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CASE REPORT

CO-INFECTION OF DENGUE VIRUS BY SEROTYPES 1 AND 4 IN PATIENT FROM MEDIUM SIZED CITY FROM BRAZIL

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SUMMARY

The natural co-infection with dengue virus can occur in highly endemic areas where different serotypes have been observed for many years. We report one case of DENV-1/DENV-4 co-infection in human serum detected by molecular tests. Phylogenetic analysis of the sequences obtained indicated the presence of genotype V and II for DENV-1 and DENV-4, respectively.

KEYWORDS: Brazil; Dengue; Co-infection; *Flavivirus*.

INTRODUCTION

Dengue is a major public health issue in tropical and subtropical countries[18,33]. Dengue viruses (DENVs) belong to the *Flavivirus* genus, Flaviviridae family, and are single stranded RNA viruses, with four immunologically related serotypes (DENV-1 to 4) and each serotype is phylogenetically divided into different genotypes[21,45] that have a increased genetic diversity as a consequence of the exponentially growing human circulation[21,44,46].

Infection by any DENV serotype can cause different clinical diseases that range from an acute self-limited febrile illness, the dengue fever (DF) to life-threatening syndromes characterized by hemorrhagic and capillary leak, the dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS)[17,29]. DENV infections cause 25 to 100 million cases of DF and 250,000 cases of DHF/DSS per year in the world. Moreover, it is estimated that 2.5 to 3 billion people are at risk of infection in more than 100 countries[23,49].

Historically, the state of São Paulo, Brazil, has been presenting dengue outbreaks since 1990 when DENV-1 was introduced in the state. Subsequent DENV introductions occurred in 1997 and 2002, when DENV-2 and DENV-3 caused huge epidemics, with an increasing number of mild and severe cases[16,36,38].

DENV-4 had a brief circulation in the country in 1982, with a focal epidemic at the Northwestern region of the Brazilian Amazon. No further cases of DENV-4 infection had been registered in the country until 2008, when the virus was detected in three patients from Manaus, who had no international traveling history[14].

DENV-4 reemerged in the country in 2010 and 2011 in the municipalities of Boa Vista and Cantá in Roraima State[42]. The virus then spread to different geographic regions of Brazil with cases reported in the northern states (Roraima, Amazonas, Pará)[4], northeastern states (Bahia, Pernambuco, Piauí) and southeastern states (Rio de Janeiro, São Paulo)[32,40].

São José do Rio Preto (SJRP), which is located in the northwestern region of São Paulo State, has been presenting an endemic circulation of dengue for 10 years and all four serotypes have been detected in the city[34,40]. In regards to dengue serotypes circulating in the years 2011-2012, 527 samples were examined and 359 (68%) were positive for dengue virus (311 for DENV-1, 23 for DENV-2, 24 for DENV-4, 1 for DENV-1 and DENV-4 (unpublished). In this manuscript we report a DENV-1/DENV-4 co-infection in a patient from SJRP.

CASE REPORT

The sample RP/BR/2012/507 was obtained from a patient with DF in SJRP, São Paulo, Brazil, in March 12th 2012. His serum was NS1 enzyme-linked immunosorbent assay (Bio-Rad) positive. RNA extraction was performed with QiAmp Viral RNA (QIAGEN, Germany). RNA was examined using Multiplex-Nested-PCR (M-N-PCR) to detect DENV 1-4[8]. The first RT-PCR was performed using *Flavivirus* generic primers based on non-structural protein (NS5). In the second PCR, nested assays

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based on multiplex or conventional systems were used with species-specific primers to detect and identify dengue viruses (DENV1-4). PCR products were loaded onto a 1.5% agarose gel and visualized under ultraviolet light. Amplicon sizes were determined by comparison with a 100 bp DNA ladder (Invitrogen). Precautions to avoid contamination were followed, positive and negative controls were used in all reactions, and the procedure was reproduced several times[7]. Another protocol[27] that amplifies the capsid protein (C) was also performed to confirm the presence of DENV-1 and DENV-4 in the same sample (Fig. 1).

