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Phylogenetic relationships of dengue-1 viruses from Argentina and Paraguay

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Summary. We sequenced the Capsid-pre Membrane (C/prM) and the Envelope-Nonstructural protein 1 (E/NS1) regions of 24 recent isolates of dengue-1 (DEN-1) from South America. This included 12 Argentinean and 11 Paraguayan DEN-1 strains isolated in 2000 plus a Paraguayan strain isolated in 1988. These sequences were compared with published sequences of DEN-1 isolated worldwide to determine the origin of these isolates. Pairwise comparisons of strains from Paraguay and Argentina revealed a nucleotide divergence of 0–5% in the E/NS1 region and 0–3% in the C/prM region. Our results showed that these viruses belong to the same genotype, but can be separated into two clades. Interestingly, both clades circulated simultaneously in the same geographic area during the 2000 outbreaks. Amino acid differences were found between both clades in the C/prM region at position 100 (Lys vs. Arg) and in the E/NS1 region at positions 722 (Ala vs. Thr). Although the geographic movement of DEN-1 virus can not be unequivocally traced from the genetic relationship determined here, our results suggest that the recent epidemics in Argentina and Paraguay were due to the re-emergence of a previously circulating strain, or to the virus circulating unnoticed, rather than to the introduction of a new genotype.

Introduction

Dengue (DEN) virus infections are a serious public health problem in all tropical and subtropical areas of the world, with up to 100 million infections occurring

annually [7, 9]. Dengue viruses are transmitted between humans principally by the mosquito *Aedes aegypti* [7]. They belong to the family *Flaviviridae*, genus *Flavivirus*, and are grouped in one of eight flavivirus antigenic complexes [3]. Infections by any of the four serotypes can result in either subclinical manifestations or a febrile disease – dengue fever (DF) – or a fulminating illness – dengue hemorrhagic fever (DHF) – which can progress to dengue shock syndrome and death [7]. The mechanism(s) by which DEN viruses cause severe disease are still unknown. Several investigators have suggested explanations including viral factors (e.g., differences in virulence) [7], and host-immunological mechanisms such as cell-mediated immunity, and antibody-dependent enhancement due to cross-reaction in secondary infections [9]. However, because studies relating DEN virulence markers like mouse neurovirulence or cell-culture growth are of limited value in assessing human disease potential, the viral determinants of severe disease are still unclear.

The DEN virus genome is a positive-sense single-stranded RNA approximately 11,000 nucleotides long, encoding 10 distinct proteins. It has a type 1 cap at the 5′ end of the RNA but lacks a polyadenylic acid tract at the 3′ end [30]. The three 5′ proteins are structural: capsid (C), membrane (M) and envelope (E), while the remaining proteins are nonstructural (NS) proteins. The gene order is 5′-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′, expressed as a single polyprotein that is cleaved by both viral and cellular proteases to form the viral polypeptides [30].

Spread of DEN viruses between different geographic areas is an important feature in the epidemiology of the disease. Molecular characterization of DEN viruses has been used to define genetic variation among strains of the same serotype, and to track geographic movement of the viruses [4, 5, 6, 15, 16, 17, 18, 24, 25, 26, 29, 30]. The introduction of a novel DEN genotype in an area where another genotype of the same serotype is circulating may lead to the appearance of DHF and the displacement of the native genotype [25].

Wang et al. [32] used the E region of DEN 1–4 virus genomes to study the evolution of endemic/epidemic and sylvatic DEN viruses in West Africa and Malaysia. They concluded that sylvatic genotypes diverged from endemic/epidemic forms of DEN-2 virus on the order of $1{,}000 \pm 500$ years ago; with DEN-4 virus probably diverging 600 ± 300 years ago and DEN-1 virus diverging about 200 ± 100 years ago. As there were no sylvatic DEN-3 virus isolates from Malaysia, they were not able to compare the endemic/epidemic DEN-3 form with a sylvatic form. They concluded that the ancestors of DEN-1, 2 and 4 viruses could be either sylvatic or endemic/epidemic strains.

