Available online at www.sciencedirect.com
SCIENCE DIRECT®
Virus Research 101 (2004) 135–145
Virus Research
www.elsevier.com/locate/virusres

Complete genome sequencing of dengue virus type 1 isolated in Buenos Aires, Argentina☆

Paola R. Barrero*, Alicia S. Mistchenko

Laboratorio de Virología, Hospital de Niños Dr. Ricardo Gutiérrez., Gallo 1330, 1425 Buenos Aires, Argentina

Received 11 September 2003; received in revised form 22 December 2003; accepted 22 December 2003

Abstract

Dengue (DEN) constitutes a major viral arthropod-borne human illness. South America was last considered free of dengue two decades ago when a dramatic increase in the number of dengue fever and hemorrhagic dengue cases had been reported. Five viruses were isolated in Buenos Aires City from the 1999–2000 Paraguay outbreak. RT-PCRs obtained directly from plasma were cloned into pGemT vectors and sequences of the structural genes and NS1 were analyzed. Three viruses were full-length sequenced from RT-PCR obtained from cell-culture isolates. Excess of synonymous over non-synonymous mutations suggested that the structural proteins were under strong functional constraints while a weak purifying selection was operating in the whole polyprotein. Sequence diversity and selective pressures varied among patients but results were significantly above the procedure threshold. One sample showed small-plaque phenotype and impaired growth coupled to 3′untranslated region mutations. Phylogenetic analysis of full-length sequences split Buenos Aires isolates into two clusters within American DEN-1 genotype V: Clade I was phylogenetically linked to Brazilian samples and Clade II with samples from Paraguay and Northeastern Argentina. In Buenos Aires City, only dengue virus serotype 1 imported from Paraguay has been detected, though without evidence of local transmission.

Keywords: Dengue; Sequencing; Molecular epidemiology

1. Introduction

Dengue (DEN) constitutes a major viral arthropod-borne human infection. The spectrum of illness ranges from mild subclinical disease to a severe and occasionally fatal hemorrhagic clinical picture (Rigau-Pérez et al., 1998). Affecting more than 100 countries worldwide, it threatens 2.5 billion people in endemic areas where nearly 50 million infections occur annually, including 400,000 cases of dengue hemorrhagic fever (Guzmán and Kourí, 2002). Dengue virus is a member of the Flaviviridae family, genus *Flavivirus* and comprises of four different serotypes (DEN 1–4) into a distinct antigenic complex (DEN Complex). The enveloped virions contain single-stranded positive RNA, about 11 kb. in length that contains a 5′ type I cap and lacks a polyadenylate tail at 3′. Genomic RNA is the messenger RNA for translation of a single long open reading frame (ORF) as a large polyprotein processed co- and post-translationally by cellular and virally encoded serine-proteases into 10 discrete products—three structural proteins (capsid (C), membrane (M) and envelope (E) and seven non-structural proteins (NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5) (Rice, 1996). Surrounding the ORF there are 5′ and 3′ non-coding regions that form RNA secondary structures serving as *cis*-acting elements directing the processes of replication, translation and packaging (Proutski et al., 1999; You et al., 2001).

South America was last considered free of dengue two decades ago when a dramatic increase in the number of dengue fever and hemorrhagic dengue cases had been reported (Gubler, 2002). Dengue outbreaks were reported in Northern and Northeastern bordering countries as Bolivia, Brazil and Paraguay. Brazil reported the circulation of DEN-1 in several states from the first isolation in Rio de Janeiro in 1986 to the latest reported in 2001 in Paraná State, DEN-2 has been documented since 1990 in Rio de Janeiro and DEN-3 since 2000 in the same state (dos Santos et al., 2002; Nogueira et al., 1993; Miagostovich et al., 2002). The presence of DEN-2 was detected in Bo-

☆ The GenBank reference numbers for this work are 66AY277652-66.

* Corresponding author. Tel.: +54-11-49643118; fax: +54-11-49626770.
E-mail address: paobarrero@hotmail.com (P.R. Barrero).

0168-1702/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2003.12.033

livia in 1996 (Van der Stuyft et al., 1998). Two outbreaks of DEN-1 have been reported in Paraguay since its first isolation in 1988. The earlier epidemic caused almost 40,000 cases of dengue fever in 1989 and the later one nearly 27,000 in 1999 and 2000 (Boletín Epidemiológico República del Paraguay, 1999, 2000). This area is located along the northeastern border with Argentina where several cases occurred on both sides with almost 500 cases of DEN-1 in 2000. In addition, 341 cases of DEN-2 were reported 2 years before (1998) in the northern region of our country, posing a warning about the reemergence of dengue in Argentina (Boletín Epidemiológico Nacional, 2000, 2001).

Dengue fever compatible cases were detected in Buenos Aires City in patients coming from Paraguay in 1999 and 2000. In this work we describe the diversity found in cloned structural genes, full-length sequence analysis and phylogenetic inference of dengue virus isolated in Buenos Aires City, Argentina. This work provides one of the first attempts to accomplish full-length viral sequencing in our country.

2. Materials and methods

2.1. Samples

Acute blood samples from five patients with dengue fever syndrome were obtained between day 2 and day 7 after their temperature reached = 38 °C. All patients had previous history of travelling to Paraguay and arrived in Buenos Aires City carrying the illness. Plasma was separated and stored at −70 °C until necessary. Thirty microliter of plasma were inoculated in C6/36 *Aedes albopictus* cell line in 5% FBS-MEM Earle at 35 °C until the appearance of cytopathic effect (CPE) and then passaged twice again to prepare viral stocks. Indirect immunoflurecescence was performed to confirm the presence of dengue virus in cell cultures with monoclonal antibody H3-6 (anti-dengue complex). Total RNA was purified by the guanidinium–thiocyanate–phenol–chloroform method from plasma and cell culture supernatant (Chomczynski and Sacchi, 1987) and serotype determined by means of a previously described multiplex RT-PCR that can distinguish the four serotypes by the size of their products (Lanciotti et al., 1992).

2.2. Amplification strategy

Nineteen pairs of primers were designed based on the DEN-1 Singapore 275/90 sequence (GenBank accession number E06832) and sequences are available from authors upon request. RNA obtained from cell culture stock was used for full-length sequence experiments and RNA from plasma for cloning experiments. Products from 1.0 to 1.2 kb. were obtained by commercial one-step reverse transcription-polymerase chain reaction (RT-PCR) kit (Qiagen, Hilden, Germany). Briefly, RT was performed at 50 °C for 30 min followed by 15 min at 95 °C to inactivate Omniscript and Sensiscript Reverse Transcriptase; and to activate HotStarTaq DNA polymerase. Thirty-five cycles of 94 °C for 15 sec, 50 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 10 min were completed. Bands were visualized in 2% (w/v) agarose-*tris*-acetate-EDTA (TAE) Gel Electrophoresis, excised and purified by affinity bond and centrifugation through commercial columns (Promega, Madison, WI, USA). Quantification was performed by comparative densitometry analysis with AlphaEase software v5.04 (Alpha Innotech Corporation, San Leandro, CA, USA) using reference molecular markers (BioRad, Hercules, CA, USA).

