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Genome analysis of dengue type-1 virus isolated between 1990 and 2001 in Brazil reveals a remarkable conservation of the structural proteins but amino acid differences in the non-structural proteins☆

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Abstract

We have investigated the genetic diversity of dengue type-1 (DEN-1) virus in Brazil. The full nucleotide sequences of three DEN-1 virus isolated from DEN fever (DF) and DEN hemorrhagic fever patients in northeastern Brazil in 1997 (BR/97) and one from a DF patient in the south of Brazil in 2001 (BR/01) were compared to that of the reference strain BR/90 obtained in the city of Rio de Janeiro in 1990. Sequence analysis showed that the structural proteins were remarkably conserved between all isolates. A total of 27 amino acid changes occurred throughout the non-structural proteins. Among them, nine amino acid substitutions were specific of BR/97 and BR/01 isolates, indicating that in situ evolution of these strains had occurred. Within the BR/97 and BR/01 samples, some amino acid substitutions have been previously identified in DEN-1 virus strains sequenced so far, suggesting that recombination events might have occurred.

Keywords: Dengue virus; Genome sequencing; Molecular evolution

☆ The nucleotide sequences reported in this study have been submitted to the GenBank Data Library under Accessions no. AF 226685 (BR/90), AF 311956 (BR/97-111), AF 311957 (BR/97-409), AF 311958 (BR/97-233) and AF 513110 (BR/01-MR).

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Dengue (DEN) is the most important vector-borne viral disease in tropical countries with at least 100 million cases recorded each year (McBride and Bielefeldt-Ohmann, 2000). It is caused by four serotypes of DEN virus (DEN-1, -2, -3, and -4), a member of the *Flavivirus* genus (family Flaviviridae). DEN virus causes a spectrum of illnesses, ranging from DEN fever (DF) to DEN hemorrhagic fever (DHF) that can progress to DEN shock syndrome (Rothman and Ennis, 1999). DEN disease is one of the most serious health problems affecting the tropical and subtropical countries in the Americas (Gubler and Meltzer, 1999).

The DEN virion is composed of three structural proteins, designated C (core protein), M (membrane protein), and E (envelope protein) (Chambers et al., 1990; Rice, 1996). DEN virus genome is a ~11-kb single-stranded positive sense RNA. It contains a single open reading frame that is flanked by two untranslated regions (5′ and 3′UTR). The genomic RNA encodes a polyprotein precursor of about 3400 amino acid residues, which is co- and post-translationally processed by host-cell and virus-specified proteases to yield the individual viral proteins. The structural proteins are C, prM (the precursor of the M protein), and E, and the non-structural (NS) proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990; Rice, 1996).

The emergence of DEN hemorrhagic is particularly apparent in Brazil. About 80% of notified DEN cases in the Americas occurred in Brazil, and in the last years, more than 1 million cases have been reported in the whole country. In the last 5 years DEN virus infection has dramatically emerged in northeastern Brazil. In 1997, Pernambuco State experienced DEN epidemic with the co-circulation of serotypes 1 and 2. At least 150 000 DF and 65 DHF cases were notified.

In the South region of Brazil (more than 3000 km apart from Pernambuco State) the incidence of DEN cases in Paraná State is increasing significantly since 1999. Here, we have investigated the molecular evolution of DEN-1 virus in Brazil since 1990. The complete nucleotide sequences of DEN-1 strains BR/97-111, BR/97-233 and BR/97-409 isolated from DF and DHF patients in neighboring communities of the state of Pernambuco and BR/01-MR isolated from a DF patient of Paraná State were determined. The well-characterized Brazilian strain BR/90 was used as a reference strain from DEN-1 epidemic in South America (Després et al., 1993; Wang et al., 2000). Our data suggest that strains of DEN-1 virus recently isolated in Brazil have diverged from BR/90.