Rico-Hesse [24] defined five genotypes for DEN-1 viruses isolated worldwide using partial sequence from the E/NS1 junction region: I) America, Africa, and Southeast Asia; II) one isolate from Sri Lanka; III) one isolate from Japan; IV) Southeast Asia, the South Pacific, Australia and Mexico; V) Taiwan and Thailand.

Dengue-1 virus was first detected in Paraguay in 1988; a virus strain was isolated and the E/NS1 region was sequenced [24]. In 1989, DEN-1 virus caused an outbreak in Paraguay that produced approximately 40,000 cases of DF [1].

The most affected city was Asuncion (Federal District). In 1999–2000, a second outbreak again caused by DEN-1 virus was reported in Paraguay with at least 27,000 cases. There is evidence to suggest that more than 100,000 cases may have occurred in Asuncion district [2]. All cases were classified clinically as DF under PAHO/WHO criteria [22]. This outbreak spilled over into Argentina, where several cases occurred in the northern part of the country (Avilés et al., unpublished data). The geographic distribution of cases in Argentina covered a large area. The northeast Misiones and Formosa provinces are located close to the border with Paraguay but Jujuy province is located in the far northwest of Argentina, separated from the other provinces by 1,200 km.

Given the limited number of studies on the molecular epidemiology of DEN-1 virus [4, 5, 24], we decided to sequence multiple DEN-1 virus strains from Argentina and Paraguay. We analyzed the genomic sequences from the Capsid-pre Membrane (C/prM) and the Envelope-Nonstructural protein 1 (E/NS1) regions of DEN-1 from the 1988 Paraguayan isolate and 12 Argentinean and 11 Paraguayan isolates from the more recent outbreaks. These sequences were compared with published sequences of DEN-1 isolated from other countries worldwide, in order to determine potential origins of these isolates.

Material and methods

Viral isolation and RNA extraction

The 24 DEN-1 viral strains analyzed by RT-PCR and sequencing are listed in Table 1. All the viruses were isolated at The Instituto Nacional de Enfermedades Virales Humanas "Dr. Julio I. Maiztegui" (INEVH), Pergamino, Argentina, except for the Paraguayan strain from 1988, which was provided by Dr. Robert Shope, Department of Pathology, University of Texas – Medical Branch, Galveston, USA. The viruses were isolated from the serum samples of patients in the *Aedes albopictus* cell line, C6/36, according to published techniques [8]. The dates of onset of the infections or collection of the samples were from 3/3/00 to 3/22/00 for Paraguayan samples and 3/24/00 to 4/19/00 for Argentinean samples. The viral isolates were identified as DEN-1 virus by indirect immunofluorescence assay [8] using monoclonal serotype-specific antibodies against all four DEN virus serotypes strains obtained from PAHO/WHO. Viral RNA was extracted from the infected cells using the QIAmp Viral RNA Kit (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions.

RT-PCR amplification

For the C/prM region, RT-PCR followed by a second round of nested priming was performed as described previously [14]. Primers D1 (genome position 134) and D2 (genome position 616) for the first round PCR, and D1 and TS1 (genome position 568) for the nested second round PCR were used to obtain a fragment of 482 nt. A double amplification was used because in most cases in the first round PCR we could not observe any product. A set of primers to amplify the E/NS1 region of the genome was designed from the consensus sequences of four DEN-1 strains taken from GenBank: 16007 (Accession number AF180818), Nauru (M23027), Hawaii (X69395) and Thailand (D00502). The primer sequences are: D1F: 5′-TGAAACTAAGCTGGTTCAAG-3′ (forward) and D1R: 5′-CCCAATGGC(A/G)GCTGATA GTCTC-3′ (reverse), at nucleotide positions 2094 and 2539, respectively. This combination generated a 466-nt fragment. The PCR conditions for the E/NS1 fragment were similar to