2.3. Cloning strategy

RT-PCR products obtained directly from plasma for the structural genes and part of NS1 of five samples were cloned into high copy pGemT vectors (Promega) at a 3:1 insert-vector ratio. Competent JM109 *Escherichia coli* were transformed and plated into Luria-Bertani (LB) broth with Ampicillin (sodium salt, 100 ug/ml), 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 ug/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). White colonies were grown overnight in 20 ml LB broth with ampicillin and a commercial midiprep was performed (Qiagen). The presence of the expected insert was verified by specific PCR and restriction analysis through 1% (w/v) agarose-TAE gel electrophoresis.

2.4. Sequencing strategy

Each RT-PCR or clone was quantified and the recommended mass (300–400 ng for RT-PCR products or 500–600 ng for clones) was labeled with DyET terminators for 30 cycles according to the manufacturer's protocol in the presence of 10 pmol of each specific primer or universal primers for cloned products. Overlapping products were sequenced in a capillary automated sequencer (MegaBACE 1000, Amersham BioSciences, Amersham BioSciences, Piscataway, NJ, USA). Read lengths were optimized varying injection time and voltage. All sequences were submitted to GenBank.

2.5. Sequence analysis

Sequences and contigs were aligned with ClustalX v1.81 (Thompson et al., 1997) and polymorphisms analyzed with DNASP v3.5 (Rozas and Rozas, 1999). Amino acidic sequences were inferred using universal code. Diversity was assessed as total number of segregating sites (S)/sequenced bp (L)/number of amplification cycles. Mean diversity was measured as the number of substitutions divided by the total number of nucleotides analyzed × 100. The ω ratio was calculated as non-synonymous versus synonymous substitutions (dns/ds). Recombination was tested with RDP v1.07

(Martin and Rybicki, 2000) and Simplot v2.4 (Lole et al., 1999). RNA secondary structure for 3′UTR was predicted with RNAdraw v1.01 (Matzura and Wennborg, 1996) and RNAstructure v3.71 software (Mathews et al., 1999).

2.6. Phylogenetic inference

Distance matrix was calculated with the DNAdist and Protdist components of the PHYLIP software package v3.5c (Felsestein, 1989). Base frequencies (π), the shape parameter of γ distribution and the model of DNA substitution were calculated from the data with Modeltest v3.06 (Posada and Crandall, 1998) and its Windows front-end by Francesco Paolo Patti (Winmodeltest v4b). These parameters were entered into PAUP* v4 (Swofford, 1998) and phylogenetic relationships inferred by maximum-likelihood criteria. The derived trees were visualized with Treeview v1.5.2 (Page, 1996). DEN-1 samples from South America , including French Guyana (FGA/89:AF226687 FGA/NA:AF226686), Brazil (BR/90:AF226685, BR97-111, 409, 233:AF311956-8, BR01MR:AF513110), the Northeastern region of Argentina (297arg00:AF514889, 295arg00:AF514885) and Paraguay (280par00:AF514878) along with African strains Côte D'Ivoire strain Abidjan (AF298807) and Djibouti (AF298808), Asiatic strains Nauru Island, Western Pacific, clone WestPac (U88535), Cambodia (AF309641) and Singapore S275/90 (M87512) were taken from GenBank for analysis. DEN-2 strain (FJ11/99:AF359579), DEN-3 (strain H87:M93130) and DEN-4 (M14931) were taken as outgroups. Topology was confirmed by generating 100 pseudoreplications (bootstraps) from the sample, summarised using a 50% majority-rule consensus tree. Scores represent the number of bootstrap replicates supporting each node.

3. Results

All samples proved positive for DEN-1 by RT-PCR from plasma as well as from cell culture supernatant. CPE was observed within a week after inoculation and was similar in most samples except for ARG9928 samples where small-plaque phenotype was detected. Two more passages were done in order to obtain a viral stock with identical results.

Best-length sequences (800–1000 bp) were obtained injecting samples 112 s at 2 kV and running for 5 h at 6 kV in an automated capillary sequencer. For each fragment, two independent sequence reactions were obtained with the same primer, and a consensus was made. The same was done with the complementary primer and the resulting consensus integrated in a contig. Redundancy varied from 2 to 6 but as a rule each base was read at least twice.

3.1. Sequence analysis: structural proteins

Consensus sequences of 2887 bp from both direct and two sets of clones from five viruses were analyzed. Overall differences between direct and cloned sequencing were <1.96% at nucleotide level and <1.6% at amino acid level. From 202 substitutions, 52 were non-synonymous and 150 synonymous when compared to FGA/89. Most were located in E gene, followed by NS1, prM-M and C (Fig. 1). Comparisons between direct and cloned sequences are shown in Table 1. The number of nucleotide changes in clones (S/L/cycles) was 9.3×10^{-4} substitutions/nt/cycle. This finding exceeded (χ^2 test $P < 0.05$) the inferred method error of RT ($0.2–2 \times 10^{-4}$) and PCR ($4–9 \times 10^{-6}$) (Smith et al., 1997). In fact, it was above the experimental error calculated by Wang of 5.3×10^{-6} for DEN-3 E gene and evidenced the presence of quasispecies in our data (Wang et al., 2002a). The ω index was <1 (=0.388) and the Tajima's D-test showed that all mutations were selectively neutral ($D = -0.63$ non-significant $P > 0.1$) (Tajima, 1989). However, mean diversity measured as $S/L \times 100$ ranged from 0.44 to 2% and ω from 0.4 to 2.66.

Replacement changes are listed in Table 2. Deletions, insertions and premature polyprotein stop codons were absent. Conserved cysteines and *N*-glycosylation sites remained unchanged in most samples but ARG9920c1 lost a Cys in prM34 and ARG0048c2 lost the prM69 *N*-glycosylation site. Receptor binding site E310–314 as well as fusion peptide E106–109 sequences were conserved (Chen et al., 1996; Heinz and Allison, 2001). Half of the replacements were non-conservative changes. Five non-synonymous replacements were present in all samples mostly in the E protein (3/5) coinciding with BR/90 sequence. Two were conservative changes: C112 in the transmembrane hydrophobic domain that serves as prM signal peptide, and E379 in a DEN-complex epitope E375-399; and three were non-conservative at E180 in the E165-201 Th-stimulating peptide, E473 in the NS1 signal peptide and NS1-128, next to the glycosylation site (NS1-130).

Table 1
Comparisons between direct and cloned sequences, Samples were coded as follows: country in three-letter-code designation, year of isolation and number of protocol

Sample	Substitutions	Silent	Non-silent	ω	Mean diversity	Changes/nt/cycle
ARG9920	58	40	18	0.45	2%	3.09×10^{-4}
ARG0023	11	3	8	2.66	0.38%	5.86×10^{-5}
ARG0028	39	28	11	0.3928	1.35%	1.88×10^{-4}
ARG0044	8	5	3	0.6	0.27%	4×10^{-5}
ARG0048	20	11	9	0.818	0.69%	1.06×10^{-4}