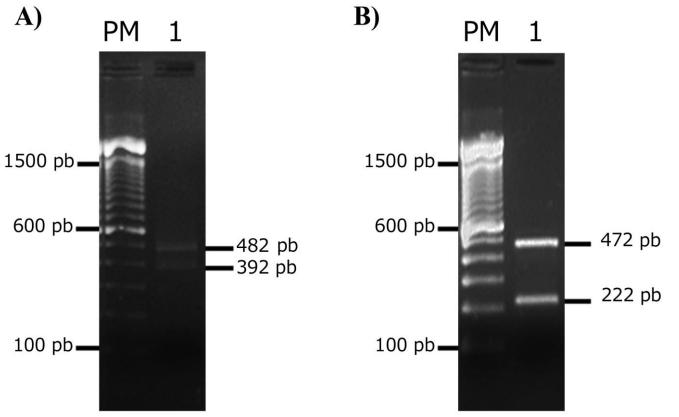

Fig. 1 - Electrophoresis in agarose gel showing the amplified products on two methodologies. PM = DNA ladder = 100 bp and 1 = Co-infection Sample RP/BR/507/2012, dengue 1 and 4 strains. (A) Lanciotti (1992) PCR assay showing the amplicon of 482 bp and 392 bp DENV-1 of DENV-4; (B) Bronzoni (2005) PCR assay showing the amplicon of 472 bp and 222 bp DENV-1 of DENV-4.

The fragments amplified by confirmatory method were purified and sequenced using the BigDye v3.1 (Applied Biosystems, Foster City, CA, USA) in an ABI3130 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were analyzed using the DS Gene 2.0 Software (Accelrys, USA) and confirmed as DENV-1 and DENV-4. These sequences were deposited at GenBank (Accession numbers: DENV-1 JQ950328 and DENV-4 JQ950329) (Table 1). Thirty-one sequences of dengue virus serotype 1 and thirty-three sequences of serotype 4 that correspond to the Capsid Protein gene (C) of Dengue viruses were used for phylogenetic analysis (Table 1). Multiple sequence alignment was conducted using ClustalW as implemented in BioEdit 7.1.3[19].

Fragments of 365 bp (from DENV-1) and 257 bp (from DENV-4) with sequences that encode the capsid gene were used in the alignment. Using as reference the DENV-1 Mochizuki strain by Japan (Genbank access number AB074760.1) and DENV-4 H778494 strain for Brazil (Genbank access number JQ513335.1) we identify relevant amino acid substitutions: two substitutions were identified in polyprotein of DENV-1, R97G (Arginine to Glycine) and M107V (Methionine to Valine), as for DENV-4 polyprotein, three aminoacid substitutions were identified: F49L (Phenylalanine to Leucine), R53F (Arginine to Phenylalanine), S57F (Serine to Phenylalanine) (Fig. 2A and 2B).

The phylogenetic tree was designed with tools of MEGA 5 software (http://www.megasoftware.net). The statistical method was Neighbor-Joining with Tamura-Nei model[41] and the phylogeny analysis was tested by bootstrap method with 1000 replications.

According to the phylogenetic tree created by genotyping of DENVs strains from the co-infection detected in SJRP, DENV-1 that circulated

Table 1
DENV-1 and DENV-4 sequences used for phylogenetic analysis, including serotype and genotype, isolation sites, isolation year, strain and GenBank accession numbers

Serotype and Genotype	Genbank Accession	Isolation site	Strains	Isolation Year
Serotype 1				
D1G1	AB074760.1	Japan	Mochizuki	2001
D1G1	AF180817.1	USA	16007	1999
D1G1	JN054255.1	Sri Lanka	DV1_SL_2010b	2010
D1G1	GQ398255.1	Singapore	SG/07K3640DK1/2008	2008
D1G1	DQ193572.1	Fujian/China	Fj231/04	2004
D1G2	FJ196841.1	Guangzhou/China	GD54/03	2003
D1G2	FJ196842.1	Guangzhou/China	GD66/03	2003
D1G2	EF440432.1	Australia	ET243	2000
D1G2	EF025110.1	China	71/02GZ	2006
D1G3	AB074761.1	Japan	A88	2002
D1G3	DQ285561.1	Seychelles/ France	1480/04	2004
D1G4	U88535.1	Nauru Island	Clone WestPac	1997
D1G4	M23027.1	Nauru Island	Nauru Island	1994
D1G5	FJ850087.1	Northern Brazil	BR/BID-V2395/2006	2006
D1G5	AF226685.2	Rio de Janeiro/Brazil	Den1BR/90	2000
D1G5	AF513110.1	Curitiba/Brazil	BR/01-MR	2002
D1G5	FJ850075.1	Northern Brazil	BR/BID-V2381/2002	2002
D1G5	FJ850090.1	Northern Brazil	BR/BID-V2398/2007	2007

Table 1
DENV-1 and DENV-4 sequences used for phylogenetic analysis, including serotype and genotype, isolation sites, isolation year, strain and GenBank accession numbers (cont.)

