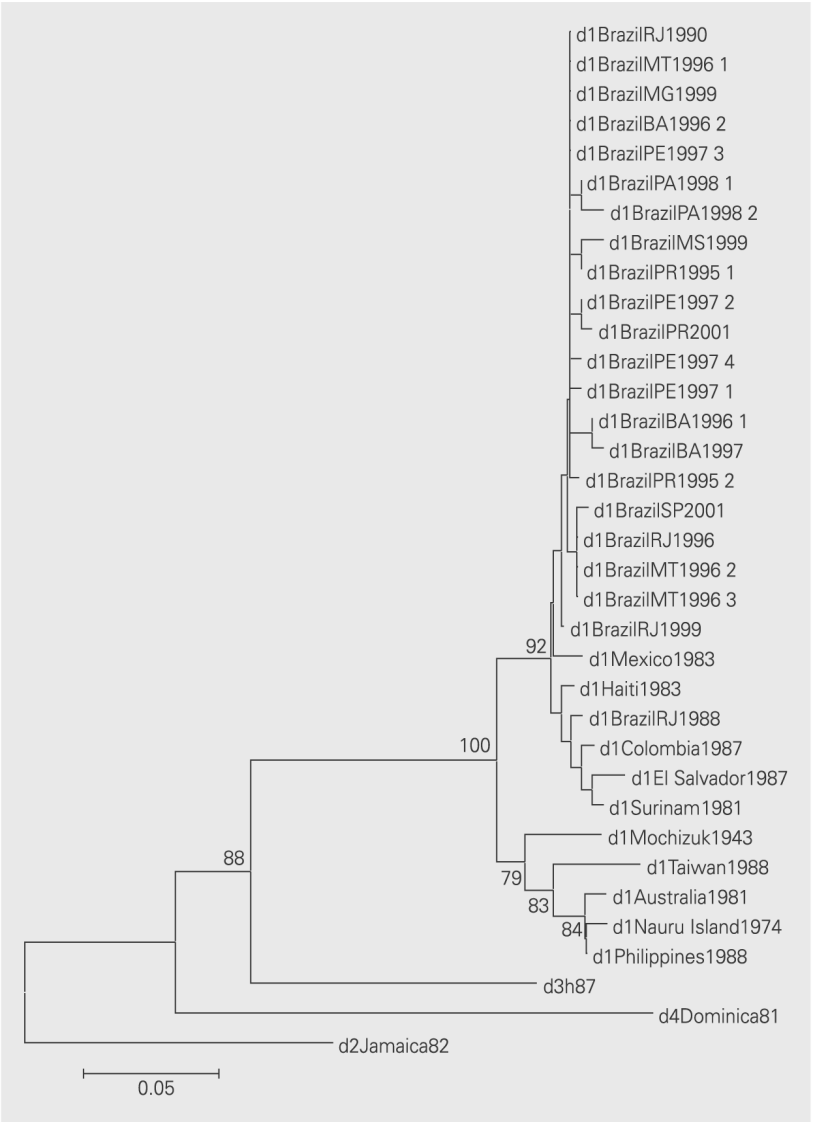

Figure 1. Phylogenetic relationships among dengue-1 (DEN-1) viruses. Phylogenetic tree generated by neighbor-joining analysis of nucleotide sequences from the E/NS1 junction of 32 strains of DEN-1 and representatives of serotypes 2, 3 and 4. Viruses are listed by strain abbreviation for state and year of isolation (see Table 1). Horizontal branch lengths are drawn to scale. Bootstrap values (500 replicates) are shown for some key nodes that connect the genotypic groups of DEN-1.

