Article/Artigo

Genetic diversity of dengue virus serotypes 1 and 2 in the State of Paraná, Brazil, based on a fragment of the capsid/premembrane junction region

Diversidade genética dos vírus dengue sorotipos 1 e 2, no Estado do Paraná, Brasil, baseada no fragmento da junção do gene capsídeo/pré-membrana

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ABSTRACT

Introduction: The precise identification of the genetic variants of the dengue virus is important to understand its dispersion and virulence patterns and to identify the strains responsible for epidemic outbreaks. This study investigated the genetic variants of the capsid-premembrane junction region fragment in the dengue virus serotypes 1 and 2 (DENV1-2). **Methods:** Samples from 11 municipalities in the State of Paraná, Brazil, were provided by the Central Laboratory of Paraná. They were isolated from the cell culture line C6/36 (*Aedes albopictus*) and were positive for indirect immunofluorescence. Ribonucleic acid (RNA) extracted from these samples was submitted to the reverse transcription polymerase chain reaction (RT-PCR) and nested PCR. **Results:** RT-PCR revealed that 4 of the samples were co-infected with both serotypes. The isolated DENV-1 sequences were 95-100% similar to the sequences of other serotype 1 strains deposited in GenBank. Similarly, the isolated DENV-2 sequences were 98-100% similar to other serotype 2 sequences in GenBank. According to our neighbor-joining tree, all strains obtained in this study belonged to genotype V of DENV-1. The DENV-2 strains, by contrast, belonged to the American/Asian genotypes. **Conclusions:** The monitoring of circulating strains is an important tool to detect the migration of virus subtypes involved in dengue epidemics.

Keywords: Concurrent infection. RT-PCR. *Flavivirus*. Genetic variation.

RESUMO

Introdução: A identificação precisa da variante genética do vírus da dengue é importante para compreender a dispersão, virulência e identificação das cepas responsáveis pelas epidemias. O objetivo da pesquisa foi investigar a variação genética do fragmento da junção do gene capsídeo/pré-membrana dos sorotipos 1 e 2. **Métodos:** Amostras de onze municípios do Estado Paraná, Brasil, foram cedidas pelo Laboratório Central do Paraná e consistiam em isolados de cultura de células da linhagem C6/36 (*Aedes albopictus*), positivos para técnica de imunofluorescência indireta. O *Ribonucleic acid* (RNA) dessas amostras foi extraído, seguido da transcrição reversa, reação em cadeia da polimerase (PCR) e *nested* PCR**. Resultados:** Co-infecção por DENV-1 e 2 (virus da dengue 1 e 2) foi observada em quatro pacientes, através da técnica *Reverse transcriptase-polymerase chain reaction* (RT-PCR). Para o DENV-1 a porcentagem de similaridade variou de 95 a 100% comparando com cepas do Genbank. Para o DENV-2 a porcentagem de similaridade variou de 98 a 100%. De acordo com o cladograma gerado, todas as cepas deste estudo se agruparam no genótipo V para DENV-1. Para o DENV-2 foi encontrada a cepa referente ao genótipo asiático/americano. **Conclusões:** O monitoramento das cepas circulantes torna-se uma ferramenta importante na detecção da migração dos subtipos do vírus da dengue envolvidos em epidemias.

Palavras-chaves: Infecção simultânea. RT-PCR. *Flavivirus*. Variação genética.

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INTRODUCTION

The virus that causes dengue fever belongs to the genus *Flavivirus* (*Flaviviridae*). Its 11kb RNA genome contains an open reading frame (ORF) that encodes 3 structural and 7 non-structural proteins. The structural proteins are: capsid (C), membrane (M), and envelope (E); and the non-structural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5[1].

Dengue virus exhibits substantial genetic diversity, most notably in the existence of 4 distinct serotypes and several genotypes. Attempts to identify these genetic variants have been made by sequencing certain regions of the viral genome. For instance, the E protein, which is the main target of virus selection in nature, has been sequenced often[2-4]. However, Singh and Seth[5] suggested that fragments of the capsid-premembrane junction region (CprM) might be a faster and cheaper alternative for sequencing short regions. Since then, CprM has been used by a number of authors[6-8]. Genes that code for non-structural proteins have also been sequenced and used in phylogeny studies[9]. Ascertaining the molecular diversity of the dengue virus is important to assess the impact of these genetic variants in the human population, as well as the virulence, distribution, and origin of the various strains.