The DEN-1 virus strains used in this study represented single or low passage isolates obtained from sera of adult patients. Strains BR/97-233 and BR/97-409 from DF cases and BR/97-111 from a DHF case in neighboring communities of Pernambuco state during an outbreak in 1997 were analysed. Strain BR/01-MR was obtained from a DF case in Paraná State in 2001 (Fig. 1). DEN virus strains isolated from human viremic sera and identified as DEN type 1 virus were used to infect mosquito C6/36 cells. Total RNA was extracted from infected C6/36 cells using phenol–chloroform standard extraction after cell lysis/clarification. The derivation of the DEN-1 virus BR/90 has been previously described (Després et al., 1993). Monolayers of mosquito cell line were used to prepare highly purified BR/90 virus stocks from which genomic RNA was extracted as previously described (Després et al., 1993; Duarte dos Santos et al., 2000).

Viral RNA was used as template in reverse transcriptase PCR. Specific primers were used to produce overlapping cDNA products (two to six fragments) from the viral RNA using expand reverse transcriptase (Roche Molecular Biochemicals) as previously described (Després et al., 1993; Duarte dos Santos et al., 2000). Both strands of the amplified cDNA products were directly sequenced as described in Thermo Sequenase manual (Amersham).

Sequences from the 5′ and 3′UTR of the BR/90 genome were obtained after uncapping and RNA ligation as described elsewhere (Duarte dos Santos et al., 2000). For 1997 and 2001 samples, the 5′ end of the 5′UTR (27 nucleotides) and the 3′ end of the 3′UTR (29 nucleotides) are not known. It is presumed that these short nucleotide sequences are identical to those from DEN-1 virus strains BR/90, FGA/89 (GenBank accession number no.

Fig. 1. Geographic map from Brazil highlighting the states where DEN-1 viruses have been isolated. The distances are indicated in kilometres.

AF 226687) and FGA/NA d1d (AF 226686) (Duarte dos Santos et al., 2000).

Homology searches and comparison of all sequences obtained were done using the Laser Gene program (DNA Star Inc.). The secondary RNA structure prediction programs were based on an algorithm developed by Zucker et al. (1999).

The prototype strain BR/90 isolated from epidemic DEN-1 in Rio de Janeiro in 1990 and the 1997 and 2001 isolates displayed 1.2 and 1.4% of nucleic acid changes, respectively. More than 90% of the nucleotide differences were transitions.

A surprising finding of this study was the observation that the C, prM and E proteins were conserved in almost all Brazilian DEN-1 virus strains, with the exception of BR/97-233 (1 amino acid difference in E-180) and BR/01-MR (2 amino acid differences prM-29 and E-338) (Table 1). However, within the NS proteins, a total of 27 amino acid substitutions distinguished BR/97 and BR/01 samples from the reference strain BR/90 (Table 1). Twelve of these changes (44.4%) were localized in the NS1–NS2A proteins (Table 1). The amino acid substitutions at positions NS1-92, NS2A-29, NS2A-144, NS2A-170, NS2A-212 and NS2A-213 are non conservative (Table 1). The amino acid substitution at position NS3-465 in 1997 and 2001 virus samples is located in the C-terminal region of NS3. DEN-1 NS3 protein has several activities associated with virus replication, including NTPase, helicase, and triphosphatase, which are located within a large C-terminal domain (Cui et al., 1998). Residue NS3-465 is immediately above the conserved sequence SAAQRRGRIRGR^{464}, encompassing motif VI of the RNA helicase superfamily in the RNA binding domain (Kadare and Haenni, 1997). We have used the known 3D structure of the helicase domain of protein NS3 from Hepatitis C virus (HCV) to map the Ser^{465} in DEN-1 NS3 protein in

Table 1
Summary of amino acid sequence differences among the DEN-1 isolates and the reference strain