The phylogenetic trees generated by neighbor joining analysis of nucleotide sequences are presented in Figures 1 and 2. Some reference nucleotide sequences of different genotypes of both DEN-1 and DEN-2 viruses isolated in other countries and published in the literature, as well as nucleotide sequences from Brazilian isolates available in the GenBank database, were also included for phylogenetic purposes. Brazilian DEN-1 strains segregated into one large group along with reference strains from the Americas and the Caribbean (Colombia, Surinam, El Salvador, Haiti, and Mexico), whereas Brazilian DEN-2 segregated into a group along with reference strains from the Americas and Southeast Asia (Jamaica, Thailand, and Vietnam). Bootstrap values of statistical significance were obtained for these groups. There was no segregation of Brazilian DEN-1 viruses according to regions or states but some nucleotide variations were observed between viruses from the same regions. However, for Brazilian DEN-2 viruses most strains isolated in 1990 from the Southeast region presented a distinct segregation pattern from the viruses isolated more recently. The Brazilian DEN-1 strains analyzed in this study presented 97.9% similarity amongst themselves and 96.8% similarity to other samples belonging to the American/Caribbean genotype, the same genotype as the sample isolated in Brazil in 1988 (6). Brazilian DEN-2 isolates presented 97.3% similarity amongst themselves and 94.2% similarity to other samples of the Asian/American-Asian genotype, the same genotype of two viruses isolated in Brazil in 1990 and 1991 (16).

Discussion

Epidemics of DF reemerged in Brazil in 1981 when an outbreak caused by DEN-1 and DEN-4 viruses occurred in the Northern region (Roraima State). Subsequently, in 1986, the first outbreak of greater proportions caused by DEN-1 occurred in the met-

ropolitan area of Rio de Janeiro and then spread towards the urban areas in the Northeast and Midwest regions of Brazil. In 1990, a new epidemic broke out in Rio de Janeiro, now related to the introduction of DEN-2. Since then, with the spread and circulation of more than one serotype, several Brazilian regions have reported outbreaks with severe illness and deaths (17,24-26). From 1995 to 2001, an increasing number of DF and DHF cases has been observed in several urban areas of the country (17). Recently, DEN-3 has been isolated in Brazil but its association with DHF has not been clearly established (27,28).

In a country of continental proportions and incredible heterogeneity of people and environment such as Brazil, phylogenetic studies of dengue isolates must represent all regions of the country. The present study contributes to this viewpoint in the sense that viral strains from Brazilian endemic regions poorly studied before (North and South) were also analyzed. Although all strains were isolated from patients with DF, they represent a sample of the dengue viruses circulating in Brazil during the study period, and we assume that they would also carry the ability to cause DHF/DSS. Supporting this assumption, evolutionary divergence of DF- versus DHF-associated viruses (serotype 2) from Thailand was not observed, denoting that one particular strain has the potential to cause both DF and DHF in different hosts (13).

Different genomic regions of Brazilian dengue viruses have been previously studied by comparative analysis of partial nucleotide sequences for both serotypes 1 and 2. For DEN-1, comparative analysis of a protein E gene segment (nucleotides between 52 and 288) of two DEN-1 samples isolated in Brazil, one in 1986 and another in 1990, classified them into the American/Caribbean genotype (9). Analysis of the DEN-2 E protein gene (nucleotides 1685 to 2504) of twelve samples isolated between 1990 and 1995 in the States of Rio de Janeiro, Ceará, Bahia, and Alagoas classified them into the Asian/American-Asian genotype (18). Similar results were also obtained by nucleotide sequence analysis of another E protein gene segment (nucleotides 85 to 282) of three DEN-2 samples isolated in Brazil in 1990 (7). Nucleotide sequence analysis of the NS5/3'NC junction area of two DEN-1 samples isolated in 1990 and 1994 from Rio de Janeiro and São Paulo States, respectively, classi-