In this study we genetically characterized the capsid-premembrane junction region fragment of the dengue virus in patients who tested positive for dengue in 2010.

METHODS

The samples, which were provided by the Laboratório Central do Paraná, were isolated from the cell culture line C6/36 (*Aedes albopictus*) and were positive for indirect immunofluorescence (IFA). The human blood samples were collected from

March-May, 2010, from autochthonous cases in the State of Paraná, Brazil. Until June 2010, 147 (37%) municipalities had 15,012 autochthonous cases of dengue[10]. Municipalities were sampled in the western region of the State of Paraná (Foz do Iguaçu, 25°32′48″S, 54°35′17″W; São Miguel do Iguaçu, 25°20′49″S, 54°14′20″W; Realeza, 25°48′45″S, 53°33′45″W; Toledo, 24°41′15″S, 53°41′15″W; Pato Bragado, 24°41′15″S, 54°11′15″W; and Nova Aurora, 24°33′45″S, 53°18′45″W) and northern region (Maringá, 23°26′15″S, 51°56′15″W; Assaí, 23°18′45″S, 50°48′45″W; Planaltina do Paraná, 23°03′45″S, 52°56′15″W; Sertanópolis, 23°03′45″S, 51°03′45″W; and Alvorada do Sul, 22°48′45″S, 51°11′15″W) (**Figure 1**).

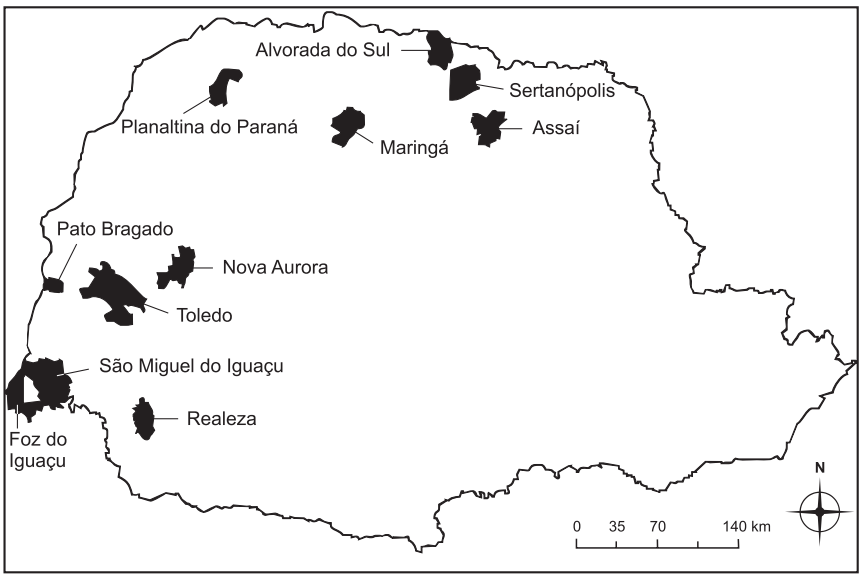

FIGURE 1 - Municipalities of the State of Paraná (Brazil) where blood suspected to be infected with the dengue virus was collected.

RNA extraction from the cell supernatant was conducted using a QIAmp Viral Mini Kit (Qiagen, USA), following the manufacturer's protocol. The primers were the same as in Lanciotti et al.[11]. To obtain cDNA, 2,000ng of viral RNA and 1µL of primer D2 (50pmol) were placed in a thermocycler for 5 min at 70°C. The sample was kept on ice and 5µL of 5× buffer, 0.5µL of dNTPs (200mM), and 20U of AMV reverse transcriptase (Promega) were added. The volume of the mixture was brought to 25µL by adding DEPC-treated water (0.1%). The sample was kept in a thermocycler for 90 min at 42°C and 15 min at 70°C. The negative control contained water instead of RNA.