Serotype and Genotype	Genbank Accession	Isolation site	Strains	Isolation Year
Serotype 1				
D1G5	FJ850084.1	Northern Brazil	BR/BID-V2392/2005	2005
D1G5	JX502769.1	Brazil	RR_DENV1_09007	2009
D1G5	FJ850070.1	Northern Brazil	BR/BID-V2374/2000	2000
D1G5	FJ850071.1	Northern Brazil	BR/BID-V2375/2000	2000
D1G5	AB519681.1	Brasilia/Brazil	SB 01057805 (DF02)	2002
D1G5	FJ850081.1	Northern Brazil	BR/BID-V2389/2004	2004
D1G5	HM043709.1	Brazil	55/2009ES	2009
D1G5	JN086990.1	Brazil	A	2010
D1G5	JN086991.1	Brazil	B	2010
Serotype 4				
D4G1	FJ196850.1	Guangzhou/China	GD09/1990	1990
D4G1	AF289029.1	Guangzhou/China	Guangzhou B5	2000
D4G1	AF177542.1	Mindanao/Philippines	Mindanao/BDJ/1995	1995
D4G1	GQ868594.1	Philippines	PH/BID-V3361/1956/1956	1956
D4G2	EU854295.1	Puerto Rico	US/BID-V1083/1986	1986
D4G2	GQ868582.1	Santander/Colombia	CO/BID-V3409/2001	2001
D4G2	AH011968.1	Costa Rica	D4.108_1996CR	1996
D4G2	JN559741.2	Boa Vista/Brazil	ROR7365	2010
D4G2	JN983813.1	Boa Vista/Brazil	Br246RR/2010	2010
D4G2	HQ822126.1	Roraima/Brazil	Br347RR	2010
D4G2	HQ822125.1	Roraima/Brazil	Br272RR	2010
D4G2	JN092557.1	Brazil	SPH318527	2011
D4G2	JN712226.1	São José do Rio Preto/Brazil	RP/BR/2011/131	2011
D4G2	JN712225.1	São José do Rio Preto/Brazil	SJRP/BR/2011/167	2011
D4G2	JQ513335.1	Belém/Brazil	BeH778494	2011
D4G2	JN092556.1	Brazil	SPH319325	2011
D4G2	JN092555.1	Brazil	SPH319268	2011
D4G2	JN092556.1	Brazil	SPH319325	2011
D4G2	JQ513333.1	Boa Vista/ Brazil	ROR7542	2010
D4G2	JQ513340.1	Boa Vista/ Brazil	ROR7591	2010
D4G2	AY152252.1	San Juan/ Puerto Rico	D4.69_1987	1987
D4G2	AY152084.1	San Juan/ Puerto Rico	D4.84_1994	1994
D4G2	AY152056.1	San Juan/ Puerto Rico	D4.17_1998	1998
D4G2	AY152196.1	San Juan/ Puerto Rico	D4.28_1992	1992
D4G2	AY152336.1	San Juan/ Puerto Rico	D4M.5_1982	1982
D4G2	AY152360.1	Dominica	D4M.44_1981DM	1981
D4G3	HQ875339.1	Singapura	SG(EHI)D4/2641Y08	2008
D4G3	GQ398256.1	Singapura	SG/06K2270DK1/2005	2005
D4G3	AY618993.1	Bangkok /Thailand	ThD4_0734_00	2000
Serotypes 1 to 4 using as outgroups				
DENV-1	AB074760.1	Japan		2001
DENV-3	AF317645.1	Guangxi/China		2000
DENV-2	HM181971.1	São Paulo/Brazil		2008
DENV-4	AF326573.1	Dominica		2000