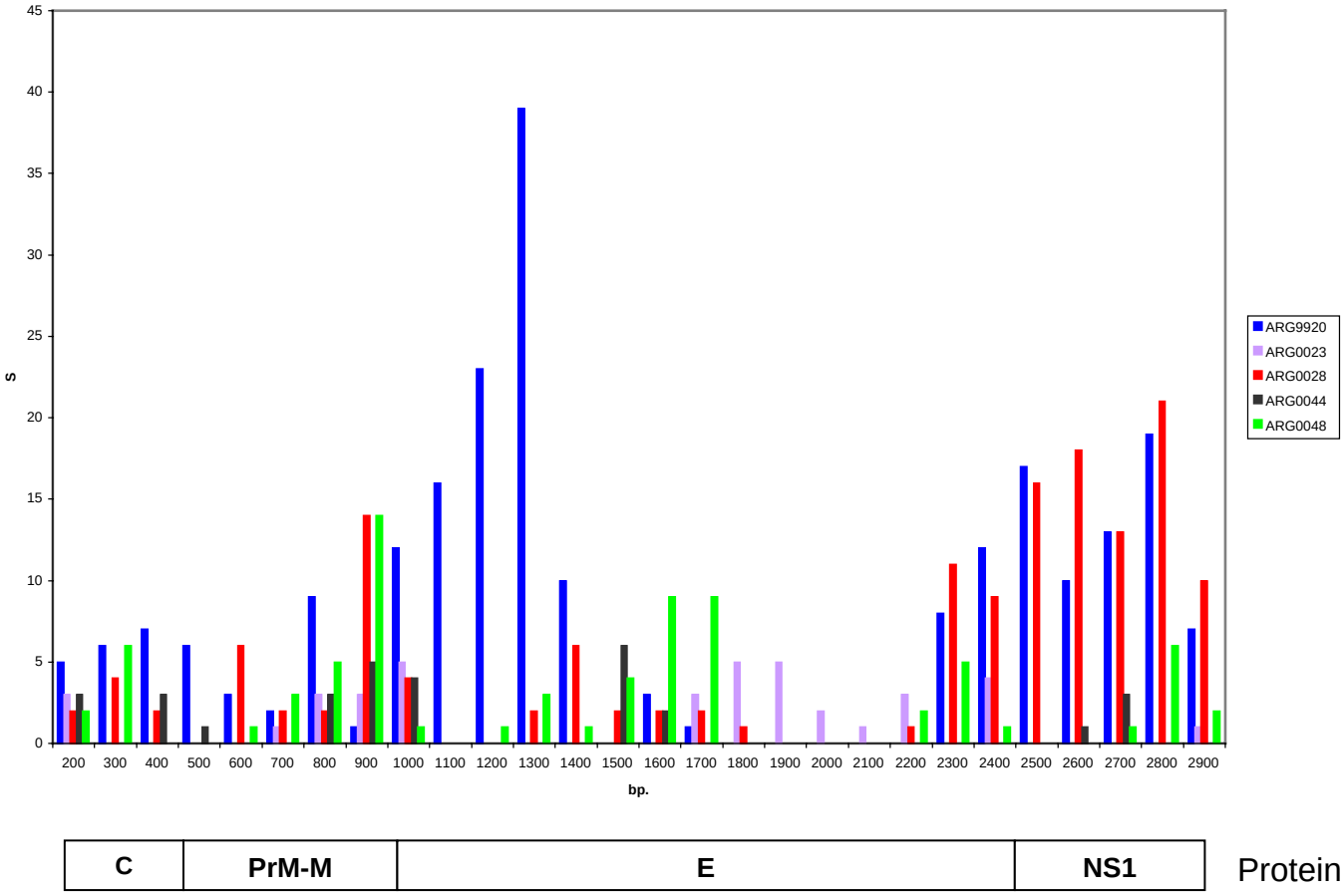


Fig. 1. Clone polymorphisms: polymorphic sites (S) were plotted against bp in a 100-site sliding window with a 25 bp step size. Synonymous and non-synonymous mutations were included. Each sample along with its clones was assessed separately. Positions are relative to genomic RNA and referred to the corresponding protein in the box below. Sequences that shared the same mutations as well as those with unique changes are detailed in Table 2.

3.2. Sequence analysis: full-length sequences

Three viruses were full-length sequenced (ARG9920, ARG0028 and ARG0048). A total of 10735 nt. were analyzed, and the single ORF was located at positions 95–10271 accounting for 3392 aa. Presence of functional protein domains was evaluated in database of conserved domain alignment (CDART v 1.62 by Marchler-Bauer et al., 2003). Percentage G + C was 53% and a slight codon usage was detected (0.272 ± 2) mostly at synonymous sites. Forty-nine different codons were used for translation from 61 available tRNA. Differences found were <3.11% at nucleotide level and <1.38% at amino acid level.

Similarity between samples and FGA/89 was above 92% and percentages varied according to the gene (Fig. 2). From 440 substitutions, 61 were non-synonymous and 379 synonymous with an ω index <1 (=0.16). Tajima's *D*-test corroborated that all mutations were selectively neutral ($D = 0.3571$ non-significant $P > 0.1$) (Tajima, 1989).

Replacement changes were mostly found in NS5 (16/61), E (13/61) and NS1 (10/61) and are listed in Table 3. Half of the non-silent changes were conservative. Ten replacements were fixed, six described in the previous section and four located in NS4 (2/4) and NS5 (2/4). Remarkably, NS5-375 and NS5-399 mutations were identical to those mutations found in Brazilian samples from 1990–1997 (dos Santos et al., 2002). NS5-135 showed a multiple replacement site, a non-conservative change in ARG9920 and ARG0048, and a conservative change in ARG0028.

Multiple antigenic sites were affected in E protein as non-neutralizing linear epitope E37-46, B/T epitope E35-55 and E79-99 in Domain I, and E333-368 in Domain III as well as E375-399 DEN-complex epitope (Roehrig et al., 1994; ianmin et al., 1995; Rey et al., 1995; Falconar, 1999). E390 distinctive marker between Asiatic and American strains in DEN-2 rendered a Ser as one of the described cosmopolitan variants (Leitmeyer et al., 1999). On the other hand, NS1 was affected into NS1-57-126 and NS1-111-116 epitopes defined by monoclonal antibodies and Phage display, respectively, (Yao et al., 1995; Wu et al., 2001).

Non-conservative changes took place in viral protease domain (Dom) II (NS3-97-175) as well as in NS5 S-adenosyl-methyl transferase (SAM) Dom I (NS5-1-54), II

Table 2
Replacement changes: structural proteins and NS1, direct (dir) and cloned (c) sequences were compared with FGA/89