Table 1. Dengue 1 virus isolates used in the molecular epidemiology study

Isolate	Year isolated	Country	Locality	Region or province
259par00	2000	Paraguay	Asunción	Central
264par00	2000	Paraguay	Caaguazú	Caaguazú
269par00	2000	Paraguay	Ciudad del Este	Alto Paraná
274par00	2000	Paraguay	Capiatá	Central
275par00	2000	Paraguay	Fernando de la Mora	Central
276par00	2000	Paraguay	San Lorenzo	Central
280par00	2000	Paraguay	Lambaré	Central
281par00	2000	Paraguay	Lambaré	Central
282par00	2000	Paraguay	Lambaré	Central
283par00	2000	Paraguay	Lambaré	Central
284par00	2000	Paraguay	Areguá	Central
291arg00	2000	Argentina	Eldorado	Misiones
292arg00	2000	Argentina	Puerto Esperanza	Misiones
293arg00	2000	Argentina	Eldorado	Misiones
294arg00	2000	Argentina	9 de Julio	Misiones
295arg00	2000	Argentina	Libertad	Misiones
296arg00	2000	Argentina	9 de Julio	Misiones
297arg00	2000	Argentina	Wanda	Misiones
298arg00	2000	Argentina	Puerto Esperanza	Misiones
299arg00	2000	Argentina	Clorinda	Formosa
300arg00	2000	Argentina	San Pedro	Jujuy
301arg00	2000	Argentina	San Pedro	Jujuy
302arg00	2000	Argentina	Clorinda	Formosa
2000	1988	Paraguay	Asunción	Central

those used for the C/prM fragment as described [14], but with an annealing temperature of 50 °C instead of 55 °C, and 40 cycles instead of 35.

DNA purification and sequencing

PCR amplified DNA products were separated on 2% agarose gels, and bands of the predicted size were excised from the gel and purified using a Geneclean^{TM} Kit (Bio 101) (Vista, CA) according to the manufacturer's instructions. Purified PCR products were sequenced using the Big Dye^{TM} Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on the ABI PRISM 310 Genetic Analyzer. Amplifying primers were also used for sequencing.

Computer analysis

Sequence alignments were performed using Auto Assembler (Applied Biosystems) and MegAlign program (DNASTAR, Lasergene). The aligned files were converted into text files using BBEdit Lite 4.6, and imported into PAUP 3.1.1 [28]. Protein translations were made using ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB). We removed primer generated ends from the sequences prior to alignments.

Phylogenetic analysis of nucleotide sequences was carried out by the maximum parsimony method using PAUP, version 3.1.1 [28]. Published sequences used in the analysis are listed in

Table 2. Dengue viral sequences from GenBank used in the phylogenetic analysis

Virus	Strain	Origin	Year isolated	Accession number
DEN-1	BR/90	Brazil	1990	S64849
DEN-1	CV1636/77	Caribbean (Jamaica)	1977	D00501
DEN-1	FGA/89	French Guiana	1989	AF226687
DEN-1	16299	Nauru	1974	M23027
DEN-1	S275/90	Singapore	1990	M87512
DEN-1	836-1	Philippines	1984	D00503
DEN-1	VN93/1	Vietnam	1993*	Z74047
DEN-1	AHF82-80	Thailand	1980	D00502
DEN-1	5189–97	Peru	1997*	AF193860
DEN-2	NGC	New Guinea	1944	AF038403

*These sequences are unpublished. They can be found in GenBank but the year of isolation is not given. We inferred the year from the name of the strains.

Table 2. Analysis was carried out by a heuristic search, using a 4:1 weighing of transversions over transitions. Such weighing is predicted to improve the effectiveness of the maximum parsimony method for estimation of the correct phylogeny [11, 5]. The resulting unrooted trees were outgrouped to DEN-2 (New Guinea C) sequence. Bootstrap confidence limits were calculated by 1,000 heuristic search repetitions. A bootstrap value of 70 or higher at a particular node or branch point has been shown to correspond to a probability of 95% or greater that the node (and corresponding clade) is real [10].