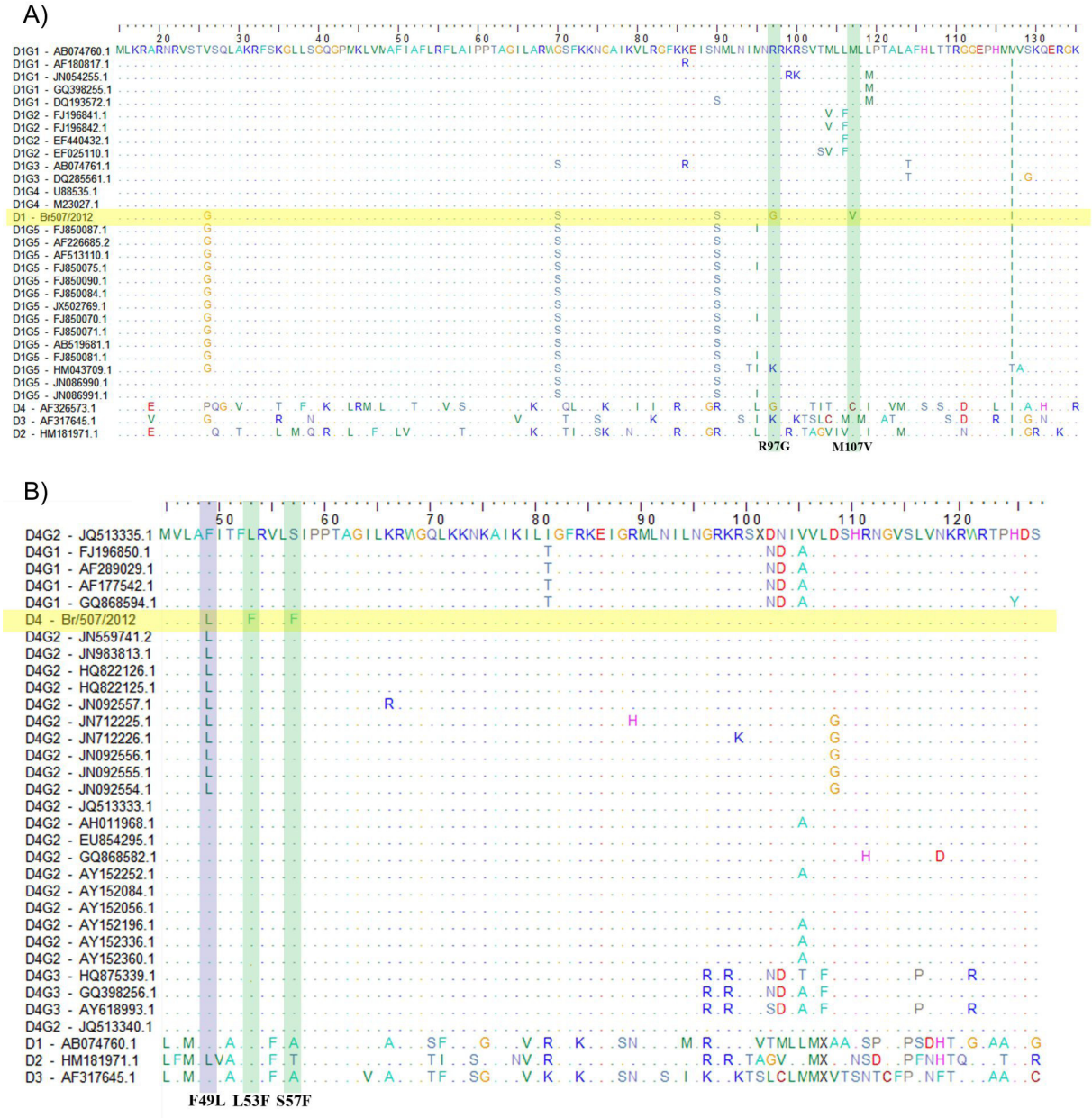

Fig. 2 - (A) Alignment of thirty-one amino acid sequences of Capsid Protein (C) of samples obtained at Genbank and the DENV-1 sequence isolated in this work. The strain RP/BR/2012/507a is shown in yellow and amino acid substitutions identified are marked in green. Only amino acids are shown as distinct from the reference sample (AB074760.1). **(B)** Alignment of thirty-three amino acid sequences of Capsid Protein (C) of samples obtained at Genbank and the DENV-4 sequence isolated in this work. The strain RP/BR/2012/507b is shown in yellow and amino acid substitutions identified are marked in green. The amino acid substitution F49L is marked in blue. Only amino acids are shown as distinct from the reference sample (JQ513335.1).

in the patient belongs to genotype V (American-African group) (Fig. 3A) and DENV-4 belongs to genotype II (American group) (Fig. 3B).