The amplification reaction was conducted using 3µL of cDNA, 2.5µL of 10× buffer, 1µL of primer D1 (20pmol), 1µL of primer D2 (20pmol), 1.5µL of $MgCl_2$ (25mM), 0.5µL of dNTPs (200mM), and 3U of AmpliTaq Gold DNA polymerase (Applied Biosystems). The volume of the mixture was brought to 25µL by adding DEPC-treated water (0.1%). The following cycling program was used to obtain products: 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min.

For nested PCR, 3µL from the first amplification reaction (undiluted and diluted 1/100), 2.5µL of 10× buffer, 1µL of primer D1 (20pmol), 1µL of each primer TS1, TS2, TS3, and TS4 (20pmol), 1.5µL of $MgCl_2$ (25µM), 0.5µL of dNTPs (200µM), and 3U of AmpliTaq Gold DNA polymerase (Applied Biosystems) were combined. The mixture was brought to 25µl by adding DEPC-treated water (0.1%). The following cycling program was used to obtain products: 20 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The amplified products were visualized on a 2% agarose gel.

The purification reaction was performed using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The samples were sent to the Center for Human Genome Studies (University of São Paulo-São Paulo/SP) for sequencing on an ABI 3730 DNA Analyzer (Applied Biosystems). Consensus sequences were obtained using the Staden Package version 1.5[12]; sequence alignment and translation to amino acids were performed in BioEdit version 7.0.0[13], a ClustalW tool[14]. The sequences obtained from our samples were compared with 39 sequences from various parts of the world available from GenBank. Phylogenetic and sequence similarity analyses were performed using the neighbor-joining method implemented by the program Mega 4[15], using the Kimura 2-parameter model and 1,000 bootstrap replications.

RESULTS

RT-PCR identified 4 human blood samples that were co-infected with DENV-1 and -2. When the samples were amplified using the first PCR product without dilution, 2 bands of different intensity were visualized in the gel, showing the serotypes with the highest and lowest number of copies. However, when the first PCR product was diluted 100 times, only one band was detected, indicating the predominant serotype (**Table 1** and **Figure 2**).

Analysis of the 368-bp sequences obtained from CprM revealed 2 strains of DENV-1 with 14 polymorphic sites: GenBank: JN086990 (Foz do Iguaçu and Sertanópolis); GenBank:JN086991(Planaltina do Paraná). Analysis of the 360-bp sequences obtained from the same gene revealed 1 strain of DENV-2:GenBank: JN086992 (Alvorada do Sul and Pato Bragado). The 2 DENV-1 strains were distinguished by 13 transitions and 1 transversion. Transitions: G↔A (sites 207 and 351); T↔C (sites 81, 267, 306, 309, and 360); A↔G (site 180); C↔T (sites 69, 276, 297, 350, and 354). Transversion: G↔C (site 264). Changes were observed in codons 69 (Met→Ile) and 117 (Ala→Val) of DENV-1, corresponding to nucleotide substitutions

TABLE 1 - Samples from patients in 11 municipalities of the State of Paraná, Brazil, with the dengue virus detected by RT-PCR and indirect immunofluorescence.

		RT-PCR		
Region	**Samples**	**undiluted**	**diluted 1/100**	**IFA**
Northern	Maringá	DENV-2	DENV-2	DENV-2
	Assaí	DENV-1	DENV-1	DENV-1
	Planaltina do Paraná	DENV-1	DENV-1	DENV-1
	Sertanópolis	DENV-1	DENV-1	DENV-1
	Alvorada do Sul	DENV-2	DENV-2	DENV-2
Western	Pato Bragado	DENV-1/DENV-2	DENV-2	DENV-2
	Realeza	DENV-1/DENV-2	DENV-2	DENV-2
	Toledo	DENV-2	DENV-2	DENV-2
	São Miguel do Iguaçu	DENV-1/DENV-2	DENV-1/DENV-2	DENV-1
	Nova Aurora	DENV-1/DENV-2	DENV-1/DENV-2	DENV-1
	Foz do Iguaçu	DENV-1	DENV-1	DENV-1

RT-PCR: reverse transcriptase-polymerase chain reaction; **IFA:** immunofluorescence assay; **DENV:** dengue virus serotype.