Results

We compared DEN-1 virus sequences from Argentina and Paraguay with sequences from DEN-1 from other areas of the world in the C/prM and E/NS1 regions of the genome. A 333-nucleotide fragment from the C/prM region (positions 193 to 525) and a 333-nucleotide fragment from the E/NS1 region (positions 2133 to 2465) was sequenced to determine the relationship between the DEN-1 isolates. These nucleotide sequences were compared to published DEN-1 sequences.

Comparison of nucleotide and amino acid sequences

Pairwise comparisons of selected representative strains from Paraguay and Argentina revealed a maximum nucleotide divergence between strains of 5% in the E/NS1 region, and 3% in the C/prM region. (Tables 3 and 4). Sequences of these strains clustered into one of two groups – sequences that were identical or nearly identical to the Paraguay 88 strain (Paraguay 88-like) and sequences that were identical or nearly identical to the 269par00 strain (269par00-like). Amino acid sequence comparisons between selected Paraguay 88-like strains and selected 269par00-like strains showed a divergence of 2% in the E/NS1 region and 1–2% in the C/prM region (Tables 3 and 4). Using 6% nucleotide divergence in the E/NS1 region as a cut-off point for the definition of a DEN virus genotype within a serotype [24], we consider that our isolates belong to two clades, or subgroups,

Table 3. Nucleotide and amino acid homology between typical DEN-1 virus strains used in this study for the envelope/non structural protein 1 region (%)*

		Nucleotide percent identity				
		269par00	284par00	Caribbean77	FGA/89	Paraguay88
Amino acid percent identity	269par00		99.4	91.3	95.8	97.3
	284par00	100.0		90.7	95.2	96.7
	Caribbean77	95.5	95.5		90.7	91.3
	FGA/89	98.2	98.2	95.5		97.3
	Paraguay88	98.2	98.2	95.5	98.2	

*Nucleotide position of the analyzed region: 2133–2465.

of the same genotype. In the E/NS1 region, comparison of Paraguay 88-like or 269par00-like strains with Caribbean 77 or Nauru strains showed a nucleotide divergence of 9% and an amino acid divergence of 5%, indicating that Caribbean 77 and Nauru represented a different genotype. The C/prM region comparisons were similar, although nucleotide and amino acid identities were higher, indicating that this region is more conserved between genotypes.

Phylogenetic analysis

Figure 1 shows the result of phylogenetic analysis of the combined C/prM and E/NS1 regions. Two equally parsimonious trees were obtained, with the only differences being minor rearrangements in the terminal branches. Both trees had three clades. The first clade (clade I) was comprised of strains from Argentina and Paraguay isolated during the 2000 outbreak, the strain from Paraguay isolated in 1988, and the strain from French Guiana isolated in 1989. The second clade (clade II) contained only strains from Argentina and Paraguay isolated in the 2000 outbreak. The third clade (clade III) was comprised of published sequences from Asia, the Pacific and the Caribbean. Both trees obtained with the combined genome regions show the same grouping of isolates as the E/NS1 and C/prM

Table 4. Nucleotide and amino acid homology between typical DEN-1 virus strains used in this study for the Capsid/premembrane region (%)*

		Nucleotide percent identity				
		269par00	284par00	FGA/89	Nauru	Paraguay88
Amino acid percent identity	269par00		99.7	97.0	95.2	97.3
	284par00	100.0		96.7	95.5	97.0
	FGA/89	98.2	98.2		94.9	98.5
	Nauru	97.3	97.3	97.3		95.2
	Paraguay88	99.1	99.1	99.1	98.2	

*Nucleotide position of the analyzed region: 193–525.