ORF	Protein	aa	FGA/89	ARG9920			ARG0023			ARG0028			ARG0044			ARG0048		
				dir	c1	c2	dir	c1	c2	dir	c1	c2	dir	c1	c2	dir	c1	c2
37	C	37	L	*	*	M	*	*	*	*	*	*	*	*	*	*	*	*
53		53	F	*	*	*	*	*	*	*	*	*	*	*	*	*	*	S
84		84	F	*	*	L	*	*	*	*	*	*	*	*	*	*	*	*
100		100	R	K	K	K	K	K	K	*	*	*	K	K	K	K	K	K
101		101	S	*	P	*	*	*	*	*	*	*	*	*	*	*	*	*
112		112	V	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
114		114	A	*	*	V	*	*	*	*	*	*	*	*	*	*	*	*
145	PrM	31	V	I	I	I	I	I	I	*	*	*	I	I	I	I		I
148		34	C	*	R	*	*	*	*	*	*	*	*	*	*	*	*	*
164		50	T	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*
183		69	N	*	*	*	*	*	*	*	*	*	*	*	*	*	*	I
200		86	H	*	*	*	*	*	R	*	*	*	*	*	*	*	*	*
223	M	18	T	*	A	A	*	*	*	*	*	*	*	*	*	*	*	*
230		25	A	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*
190		45	I	T	T	T	T	T	T	*	*	*	T	T	T	*	T	T
192		47	L	*	*	*	*	F	F	*	*	*	*	F	F	*	*	*
207		62	I	*	*	*	*	*	N	*	*	*	*	*	*	*	*	*
289	E	9	R	*	*	*	*	*	*	*	*	*	K	*	*	*	*	*
317		37	N	D	D	D	D	D	D	*	*	*	D	D	D	D	D	D
324		44	E	Q	*	*	*	*	*	*	*	*	*	*	*	*	*	*
368		88	A	*	S	S	S	S	S	*	*	*	S	S	S	S	S	S
376		96	V	F	F	F	F	F	F	F	F	F	F	F	F	F	F	*
394		114	L	I	*	*	*	*	*	*	*	*	*	*	*	*	*	*
444		164	I	*	*	*	*	*	*	*	*	*	*	*	*	*	L	*
460		180	T	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
488		188	R	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*
499		219	P	*	A	*	*	*	*	*	*	*	*	*	*	*	*	
513		234	Q	*	*	*	*	*	*	P	*	*	*	*	*	*	*	*
545		265	T	*	*	*	*	*	I	*	*	*	*	*	*	*	*	*
577		297	M	*	*	*	*	*	*	T	T	T	*	*	*	*	*	*
591		311	E	*	*	*	*	D	*	*	*	*	*	*	*	*	*	*
618		338	S	*	*	*	*	*	*	L	L	L	*	*	*	*	*	*
619		339	T	A	A	A	A	A	A	*	*	*	A	A	A	A	A	A
659		379	I	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**	**V**
667		387	L	*	*	*	*	*	S	*	*	*	*	*	*	*	*	*
673		393	K	*	*	*	*	*	*	*	*	*	*	*	*	*	*	R
694		414	I	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T
722		442	T	A	*	*	A	A	A	*	A	A	A	A	A	A	A	A
753		473	A	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**
761		481	A	*	V	V	*	*	*	V	*	*	*	*	*	*	*	*
767		487	L	*	*	P	*	*	*	*	*	*	*	*	*	*	*	*
800	NS1	24	V	*	*	*	I	I	I	*	*	*	*	*	*	*	*	*
823		48	K	*	*	*	*	*	*	R	*	*	*	*	*	*	*	*
859		84	M	*	I	I	*	*	*	I	*	*	*	*	*	*	*	*
867		92	D	*	N	N	*	*	*	N	*	*	*	*	*	*	*	*
880		105	R	*	*	*	*	*	*	*	*	*	*	*	*	*	*	8
887		112	K	R	*	*	R	R	R	*	R	R	R	R	R	R	R	R
903		128	T	**I**	**I**	**I**	**I**	**I**	**I**	**I**	**I**	**I**	**I**	**I**	**I**	**I**	**I**	**I**
906		131	T	S	*	*	S	S	S	*	S	S	S	S	S	S	S	S
920		145	D	*	N	N	*	*	*	*	*	*	*	*	*	*	*	*
921		146	D	*	*	*	*	*	*	E	*	*	*	*	*	*	*	*
926		151	N	*	*	*	*	T	*	*	*	*	*	*	*	*	*	

Positions are referred to the polyprotein (ORF) and to the corresponding protein (aa). Non-synonymous mutations are expressed in single-letter code. Fixed replacements are marked in bold and absence of replacement as (*).

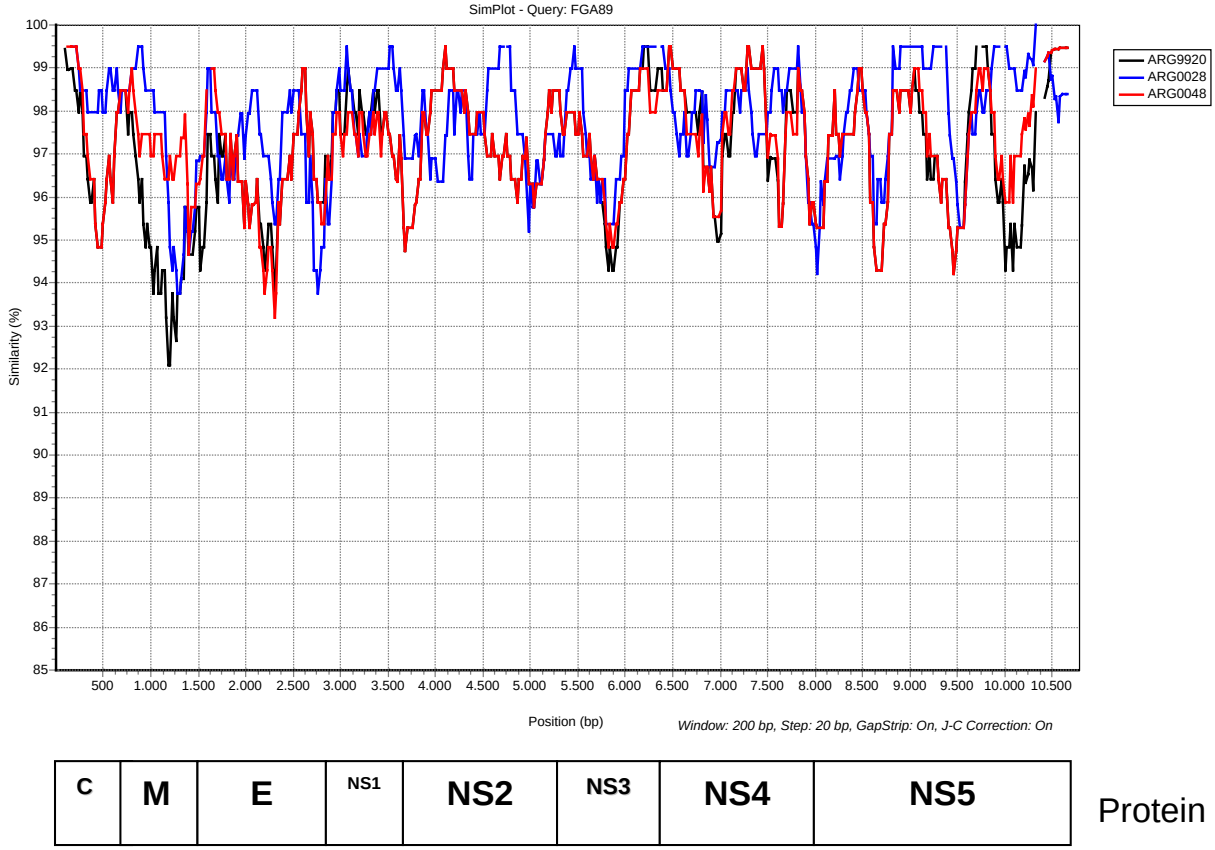


Fig. 2. Similarity plot. Full-length sequences were compared to FGA/89 through a 200 bp window with 20 bp step and similarity (%) was plotted against position (bp). Synonymous and non-synonymous mutations were included. Each sample was assessed separately. Positions are relative to genomic RNA and referred to the corresponding protein in the box below.

(NS5-55-222), and III (NS5-223-267) and nuclear localization signal I–II in the linker between SAM and RNA dependent RNA polymerase (RdRp) (Brinkworth et al., 1999; Johansson et al., 2001; Egloff et al., 2002).

No evidence for recombination was detected when sequences were analyzed by applied methods.