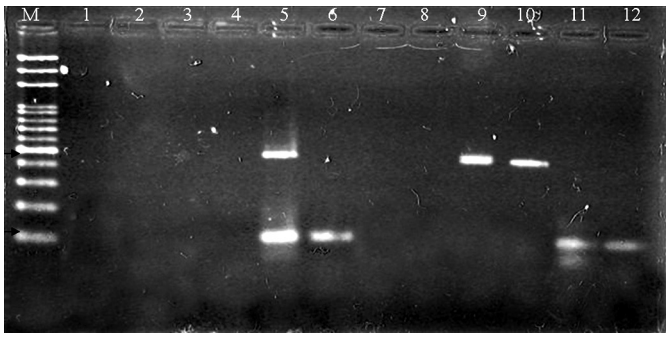

FIGURE 2 - Agarose gel (2%). M:100-pb ladder (Amresco); 1-4 Non-amplified RNA samples; 5: undiluted (co-infection with DENV-1 and -2); 6: diluted 1/100 (DENV-2); 7-8: negative control; 9: undiluted (DENV-1); 10: diluted 1/100 (DENV-1); 11: undiluted (DENV-2); 10: diluted 1/100 (DENV-2). DENV-1 and DENV-2: dengue virus serotype 1 and 2, respectively.

at 207 and 350 and 351, respectively. Amino-acid substitutions occurred between amino acids with similar biochemical properties.

The DENV-1 sequences obtained by us were 95-100% similar to the sequences of the other serotype 1 strains deposited in GenBank. Similarly, our DENV-2 sequences were 98-100% similar to other serotype 2 sequences on that database. According to our neighbor-joining tree, all strains obtained in this study belong to genotype V of DENV-1. The DENV-2 strain belonged to the American/Asian genotypes (**Figures 3** and **4**).

DISCUSSION

Co-infection with multiple dengue virus serotypes is common in hyper-endemic areas where multiple serotypes circulate[6,16], most especially when there is a high density of vectors and people susceptible to dengue. Concomitant infection with DENV-1 and 2 in human blood was reported in Brazil by Rocco et al.[17], Santos et al.[18], and Cunha et al.[19]. Furthermore, simultaneous infections by different dengue virus in mosquitoes have been reported[20]. Female *A. aegypti* may be able to transmit both viruses simultaneously[17]. Pessanha et al.[21] detected *A. aegypti* larvae that were positive for more than 1 serotype, also showing that dual infection can be passed on to offspring via transovarial transmission.

The RT-PCR technique used in our study was more efficient than IFA to detect co-infection. Repeated trials have confirmed these results obtained with RT-PCR. Therefore, if we had only used IFA in this study, the detection of serotypes would have been underestimated.

The replacement of methionine and alanine (amino acid 69) with isoleucine and valine (amino acid 117), respectively, observed in our results, had been previously identified in the JN086991 strain of DENV-1 isolated in samples from Rio de Janeiro (GenBank: HQ026762) and São Paulo (GenBank: GU131863). The 2 strains identified clustered with other genotype V sequences, the only genotype circulating in Brazil thus far[22]. In 2001, Santos et al.[23] also sequenced a third stain in the State of Paraná, besides the 2 strains found by us, totaling 3 strains in that state.

Two subclades, A and B, clustered in the neighbor-joining tree of the DENV-1 sequences with 96% bootstrap support. The DENV-1 strain JN086990 is more closely related to strains isolated in French Guiana (GenBank: EU482591), Venezuela (GenBank: EU518605), and Puerto Rico (GenBank: FJ850103). The JN086991 DENV-1