Gene	Position no.[a]	BR 90	BR/97-111	BR/97-409	BR/97-233	BR/01 MR
PrM	29	A	A	A	A	G
E	180	A	A	A	T	A
E	338	S	S	S	S	L
NS1	48	K	R	R	R	R
NS1	84	M	I	I	I	I
NS1	92	D	N	N	N	N
NS1	246	I	M	M	M	M
NS1	293	N	S	S	S	S
NS2A	29	R	R	R	R	G
NS2A	81	F	F	F	F	L
NS2A	97	A	T	T	T	T
NS2A	144	H	Q	Q	Q	Q
NS2A	170	M	M	T	M	M
NS2A	212	N	N	N	Y	N
NS2A	213	K	E	E	E	E
NS3	174	G	G	G	E	G
NS3	465	S	N	N	N	N
NS4A	4	G	G	S	G	G
NS4B	28	M	I	M	M	M
NS4B	34	R	H	H	H	H
NS4B	90	I	L	L	L	L
NS5	59	T	T	T	A	T
NS5	114	V	I	I	I	I
NS5	135	M	M	I	M	M
NS5	375	V	V	V	V	M
NS5	399	T	T	T	T	I
NS5	629	L	F	F	F	F
NS5	635	T	S	S	S	S
NS5	784	I	V	V	V	V
NS5	785	D	D	D	D	N

[a] The amino acid residue number corresponds to the position in the respective proteins.

the context of the structure of the folded polypeptide (Duarte dos Santos et al., 2000; Yao et al., 1997). As shown in Fig. 2, the RNA binding domain forms the blue patch underneath the amino acid substitution at position NS3-465 (black star) at the entrance of a groove which has been shown to contact nucleic acids. Twelve of the 27 amino acid substitutions between Brazilian samples are localized in the NS4B-NS5 proteins (Table 1). The amino acid substitutions at positions NS5-59, NS5-114 and NS5-135 are located in the putative methyltransferase domain of NS5 (Koonin, 1993). The amino acid substitutions at positions NS5-629, NS5-635 and NS5-784 and NS5-785 surround the highly conserved RNA polymerase motif GDD^{664} (Tan et al., 1996).

The 5′UTRs of the BR/97 and BR/01 samples were identical to that of the reference BR/90 strain. Within the 3′UTR, six nucleotide differences at positions 10 314, 10 319, 10 321, 10 535, 10 567 and 10 619 were observed between the samples (Fig. 3A). The predicted RNA structure formed by the complete 3′UTR of the DEN-1 viruses strains is shown in Fig. 3B. Structural prediction by computer analysis showed that nucleotide differences at positions 10 314 [T→C] and 10 319 [T→C] between BR/90 and BR/97-111 have no apparent effect on the predicted secondary RNA structure of the 3′UTR. However, the nucleotide difference at position 10 321 [A→G] in BR/97-233 and BR/97-409 might introduce a minor change in the predicted secondary structure formed by the 5′