DISCUSSION

In this work we investigated a DENV-1/DENV-4 co-infection event in a patient from a public healthcare facility of SJRP (São Paulo, Brazil) in 2012. The co-infection can worsen the patient's condition and recovery, but in this case the patient doesn't have a severe disease.

The initial diagnosis was available by commercial ELISA assays to detect DENV NS1 protein in acute plasma. This assay provides additional dengue diagnostic tool to the existing approaches of PCR, antibody capture ELISA and virus isolation[5,6,10,12,13,20,24,25,28]. Even though NS1 is a sensitive methodology for the detection of DENV it should never be used as the only methodology for DENV detection because it doesn't identify different serotypes[39].

The imunofluorescence assay is a gold standard method for Dengue

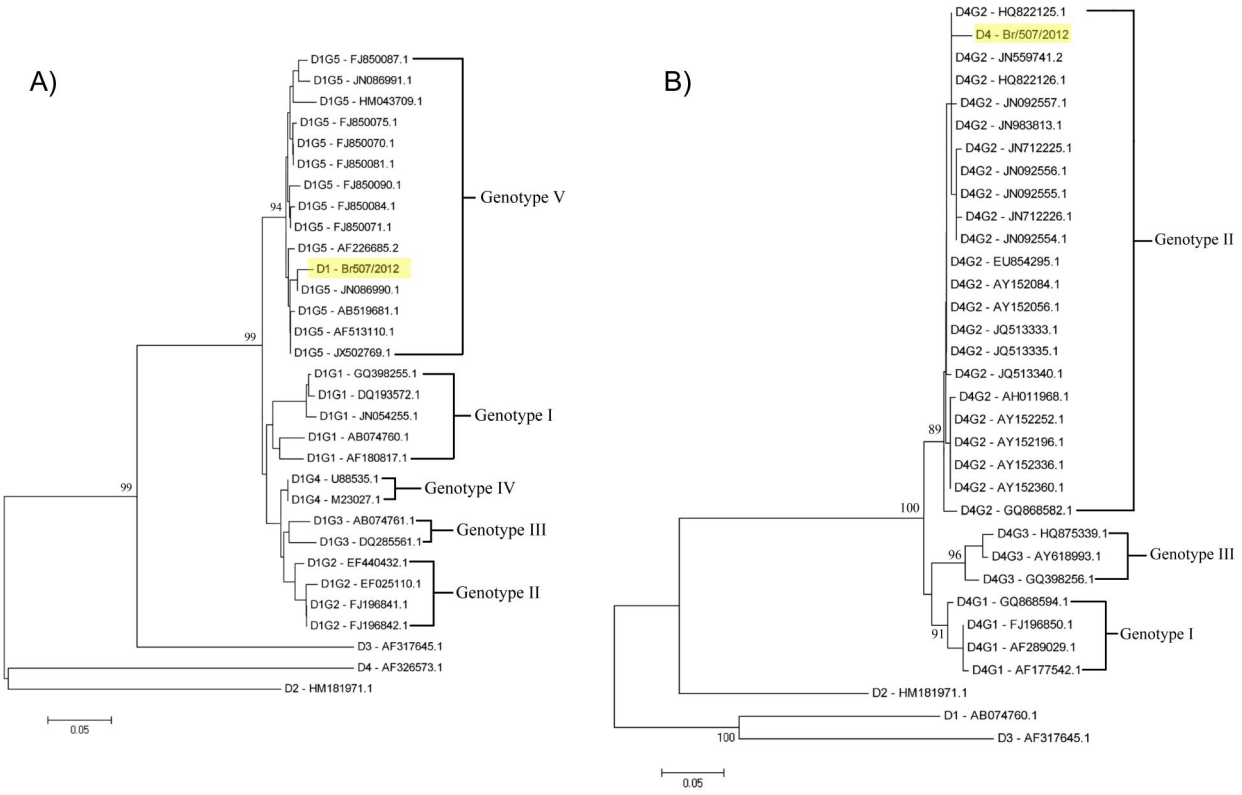

Fig. 3 - (A) Neighbor-Joining phylogenetic tree of 31 sequences of DENV-1 and the sequence RP/BR/2012/507a, inferred from Capsid gene (365 bp). RP/BR/2012/507a is highlighted in gray, the five DENV-1 genotypes are indicated with "}" and DENV serotypes 2, 3 and 4 are used as outgroups. "D" stands for "Dengue" and "1" represents the serotype. Genotype "G" is represented after the serotype, with the number of the genotype. Bootstrap values are shown for key nodes and the scale bar represents 0.05 nt changes per site. **(B) -** Neighbor-Joining phylogenetic tree of 33 sequences of DENV-4 and the sequence RP/BR/2012/507b, inferred from Capsid gene (257 bp). RP/BR/2012/507b is highlighted in gray, the three DENV-4 genotypes are indicated with "}" and DENV serotypes 1, 2 and 3 are used as outgroups. "D" stands for "Dengue" and "4" represents the serotype. Genotype "G" is represented after the serotype, with the number of the genotype. Bootstrap values are shown for key nodes and the scale bar represents 0.05 nt changes per site.

Diagnosis and can be used by serotype strains[31]. But IFA is not capable of detecting the co-infections with other arboviruses, furthermore, antibodies can be cross-reactive with Flaviviruses[2,30]. Therefore the PCR remains extremely essential for disease surveillance, especially if followed by nucleotide sequencing, which can lead us to a more robust interpretation of the epidemiological context of the infection.

In order to provide timely information for patient management, and early public health control of dengue outbreaks, it is important to establish the diagnosis of an acute dengue virus infection during the first few days after manifestation of clinical symptoms[37].