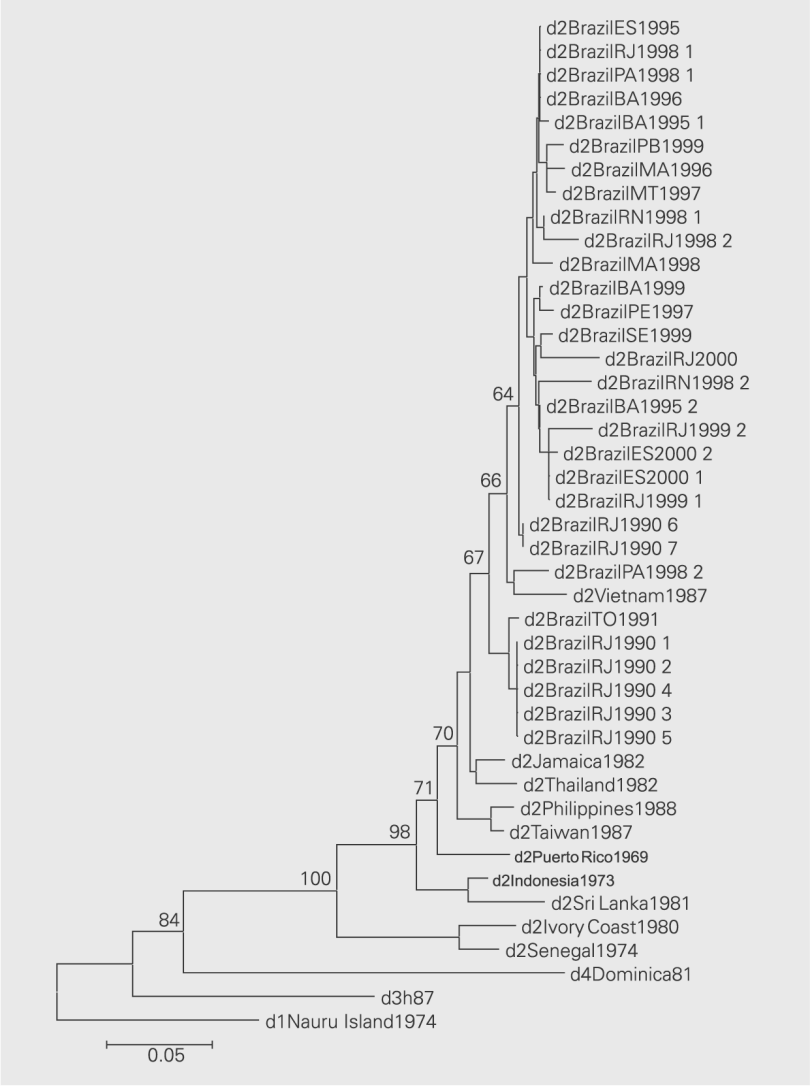

Figure 2. Phylogenetic relationships among dengue-2 (DEN-2) viruses. Phylogenetic tree generated by using neighbor-joining analysis of nucleotide sequences from the E/NS1 junction of 40 strains of DEN-2 and representatives of serotypes 1, 3 and 4. Viruses are listed by strain abbreviation for state and year of isolation (see Table 1). Horizontal branch lengths are drawn to scale. Bootstrap values (500 replicates) are shown for some key nodes that connect the genotypic groups of DEN-2.

fied these viruses into the American/Caribbean genotype (19). Recently, a nucleotide sequence of the entire genome from a Brazilian DEN-2 virus was determined for the first time, and a phylogenetic analysis also classified it into the Asian/American-Asian genotype (29). Nucleotide sequences of the entire genome were also determined recently from four Brazilian DEN-1 viruses (30).

Regarding the E/NS1 junction region, many authorities advocate it as one of the best segments for molecular epidemiology and phylogenetic studies of dengue viruses, since it is a genomic region not involved in immune recognition/stimulation and characterized by a uniform rate of random mutations that occur most frequently in the third base of codons (silent mutations) (6,8,12). Only two DEN-1 viruses isolated in Brazil in the mid-80's and 21 DEN-2 viruses isolated in the 90's have been previously studied regarding the E/NS1 junction region (6, 16,20). Comparing all these sequences with others of different origins and genotypes it becomes clear that, in spite of the detection of new evolutionary mutations in the genome of Brazilian dengue viruses in the last 15 years, phylogenetic analysis did not identify the circulation of new genotypes for either DEN-1 or DEN-2 in our country.