trees described below, but with much better bootstrap values. Bootstrap values for clades I, II, and III were 84, 97, and 90, respectively, indicating that these were distinct groups. In order to include additional previously published partial DEN-1 sequences in the analysis, as well as look for evidence of recombination, each of these regions was analyzed separately. Two equally parsimonious trees were obtained from analysis of the E/NS1 region alone, with the only differences being minor rearrangements in the terminal branches (data not shown). These trees were similar to the combined C/prM-E/NS1 trees, with the addition of strains from Peru and Singapore to clade I. Although clade I had a low bootstrap value (55), the confidence limit for the clade I/II node was quite high (86), indicating that these were distinct groups from clade III. We also compared DEN-1 E/NS1 sequences from Argentina and Paraguay with the shorter DEN-1 sequences published by Rico-Hesse [24] in the region of overlap. Our DEN-1 sequences again group in the same two clades (data not shown), and belong to genotype I as defined by Rico-Hesse. Numerous equally parsimonious trees were obtained from analysis of the C/prM region alone, but all trees showed the Argentinean and Paraguayan isolates divided into two clades (data not shown). These trees were similar to the combined C/prM-E/NS1 and E/NS1 trees, with the addition of strains 274par00, 283par00, 300arg00 and a 1990 isolate from Brazil to clade I, and the addition of strains 275par00, 281par00, 302arg00, 276par00, and 264par00 to clade II (we were unable to obtain E/NS1 PCR products for these 8 strains using a single round of PCR, therefore, they do not appear in Fig. 1). The bootstrap value for clade II was 96, indicating that this was a distinct group. Lower bootstrap values for clade