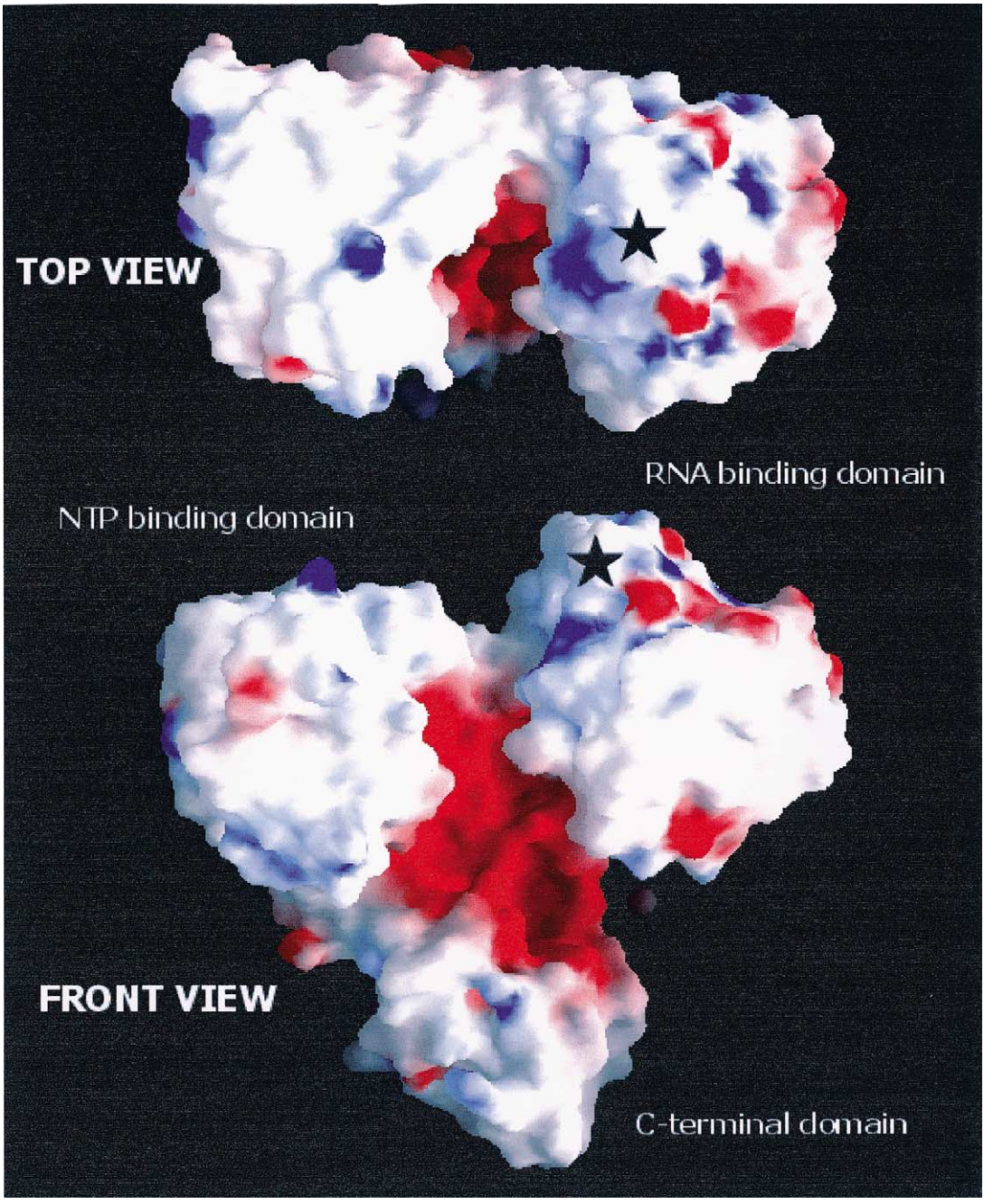

Fig. 2. Surface representation of the helicase domain of protein NS3 from HCV. The electrostatic potential is represented at the molecular surface in red and blue for negative and positive charges, respectively. Figure made with program GRASP with coordinates taken from the Protein Data Bank, accession code 8 ohm.

terminal region of the 3′UTR (Fig. 3B). The two nucleotide differences (10 535 [T→A] and 10 567 [T→G]) in BR/01-MR were previously observed in the FGA/89 virus genome (Duarte dos Santos et

A

Nucleotide position	BR/90	BR/97-111	BR/97-233	BR/97-409	BR/01-MR
10,314	T	C	T	T	T
10,319	T	C	C	C	C
10,321	A	A	G	G	A
10,535	T	T	T	T	A
10,567	T	T	T	T	G
10,619	A	A	T	A	A