Two different PCR assays were used in our study and both presented the same results. Other simultaneous *Flavivirus* infections, especially those caused by different dengue virus types, such as DENV-1 and 2[22,36], DENV-2 and 3[3,22,43,47,48], DENV-1 and 3[26], DENV-3 and DENV-4[14,15] have been reported. Hence, co-infection with distinct DENV serotypes during outbreaks may be expected.

The Nested-PCR used in our study[8] is a suitable tool for detecting *Flavivirus* co-infections. Within a short amount of time, the identification of the most common Brazilian *Flavivirus* is possible. Such techniques can be applied as a rapid diagnostic tool in clinical samples when a *Flavivirus* infection is suspected and differential diagnosis is required[34].

The nucleotide sequence was established using only one primers set (Lanciotti primers[27] - Methodology section). This assay uses type-specific primers for detection and typing of dengue viruses that presented cross reactivity among primers[27]. This trend is important for diagnosis of co-infections and also for nucleotide sequencing.

The changes in amino acids (R97G - Arginine to Glycine and M107V - Methionine to Valine) identified in DENV-1 C protein has not been reported previously. The F49L substitution perceived in DENV-4 C protein and recognized only in other Brazilian isolates[1,9,35,40] can be considered a genetic signature of Brazilian isolates and needs further investigation.

Our two phylogenetic trees present representative strains of each dengue serotypes (DENV-1 to 4) and they are correctly grouped according to what was previously described; DENV-4 was the first to diverge, followed by DENV-2 and a final split between DENV-1 and DENV-3[21].

Our phylogenetic analysis indicates that DENV-1 and DENV-4 viruses detected in co-infection were distributed in the genotypes V and

II, respectively, and it also reflects the genotypes circulating in Brazil at the present moment[11,40].

Our report demonstrates that co-infection with different serotypes of dengue virus can occur naturally. This study reinforces the importance of accurate diagnosis that really elucidate the origin of infection and emphasizes the RT-PCR has the advantage to be used for molecular characterization, when followed by nucleotide sequencing and bioinformatics analysis.

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RESUMO

Co-infecção por vírus dengue, sorotipos 1 e 4, em paciente de cidade de porte médio no Brasil

A co-infecção por dengue vírus pode ocorrer em áreas com circulação endêmica, nas quais diferentes sorotipos vêm circulando durante muitos anos. Neste trabalho relatamos um caso de co-infecção por DENV-1/DENV-4 em soro humano, detectado por testes moleculares. Análises filogenéticas das sequências obtidas indicaram a presença do genótipo V e II de DENV-1 e DENV-4, respectivamente.

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