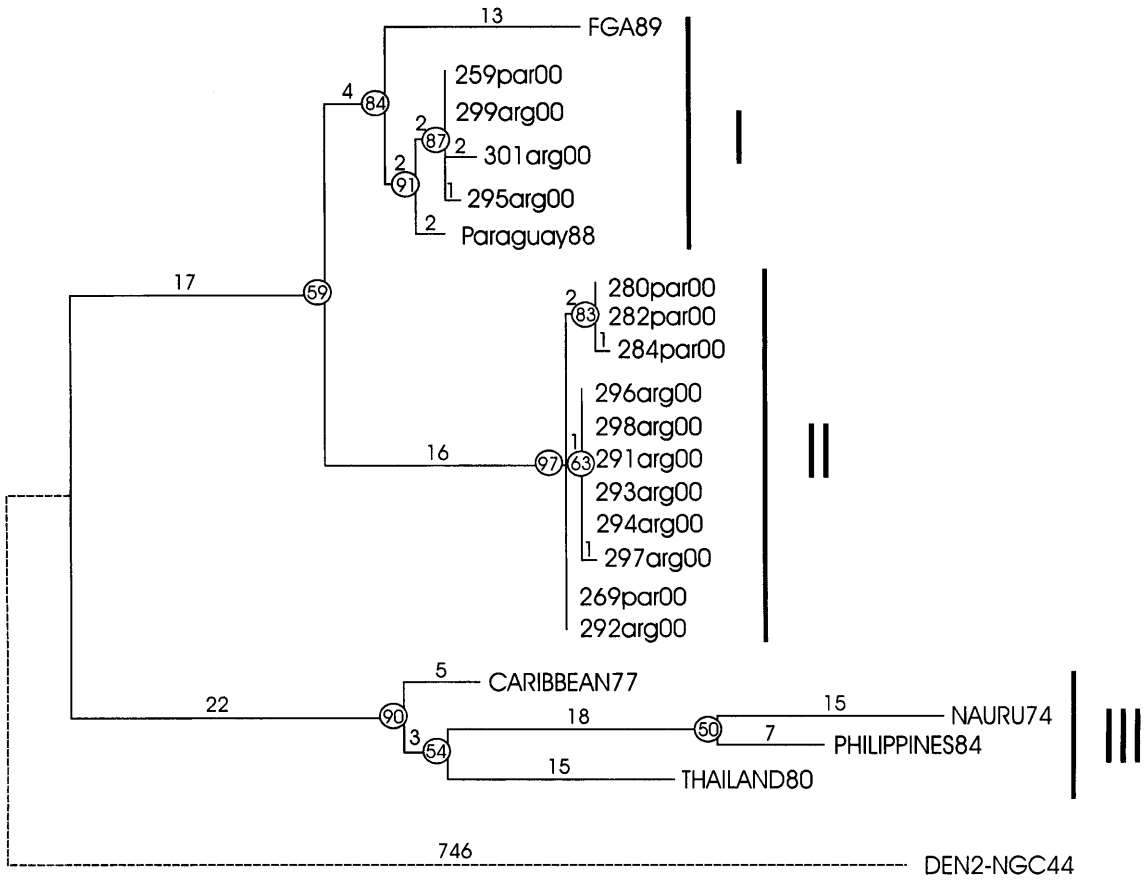

Fig. 1. Phylogenetic relationship of Argentinean and Paraguayan DEN-1 viruses to previously characterized DEN-1 viruses. Phylogenetic analysis of a 333-nucleotide sequence from the C/prM region (position 193 to 525) combined with a 333-nucleotide sequence from the E/NS1 region (position 2133 to 2465) was carried out by the maximum parsimony method using PAUP software. Horizontal line lengths are proportional to nucleotide step differences (indicated above each branch). Because the analysis was weighted 4:1 for transversions over transitions, nucleotide steps are not the number of nucleotide differences. Bootstrap confidence values greater than 50 are circled at each major branch. For clarity, bootstrap confidence values have been omitted for the terminal taxa. Vertical lines are for graphic representation only

I, clade III, and the Nauru, Singapore and Vietnam isolates in the C/prM analysis reflect the fact that this is a very conserved region of the genome.

The translated amino acids of the Argentinean and Paraguayan strains in the C/prM and E/NS1 regions (110 amino acids each) were aligned with amino acid sequences of DEN-1 virus strains deposited in GenBank (Table 2). In the C/prM region the only consistent change was a Lys 100 $\Rightarrow$ Arg change in clade II with respect to clades I and III. In the E/NS1 region the only consistent change was Ala 722 $\Rightarrow$ Thr, also in clade II with respect to clades I and III.

Figure 2 shows the geographical distribution of the two clades of DEN-1 viruses isolated from Argentina and Paraguay. Interestingly, both clades