3.3. Untranslated regions

Non-coding regions at 5′ and 3′of the unique ORF were analyzed. Upstream-untranslated region (5′UTR) was identical to the above South American strains. In contrast, several differences were identified at 3′UTR and are summarized in Fig. 3. RNA secondary structures were predicted and conserved areas named upon previously predicted Flavivirus secondary structure into A1–A4 (Proutski et al., 1997; Shurtleff et al., 2001). The hypervariable region following NS5 stop included the four imperfect repeats of the sequence UCAAACAA described for DEN-1. Strains ARG9920 and ARG0048 had identical secondary structure A3 configuration while in ARG0028 mutations 10567 and 10613 altered the conserved complementary cyclization sequence CS2 (Proutski et al., 1999) (Fig. 3c).

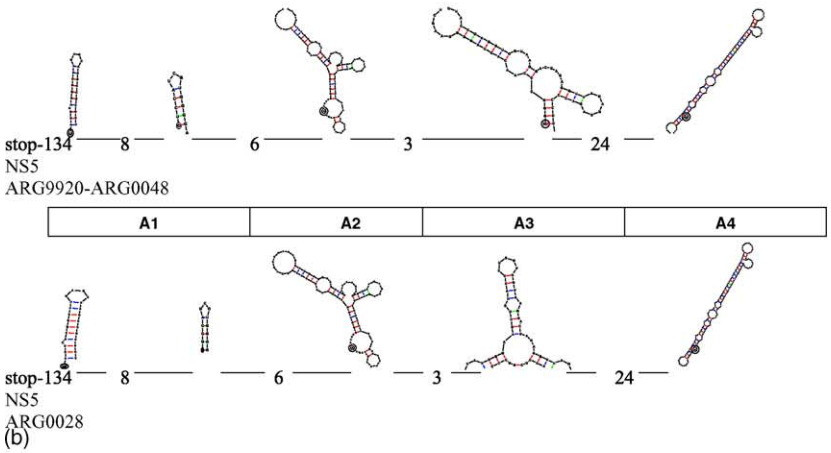
3.4. Phylogenetic analysis

Modeltest v3.06 was applied to infer the DNA substitution model from the data and the parameters were entered into PAUP* as settings for maximum-likelihood criterion. Among the 56 models tested, the best-fit model of nucleotide substitution proved to be a modification of the general time-reversible (GTR) plus gamma distribution (G) (Tamura and Nei, 1993).

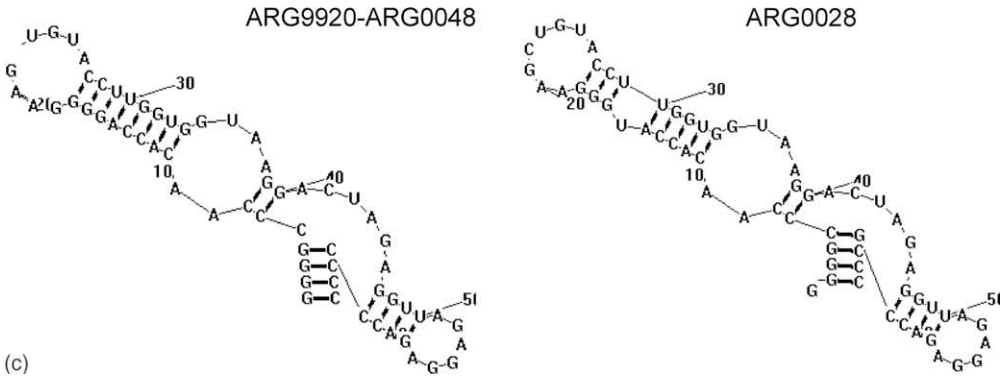
Estimated base composition frequencies were $\pi_A = 0.3803$, $\pi_C = 0.1784$, $\pi_G = 0.1539$, and $\pi_T = 0.2869$; and their relative substitution rates A ↔ C = 1, A ↔ G = 0.8460, A ↔ T = 1, C ↔ G = 1, C ↔ T = 0.1625 and G ↔ T = 1, with a γ rate at variable sites with a shape parameter (α) of 1.6368. An heuristic search for the best tree was performed and topology confirmed by 100 bootstrap replications with identical parameters. Phylogenetic relationships are shown in Fig. 4. Phylogenetic analysis of full-length divided Buenos Aires isolates into two clusters within American DEN-1 genotype V defined upon the entire E gene sequence (Goncalvez et al., 2002). Sample ARG0028 was phylogenetically linked to Brazilian samples and Argentine samples previously described as Clade I (dos Santos et al., 2002; Avilés et al., 2002). Lastly, ARG9920

Nt. position	FGA/89	ARG9920	ARG0028	ARG0048	Structure
10310	C	A	*	A	HVR
10314	A	*	C	*	HVR
10318	U	*	C	*	HVR
10320	G	A	A	A	HVR
10353	A	U	*	*	HVR
10355	G	A	*	A	HVR
10440	G	*	A	*	A1 w/o 2-D
10459	A	U	*	*	A1-A2
10535	A	*	U	*	A2
10541	U	C	*	C	A2
10567	G	*	U	*	**A3**
10613	C	*	G	*	**A3-CS2**
10620	C	*	U	*	A3-A4

(a)



(b)



(c)

Fig. 3. 3′UTR, features of the 3′UTR were analyzed (a) Nucleotide (nt) differences with FGA/89 are listed, (*) denotes the same base and the 2-D region affected is marked (A1–4). HVR stands for hypervariable region following NS5 stop codon, (w/o) for without and CS2 for cyclization sequence 2. Mutations that affect the 2-D A3 structure are in bold type. (b) Bi-dimensional structure inferred for Argentine strains, the numbers indicates the amount of nucleotides between regions marked in boxes. Drawing is not in scale. (c) Detail of A3 region, numbering refers to the region.

and ARG0048 were gathered together with samples from Paraguay and Argentine samples from former Clade II.

4. Discussion

We cloned and sequenced structural proteins from five viruses isolated in Buenos Aires City in 1999 and 2000. RT-PCR proved positive for DEN-1 from plasma and from cell culture supernatant. RNA from third passage was taken for analysis as mutations derived from this method are described to arise progressively from the 10th (Puri et al., 1997).

An overall excess of synonymous over non-synonymous mutations suggests that structural proteins are under strong functional constraints. However, several differences were observed between direct and cloned sequences, and statistical test showed that the diversity results were significantly above procedure threshold, allowing us to regard such variability as true polymorphisms. Besides, when mean diversity and ω were individually measured, a broad range was as-

Table 3
Replacement changes: non-Structural proteins, direct sequences were compared with FGA/89

ORF	Protein	AA	FGA/89	ARG9920	ARG0028	ARG0048
950	NS1	175	Y	H	H	*
1021		246	I	*	M	*
1068		293	N	*	S	*
1293	NS2A	166	K	R	*	R
1295		168	M	*	T	*
1340		213	K	*	E	*
1356	NS2B	13	I	*	V	*
1508	NS3	33	R	K	*	K
1645		170	A	T	*	T
1656		181	D	E	*	E
1714		239	M	*	I	*
1834		359	T	*	S	*
1875		399	K	R	*	R
2163	NS4A	69	M	**T**	**T**	**T**
2183		89	M	*	T	*
2193		99	S	N	*	N
2267	NS4B	23	H	Q	*	Q
2268		24	H	*	Q	*
2270		26	V	**A**	**A**	**A**
2334		90	I	*	L	*
2397		153	A	*	T	*
2533	NS5	40	E	*	*	G
2620		127	H	Y	*	Y
2628		135	I	T	*M*	T
2738		245	A	T	*	T
2868		375	M	**V**	**V**	**V**
2892		399	I	**T**	**T**	**T**
2996		503	L	F	*	F
3040		547	Q	*	R	*
3121		628	F	I	*	I
3122		629	L	*	F	*
3128		635	T	*	S	*
3137		644	D	N	*	N
3144		651	A	T	*	T
3162		669	I	V	*	V
3331		838	L	*	I	*

Positions are referred to the polyprotein (ORF) and to the corresponding protein (AA). Non-synonymous mutations are expressed in single-letter code. Fixed replacements are marked in bold, the multiple replacement in NS5-135 in italic, and absence of replacement as (*).

sessed, indicating that sequence diversity and selective pressures varied among patients as previously described (Wang et al., 2002b). Although more clones need to be sequenced to strengthen this concept, the presence of quasispecies in our data should be taken into account. Nevertheless, whether the mutant spectra are deleterious or substrates for episodic selection; or differences between samples are due to more post-infection days or to geographically restricted mutation rates remain unexplained.