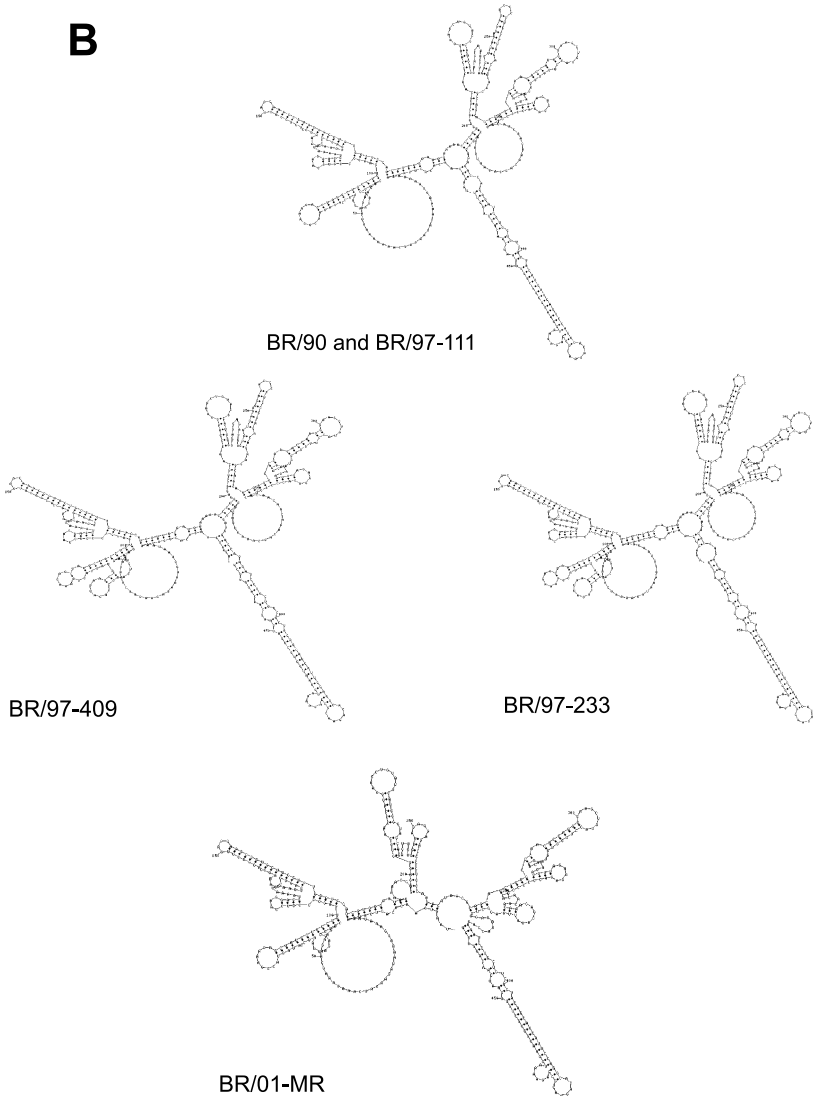

Fig. 3. In (A), nucleotide differences within the 3′UTR between the Brazilian samples. In (B), predicted RNA secondary structures formed by the complete 3′UTR (nt 10 274–10 735) of the reference strain BR/90 and BR/97-111, BR/97-409, BR/97-233 and BR/01-MR isolates.

al., 2000) and considerably change the predicted folding pattern of the 3′UTR (Fig. 3B) revealing a structure more closely related to that of the FGA/89 virus (data not shown).

After more than 50 years, DEN-1 virus was reintroduced in the state of Rio de Janeiro in 1986 and the infection behaved as virgin soil epidemic. Reference strain BR/90 of DEN-1 virus was isolated from an adult male with DF during the epidemic in the city of Rio de Janeiro in 1990 (Després et al., 1993; Nogueira et al., 1993). There is now growing evidence that genetic diversity in DEN virus might be generated by recombination, although this process is still not fully understood (Holmes and Burch, 2000; Tolou et al., 2001; Uzcategui et al., 2001). Phylogenetic analysis of South-America DEN-1 viruses suggested that BR/90 is a descendant of a single recombinant ancestor produced by genetic exchanges between Jamaica (genotype IV) and Singapore (genotype I) strains of DEN-1 virus (Holmes et al., 1999; Holmes and Burch, 2000). We have determined the full nucleotide sequences of four isolates of DEN-1 virus obtained in Brazil during outbreaks in 1997 (northeastern region) and 2001 (south region). It was surprising that BR/97 and BR/01 samples have their structural proteins remarkably conserved in comparison to the reference strain BR/90. This observation supports the conclusion that BR/90-like viruses have spread successfully through human populations and mosquito vectors since 1990. However, within the NS proteins, we identified 27 amino acid substitutions that distinguished the BR/97 and BR/01 samples from the reference strain. Interestingly, as yet none of the changes could be correlated with the disease outcome, including the conservative change observed in the NS4B-28 protein from a DHF patient. The NS1 protein is strictly conserved among BR/97 and BR/01 samples. An important observation is that the 5 amino acids substitutions in the NS1 proteins that distinguished BR/97 and BR/01 samples from BR/90 have not been described in any DEN-1 virus strain sequenced to date (Fu et al., 1992; Puri et al., 1998; Huang et al., 2000; Ishak et al. 2001; Tolou et al., 2001). The differences observed in residues NS1-84 and NS1-92 are located immediately upstream from the consensus linear epitope ^{111}HKYSWK (Yao et al., 1995). The NS1 glycoprotein is involved in the early steps of viral replication (Khromykh et al., 1999b; Lindenbach and Rice, 1999). It must be determined whether the amino acid differences between BR/97 and BR/01 samples and the reference strain BR/90 affect the antigenicity and the biological activity of NS1. The NS3 protein has one amino acid change, Ser-to-Asn at position NS3-465, that distinguished the four isolates from BR/90. The residue Asn at NS3-465 has been previously described in West Pac 74 (Puri et al., 1998), Singapore S275/90 (Fu et al., 1992), Djibouti (DI/H/IMTSSA-DJIB/98/606), Abidjan (DI/H/IMTSSA/ABID/99/1056), Cambodia (DI/H/IMTSSA-CAMB/98/658) (Tolou et al., 2001), Mochizucki, A88 (Ishak et al., 2001) and GZ/80 (Huang et al., 2000) strains. The amino acid substitution in the RNA binding domain is located immediately above the conserved region corresponding to motif VI of superfamily II RNA helicases (Kadare and Haenni, 1997). We have recently shown that amino acid substitutions in the helicase domain of the DEN-1 NS3 protein may alter the efficiency of viral RNA production by the replication complexes (RCs) and thereby affect viral growth (Duarte dos Santos et al., 2000). Thus, an Asn-to-Ser amino acid change at position NS3-465 could modulate the efficiency of RCs to catalyze the synthesis of new RNA molecules.