Although genetic and antigenic differences in dengue virus strains have become evident, the lack of animal models of the disease has made it difficult to detect differences in virulence among dengue viruses. The most accepted hypothesis to explain the development of serious illnesses by some people infected with dengue virus suggests that sequential infection with different serotypes followed by antibody-dependent enhancement of infection play a major role in the pathogenesis of DHF/DSS (2,14,15). However, DEN-1 circulating in America is capable of producing DHF/DSS even in people not affected by sequential infection. In addition, despite co-circulation of several serotypes in Central America and the Caribbean since 1970, only in 1981 was the first outbreak of DHF/DSS observed in Cuba. Epidemiological studies in Latin America have demonstrated that sequential infections and co-circulation of different serotypes in a population at risk are not sufficient for DHF/DSS occurrence and other factors related to virus, host, vector, and environment must be involved (2,31-39).

Phylogenetic studies of many different dengue virus samples have led to the association between specific genotypes and the presentation of more or less severe disease. In Cuba, the 1981 outbreak was associated with the introduction of a new DEN-2 genotype, different from the genotype that had been circulating in that country. The DEN-2 genotype circulating in Central America before 1981 was associated with DF. The introduction of a different genotype imported from Southeast Asia coincided with the appearance of DHF/DSS in four different American countries (Venezuela, Brazil, Colombia, and Mexico; 6,16). Even though specific dengue genotypes have been associated with DHF/DSS, our study shows that the growing number of DHF/DSS cases in Brazil in the last years seems not to be associated with the introduction of a new genotype. Of note, the DEN-2 genotype circulating in Brazil since the 90's is the Southeast Asian genotype but there was no explosive outbreak of DHF/DSS during the first years after its introduction into the country. There are no clear data accurately showing when the Southeast Asian DEN-2 genotype was introduced into Brazil. Rico-Hesse et al. (16) analyzed two DEN-2 viruses isolated in Venezuela and Colombia some time around 1987, and concluded that they belonged to the Southeast Asian genotype. In Brazil, this genotype was probably introduced either at the same time or at some point during 1988, like in other areas of the Americas (6,8,16, 18,20,38,40).

The present study is the largest one involving serotypes 1 and 2 of Brazilian den-

gue viruses regarding genomic variability. The 25 new nucleotide sequences determined in this study allowed us to expand considerably the number of Brazilian dengue virus strains evaluated so far regarding their genomic variability and molecular epidemiology. As a huge tropical country with many frontiers, a high rate of *A. aegypti* infestation and a growing migration flow, Brazil has been recognized as a high risk for the introduction and circulation of new serotypes and/or genotypes of dengue virus (25,27). To date, this has been true for the introduction and circulation of new serotypes, but not for new genotypes, as demonstrated by the present study. The reasons why introduction of new genotypes did not occur in Brazil in the last years are not understood. In other tropical countries the co-circulation of two or more genotypes of dengue virus is commonplace (36). Actually, we cannot rule out the possibility that new genotypes of dengue virus have already been introduced in Brazil but, for reasons not clearly defined, they were not successful in establishing themselves and consequently spreading to the whole country. Ecological, vector and host factors may be involved and should be addressed in further studies. Efforts should be directed at obtaining the complete genomic sequence of Brazilian dengue viruses so that a more detailed comparative analysis could be done. The genomic variability of Brazilian dengue virus strains isolated from patients with DHF/DSS should also be studied. Continuous monitoring of the introduction of new serotypes as well as new genotypes in Brazil is necessary so that control measures can be promptly implemented in order to reduce the potential risk for more serious epidemics.

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