Three viruses were full-length sequenced. Codon usage was slightly biased, corresponding to the described Mosquito Borne Flavivirus (Jenkins et al., 2001). Tajima's *D*-test demonstrated neutrality, and the ω ratio suggested that a weak purifying selection was operating in the whole polyprotein in agreement with other RNA viruses. This can be framed into the nearly neutral theory of molecular evolution bearing in mind short generation time, large population size and high mutation rates (Ohta and Tachida, 1990). Furthermore, RNA virus genome has mutation rates near the extreme and almost all sites are explored in a very narrow functional sequence space (Domingo and Holland, 1997). Thus, the idea that they have limited tolerance to mutations strongly supports the concept that RNA viruses are merely randomly changing rather than selectively adapting (Sala and Wain-Hobson, 2000). Although positive selection has been detected in Dengue viruses, it has not been reported in DEN-1 (Twiddy et al., 2002). Among many others, NS5-135 has been pointed out as a positive selection site in DEN-2 and seems to be a hot spot in our samples as it had multiple replacements in very short sampling time. Although the chance of appearance of new mutations is recognized by nearly neutral theory, we don't know their fate—some of them may increase fitness and will be fixed and others will be deleterious and tend to disappear in time.

Recombination has been described in dengue as a mechanism of variation but no evidence of recombination was detected in our samples (Holmes et al., 1999; Holmes and Burch, 2000; Tolou et al., 2001). As these strains derive from a relatively new recombinant parental, and samples fail to differ in many positions, the contribution of this mechanism to genetic diversity may be limited.

Two main components of the 3′UTR have been postulated as requirements for optimal minus-strand RNA synthesis—the most 3′UTR structures and sequences may act as promoters for viral replication (A4), and more proximal structures and sequences may function as enhancers for viral RNA replication and modulate its efficiency (Proutski et al., 1999). These effects in infectivity have been previously attributed to deletions in the 3′UTR and may be interpreted as a result of RNA secondary structure remodeling rather than sequence changing. In addition, mutations in the conserved complementary cyclization sequence (CS2) may alter the cyclization of 5′ and 3′UTR, proven to be essential for viral RNA replication in vitro (Khromykh et al., 2001). Mutation 10613 in ARG0028 altered the configuration of CS2 into A3 region, shortening the double strand region (stem) shaped between 5′ and 3′ sequences of that area. This vicinity of A4 that has been structurally modified by mutations may not function as a replication enhancer with the same accuracy as it does in the other Argentine viruses as clearly evidenced by their differential phenotype.

First isolation of strains FGA/89 and BR/90 defined differentiation in the CPE in mosquito cell line AP61. While BR/90 showed large syncytia in a few days, FGA/89 displayed no CPE despite producing 10 times more infectious particles. This difference in cell-to-cell fusion has been attributed to dissimilar pH requirements in cell culture, as FGA/89 required a lower pH due to increased stability of E oligomer (Desprès et al., 1993). The intermediate phenotype present in ARG0028 may be the result of sharing part of the

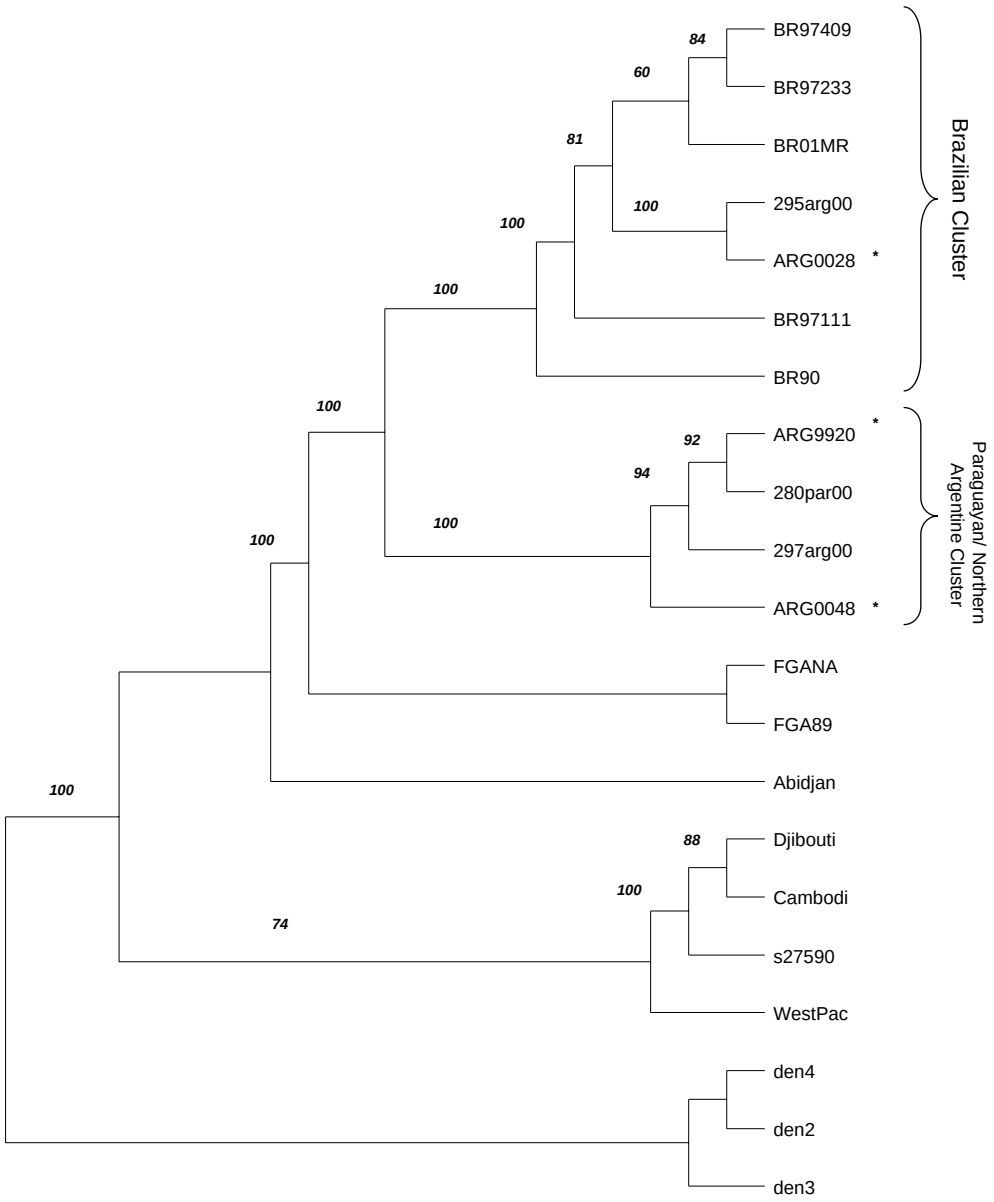


Fig. 4. Phylogenetic relationships of Argentine strains, full length sequences were used to build a ML tree with GTR + G model of DNA substitution. The result of 100 pseudoreplicates in key nodes is indicated in bold italic. (*) denotes samples sequenced in this work.