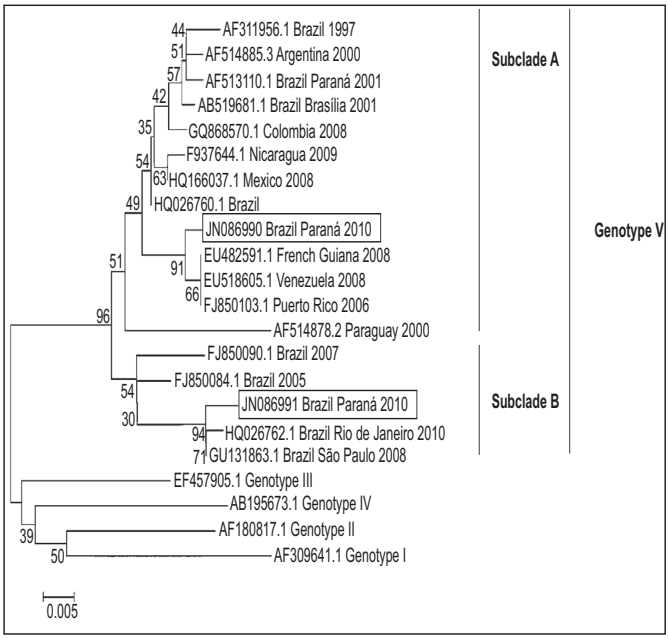

FIGURE 3 - Neighbor-joining tree for the capsid-premembrane junction region (CprM) fragment of DENV-1. The tree was constructed using the Kimura 2 parameter model and includes sequences obtained by us and from GenBank. The bootstrap values (1,000 replicates) can be found above the main nodes.

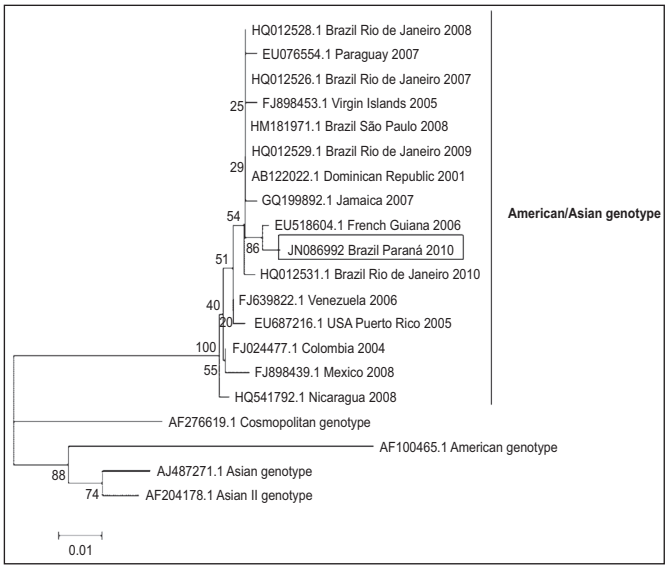

FIGURE 4 - Neighbor-joining tree of the capsid-premembrane junction region (CprM) fragment of DENV-2. The tree was constructed using the Kimura 2 parameter model and includes sequences obtained by us and from GenBank. The bootstrap values (1,000 replicates) can be found above the main nodes.

strain of subclade B clustered with the Brazilian strains isolated from 2008-2010. The DENV-1 genotype V strains are grouped in 2 different phylogenetic branches, reflecting an earlier divergence.

The DENV-2 strain JN086992 clustered with the American/Asian genotypes with 100% bootstrap support, and was closest to the strain from French Guiana (GenBank: EU518604) that was isolated in 2006. The American genotype has been associated with less virulent strains. It may have been replaced by the southern Asiatic genotype, once it has not been isolated where the southern Asiatic genotype presently circulates. The American/Asian DENV-2

genotype has predominated in Brazil during its 19 years of circulation (1991-2008). An association between certain DENV-2 genotypes and dengue hemorrhagic fever has been suggested[24,25].

Evolutionary studies have shown that the genetic diversity of the dengue virus is increasing, and that mutations are responsible for this. Genetic diversity in that virus can also be generated by recombination. For this to happen, different genotypes of the same viral serotype have to co-infect the same individual, and the genomes that infect the same cell form a hybrid RNA molecule. Simultaneous infection of *A. aegypti* by different viruses during outbreaks and epidemics, resulting in a high rate of viral replication, allow for the emergence of genetic changes. The increased genetic diversity of the dengue virus can have serious consequences, such as viruses with an expanded range of pathogenic properties and increased transmissibility and virulence[26-28].

The incidence of dengue virus subtypes, combined with multiple serotypes circulating at a given location, can generate genetic variation within the dengue virus serotypes. Studies that monitor and sequence the circulating serotypes are important to keep track of these genetic events, in order to diagnose major epidemics, and the geographical migration of these strains.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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