Four significant amino acid changes were observed between the NS5 proteins (NS5-114, NS5-629, NS5-635 and NS5-784) of BR/97 and BR/01 samples and the reference strain BR/90. Residues Ile^{114}, Thr^{635} and Val^{784} have been previously observed in West Pac 74, Singapore S275/90, Djibouti, Abidjan, Cambodia, Mochizuki, GZ/80 and A/88 NS5 proteins (Fu et al., 1992; Puri et al., 1998; Huang et al., 2000; Ishak et al., 2001; Tolou et al., 2001). The relative contribution of these changes to viral RNA capping and RNA-dependent RNA polymerase activity of NS5 remains unknown.

Within the 3′UTR, the mutation at position 10 321 is thought to introduce a minor change in the predicted RNA secondary structure of the 3′UTR in BR/97-233 and BR/97-409 isolates. The mutations in the 3′-terminal 200 nt of the BR/01-

MR isolate yielded a different 3′UTR conformation when compared to the other BR strains.

Although this is not an extensive horizontal epidemiological study, our data allowed us to demonstrate that the identified amino acid substitutions between reference DEN-1 virus BR/90 and recent isolates from epidemic DEN-1 in Brazil were essentially localized in NS1, NS2A, NS3, NS4B and NS5. It is interesting to note that some amino acid substitutions between BR/90 and BR/97 and BR/01 samples have been previously identified in DEN-1 virus strains originating from Nauru Island (West Pac 74), Asia (Singapore S275/90, Cambodia, Mochizuki, A/88, GZ/80) and Africa (Djibouti and Abidijan). These findings might be explained by recombination events with imported DEN type 1 viruses or alternatively, could be the result of multiple introductions of different viruses followed by the usual genetic drift. On the other hand, the nine amino acid substitutions specific of BR/97 and BR/01 strains indicate that evolution of DEN-1 virus in Brazil has also occurred in situ.

The active DEN virus RC is composed of the NS1, NS2A, NS3, NS4A and NS4B proteins and the viral RNA template (Khromykh et al., 1999a,b). The viral RNA helicase activity NS3 is regulated by the RNA-dependent RNA polymerase NS5 (Khromykh et al., 1999a,b). Previous reports indicate that mutations affecting viral growth may influence the pathogenicity of DEN virus (Diamond et al., 2000; Duarte dos Santos et al., 2000). We are currently investigating whether the amino acid differences that distinguish the recent Brazilian DEN-1 virus strains from references strain BR/90 alter virus replication and thereby influence the pathogenicity of DEN-1 virus.

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