BR/90 amino acid changes in E protein related to FGA/89 (E96, E180, E297, E379) but not others (E338, E391). Taken together, these additional features may be responsible for the small-plaque phenotype and impaired ARG0028 growth.

Phylogenetic analysis of full-length sequences split Buenos Aires isolates into two clusters within American DEN-1 genotype V defined upon the entire E gene sequence. Other criteria have been used for genotyping DEN-1 as E-NS1 junction or a small fragment of E protein but the overall cut-off value of less than 6% nucleotide sequence variation between genotypes operates in all (Goncalvez et al., 2002). Bootstrap values strongly supported the obtained tree topology. Our samples shared many features with BR/90 and previously characterized Brazilian samples that allowed the distinction between two clades (Clade I–II). Clade I was phylogenetically linked to Brazilian samples and Clade II to samples from Paraguay and Northeastern Argentina. This phenomenon can be explained as two different introductions to Argentina from viruses coming from two bordering countries with dissimilar mutations and features. The first one may have come from Brazil and the other from Paraguay and different in situ evolution in countries with different ethnic backgrounds may have contributed to the development of two different clades in Argentine samples.

South America was considered free of dengue two decades ago but a dramatic increase in the number of dengue fever and hemorrhagic dengue cases has since been reported. Reasons for the reemergence of dengue in the Americas may be ascribed jointly to (i) spread of different serotypes in bordering countries, (ii) permanent migration

flow of viremic travelers and (iii) increase in vector infestation due to inconstant vector control strategies and economical support (Gubler, 2002; Guzmán and Kourí, 2002). Moreover, viruses seem to take advantage of diverse mechanisms to generate genetic diversity as recombination or gene flow as well as exploiting the increasing density of human hosts and urbanization (Holmes and Burch, 2000). Two different serotypes of dengue have been circulating in Argentina for the last few years: DEN-2 in the northern region in 1998 and DEN-1 in the Northeastern area of our country in 1999–2000 (Avilés et al., 1999, 2002). Even though the DEN-2 Jamaica strain (subtype III) detected in Bolivia has not been associated with increased clinical severity, the risk of dengue hemorrhagic fever is higher in places with serotype co-circulation (Guzmán and Kourí, 2002). In Buenos Aires, only one type (DEN-1) imported from Paraguay has been detected without evidence of local transmission. All patients analyzed in this study arrived with viremia from Paraguay, detected directly from plasma in an RT-PCR assay. Although dengue viremia is high, it is also brief and conditions for optimal transmission seem to be lacking in Buenos Aires City thus arresting the spread of the virus.

Acknowledgements

PRB has a fellowship from the National Council of Investigation and Technology (CONICET) and ASM is a member of Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina. We thank Dr. R.O. Zandomeni for helping in structural gene primer design.

References

Avilés, G., Rangeón, G., Vorndam, V., Briones, A., Baroni, P., Enria, D., Sabattini, M.S., 1999. Dengue reemergence in Argentina. Emerg. Infect. Dis. 5, 575–578.

Avilés, G., Rowe, J., Meissner, J., Manzur Caffarena, J.C., Enria, D., St. Jeor, S., 2002. Phylogenetic relationships of dengue-1 viruses from Argentina and Paraguay. Arch. Virol. 147, 2075–2087.

Boletín Epidemiológico República del Paraguay, 1999. Ministerio de Salud Pública y Bienestar social. Noviembre Año 5, N 17, 1–8, Asunción.

Boletín Epidemiológico República del Paraguay, 2000. Ministerio de Salud Pública y Bienestar social. Junio Año 6, N 19, 1–8, Asunción.

Boletín Epidemiológico Nacional, 2000–2001. Ministerio de Salud República Argentina.

Brinkworth, R.I., Fairlie, D.P., Leung, D., Young, P.R., 1999. Homology model of the dengue 2 virus NS3 protease: putative interactions with both substrate and NS2B cofactor. J. Gen. Virol. 80, 1167–1177.

Chen, Y., Maguire, T., Marks, R.M., 1996. Demonstration of binding of dengue virus envelope protein to target cells. J. Virol. 70, 8765–8772.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Desprès, P., Frenkiel, M.P., Deubel, V., 1993. Differences between cell membrne fusion activities of two dengue type-1 isolates reflects modifications of viral structure. Virology 196, 209–219.

Domingo, E., Holland, J.J., 1997. RNA virus mutations and fitness for survival. Ann. Rev. Microbiol. 51, 151–178.

dos Santos, C.N.D., Rocha, C.F.S., Cordeiro, M., Fragoso, S.P., Rey, F., Deubel, V., Desprès, P., 2002. 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. Virus Res. 90, 197–205.

Egloff, M.P., Benarroch, D., Selisko, B., Romette, J.L., Canard, B., 2002. An RNA cap (nucleoside-2′-*O*-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J. 21, 2257–2768.

Falconar, A.K.I., 1999. Identification of an epitope on the dengue virus membrane (M) protein defined by cross-protective monoclonal antibodies: design of an improved epitope sequence based on common determinants present in both E and M proteins. Arch. Virol. 144, 2313–2330.

Felsestein, J., 1989. PHYLIP—phylogeny inference package. Cladistics 5, 164–166.

Goncalvez, A.P., Escalante, A.A., Pujol, F.H., Ludert, J.E., Tovar, D., Salas, R.A., Liprandi, F., 2002. Diversity and evolution of the envelope gene of dengue virus type 1. Virology 303, 110–119.

Gubler, D.J., 2002. Epidemic dengue/dengue hemorrhagic fever as a public health. Trends Microbiol. 10, 100–103.

Guzmán, M.G., Kourí, G., 2002. Dengue: an update. Lancet Infect. Dis. 2, 33–42.

Heinz, F.X., Allison, S.L., 2001. The machinery for flavivirus fusion with host cell membranes. Curr. Opin. Microbiol. 4, 450–455.

Holmes, E.C., Worobey, M., Rambaut, A., 1999. Phylogenetic evidence for recombination in dengue virus. Mol. Biol. E 16, 405–409.

Holmes, E.C., Burch, S.S., 2000. The causes and consequences of genetic variation in dengue virus. Trends Microbiol. 8, 74–77.

Jenkins, G.M., Pagel, M., Gould, E.A., Zanotto, P.M., de, A., Holmes, E.C., 2001. Evolution of base composition and codon usage bias in the genus Flavivirus. J. Mol. E 52, 383–390.

Jianmin, Z., Linn, M.L., Bulich, R., Gentry, M.K., Aaskov, J.G., 1995. Analysis of functional epitopes on the dengue 2 envelope (E) protein using monoclonal IgM antibodies. Arch. Virol. 140, 899–913.

Johansson, M., Brooks, A.J., Jans, D.A., Vasudevan, S.G., 2001. A small region of the dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-beta and the viral helicase, NS3. J. Gen. Virol. 82, 735–745.

Khromykh, A.A., Meka, H., Guyatt, K.J., Westaway, E.G., 2001. Essential role of cyclization sequences in flavivirus RNA replication. J. Virol. 75, 6719–6728.

Lanciotti, R.S., Callisher, C.H., Gubler, D.J., Chang, G.J., Vorndam, A.V., 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbiol. 30, 545–551.

Leitmeyer, K.C., Vaughn, D.W., Watts, D.M., Salas, R., Villalobos, I., Ramos, C., Ricco-Hesse, R., 1999. Dengue virus structural differences that correlate with pathogenesis. J. Virol. 73, 4738–4747.

Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C., 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. 73, 152–160.

Marchler-Bauer, A., Anderson, J.B., DeWeese-Scott, C., Fedorova, N.D., Geer, L.Y., He, S., Hurwitz, D.I., Jackson, J.D., Jacobs, A.R., Lanczycki, C.J., Liebert, C.A., Liu, C., Madej, T., Marchler, G.H., Mazumder, R., Nikolskaya, A.N., Panchenko, A.R., Rao, B.S., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Vasudevan, S., Wang, Y., Yamashita, R.A., Yin, J.J., Bryant, S.H., 2003. CDD: a curated entrez database of conserved domain alignments. Nucl. Acids Res. 31, 383–387.

Martin, D., Rybicki, E., 2000. RDP detection of recombination amongst aligned sequences. Bioinformatics 16, 562–563.

Mathews, D.H., Sabina, J., Zuker, M., Turner, D.H., 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. 288, 911–940.

Matzura, O., Wennborg, A., 1996. RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. Comput. Appl. Biosci. 12, 247–249.

Miagostovich, M.P., dos Santos, F.B., de Simone, T.S., Costa, E.V., Filippis, A.M.B., Schatzmayr, H.G., Nogueira, R.M.R., 2002. Genetic characterization of dengue virus type 3 isolates in the State of Rio de Janeiro, 2001. Brazil J. Med. Biol. Res. 35, 869–872.

Nogueira, R.M.R., Miagostovich, M.P., Lampe, E., Souza, R.W., Zagne, S.M.O., Schatzmayr, H.G., 1993. Dengue epidemic in the state of Rio de Janeiro, Brazil, 1990–1991: co-circulation of dengue-1 and dengue-2. Epidemiol. Infect. 111, 163–170.

Ohta, T., Tachida, H., 1990. Theoretical study of near neutrality. I. Heterozygosity and rate of mutant substitution. Genetics 126, 219–229.

Page, R.D.M., 1996. Treeview: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Proutski, V., Gould, E.A., Holmes, E.C., 1997. Secondary structure of the 3′ unstranlated region of flaviviruses: similarities and differences. Nucl. Acids Res. 25, 1194–1202.

Proutski, V., Gritsun, T.S., Gould, E.A., Holmes, E.C., 1999. Biological consequences of deletions within the 3′ untranslated region of flaviviruses may be due to rearrangements of RNA secondary structure. Virus Res. 64, 107–123.

Puri, B., Nelson, W.M., Henchal, E.A., Hoke, C.H., Eckels, K.H., Dubois, D.R., Porter, K.R., Hayes, C.G., 1997. Molecular analysis of dengue virus attenuation after serial passage in primary dog kidney cells. J. Gen. Virol. 78, 2287–2291.

Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from Tick encephalitis virus at 2 Å resolution. Nature 375, 291–298.

Rice, C.M., 1996. Flaviviridae: the viruses and their replication. In: Fields, B.N., Knipe, D.M., Chanock, R.M., Hirsch, M.S, Melnick, J.L, Monath, T.P., Roizman, B. (Eds.), Fields Virology, third ed. Lippincot-Raven, Philadelphia.

Rigau-Pérez, J.G., Clark, G.G., Gubler, D.J., Reiter, P., Sanders, E.J., Vorndam, A.V., 1998. Dengue and dengue haemorrhagic fever. Lancet 352, 971–977.

Roehrig, J.T., Risi, P.A., Brubaker, J.R., Hunt, A.R., Beaty, B.J., Trent, D.W., Mathews, J.H., 1994. T-helper cell epitopes on the E-glycoprotein of dengue 2 Jamaica virus. Virology 198, 31–38.

Rozas, J., Rozas, R., 1999. DNASP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15, 174–175.

Sala, M., Wain-Hobson, S., 2000. Are RNA viruses adapting or merely changing? J. Mol. E 51, 12–20.

Shurtleff, A.C., Beasley, D.W.C., Chen, J.J.Y., Ni, H., Suderman, M.T., Wang, H., Xu, R., Wang, E., Weaver, S.C., Watts, D.M., Russell, K.L., Barrett, A.D.T., 2001. Genetic variation in the 3′ non-coding region of dengue viruses. Virology 281, 75–87.

Smith, D.B., McAllister, J., Casino, C., Simmonds, P., 1997. Virus quasispecies: making a mountain out of a molehill. J. Gen. Virol. 78, 1511–1519.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). version 4. Sinauer Associates, Sunderland, Massachusetts.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. E 10, 512–526.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24, 4876–4882.

Tolou, H.J.G., Durand, J.-P., Mercier, V., de Pina, J.J., de Mico, P., Billoir, F., Charrel, R.N., Lamballerie, X., 2001. Evidence for recombination in natural populations of dengue virus type-1 based on the analysis of complete genome sequences. J. Gen. Virol. 82, 1283–1290.

Twiddy, S.S., Woelk, C.H., Holmes, E.C., 2002. Phylogenetic evidence for adaptative evolution of dengue viruses in nature. J. Gen. Virol. 83, 1679–1689.

Van der Stuyft, P., Gianella, A., Pirard, M., Holzman, A., Peredo, C., Boelaert, M., Vorndam, V., 1998. Dengue serotype 2 subtype III (Jamaica) epidemic in Santa Cruz, Bolivia. Trop. Med. Int. Health 3, 857–858.

Wang, W.K., Lin, S.R., Lee, C.M., King, C.C., Chang, S.C., 2002a. Dengue-3 virus in plasma is a population of closely realted genomes: quasispecies. J. Virol. 76, 4662–4665.

Wang, W.K., Sung, T.L., Lee, C.N., Lin, T.Y., King, C.C., 2002b. Sequence diversity of the capsid gene and the nonstructural gene NSB of dengue-3 virus in vivo. Virology 303, 181–191.

Wu, H.C., Huang, Y.L., Chao, T.T., Jan, J.T., Huang, J.L., Chiang, H.Y., King, C.C., Shaio, M.F., 2001. Identification of B-cell epitope of dengue virus type 1 and its application in diagnosis of patients. J. Clin. Microbiol. 39, 977–982.

Yao, Z.J., Kao, M.C.C., Loh, K.C., Chung, M.C.M., 1995. A serotype-specific epitope of dengue virus 1 identified by phage isplayed ramdom peptide library. FEMS Microbiol. Lett. 127, 93–98.

You, S., Falgout, B., Markoff, L., Padmanabhan, R., 2001. In vitro RNA synthesis from exogenous dengue viral RNA templates requires long range interactions between 5′- and 3′-terminal regions that influence RNA structure. J. Biol. Chem. 276, 15581–15591.