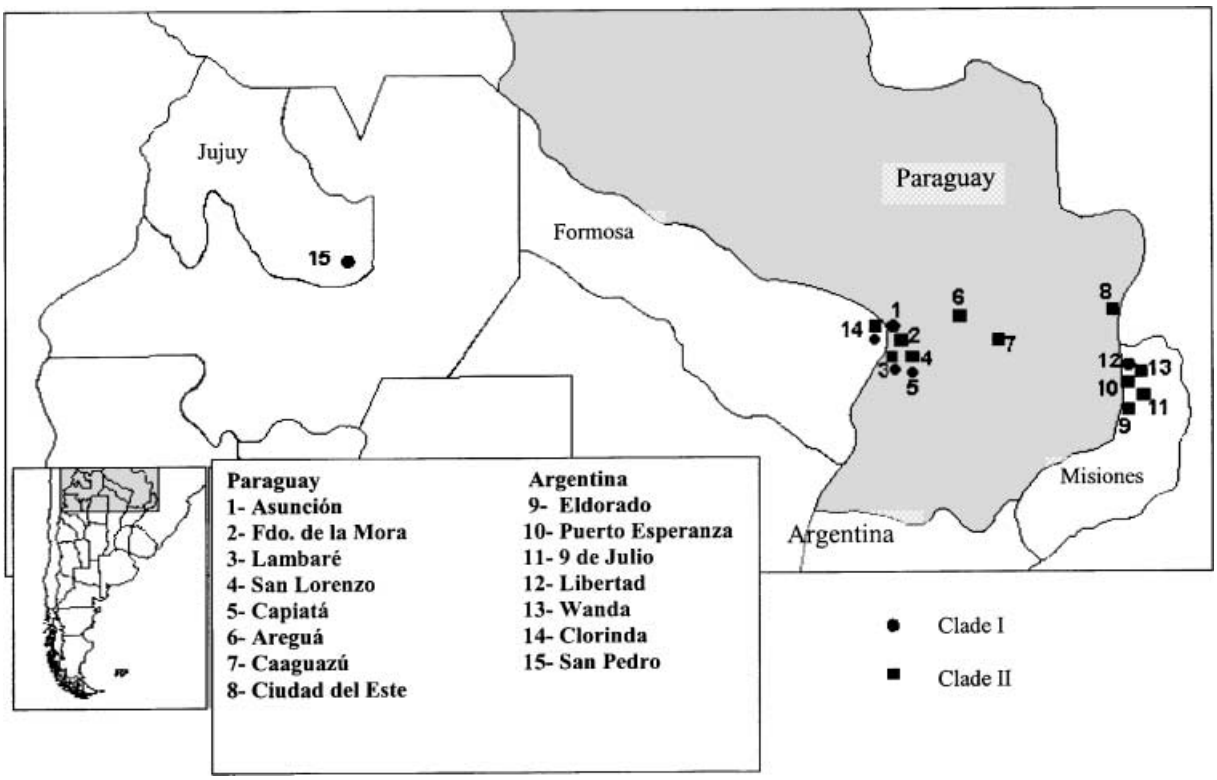

Fig. 2. Geographical distribution of the two clades of dengue-1 viruses isolated from Argentina and Paraguay in 2000

circulated simultaneously in the same geographic area during the Argentinean and Paraguayan outbreaks in 2000. In some localities such as Lambare (Paraguay) or Clorinda (Argentina), both clades were isolated together in the same location.

Discussion

This study represents the first attempt to analyze the genetic variability of DEN-1 viruses responsible for outbreaks of DEN in Argentina and Paraguay. DEN-1 virus had never been detected in Argentina prior to the cases identified in 2000. We also analyzed DEN-1 virus strains that circulated in Paraguay in 1988 and 2000. To our knowledge, these were the only times DEN-1 virus has been isolated in this region of South America.

It is impractical to compare full-length gene segment sequences when looking at a large number of samples. Several studies have shown that the phylogenetic trees obtained from small gene fragment sequences are congruent with the trees obtained from an entire gene sequence although there may be minor rearrangements in the terminal branches [12, 13, 19, 20, 21, 23, 27, 33].

The C/prM region was included in the analysis because it had been used for diagnosis, and PCR amplification products were already available for sequencing.

This region is highly conserved, yet can differentiate the four DEN serotypes [14]. It did not yield enough sequence variation among DEN-1 genotypes for robust phylogenetic comparison, however, so the E/NS1 region was separately amplified because it is a more variable region than the C/prM region. Several other phylogenetic analyses have also used this region, thus, many published sequences were available for comparison. Rico-Hesse [24] conducted a thorough analysis of the DEN-1 genome to determine the ideal region to use for phylogenetic analysis and reported that the E/NS1 junction was an informative region for this purpose.

Chungue et al. [5] analyzed 35 global DEN-1 virus strains isolated from humans and mosquitoes over a period of 50 years, using a 237 nucleotide fragment from the E protein. They found maximum nucleotide sequence variation of 6.9% over this region, and defined 3 different genotypes. In their study, all the American DEN-1 virus strains fell into the same genotype.

Chu et al. [4] observed that although there was a less than 2% change in the nucleotide sequence of DEN-1 E proteins, strains could be differentiated by the clusters of nucleotide changes and deduced amino acids in three distinct topotypes for different geographic regions (Thailand, Philippines and the Caribbean).

Our results showed that all viruses belong to the same genotype, even though they showed some variability and grouped in two different clades. It was noticeable that DEN-1 virus caused a large outbreak in Paraguay in 1989, (40,000 estimated cases), and again in 1999–2000 (reports vary between 27,000 and 100,000 cases). Possibly the pool of susceptible individuals grew in the 10 years between the two outbreaks. We found that the virus which circulated in 1988 in Paraguay was closely related to the viruses which were present in the 1999–2000 outbreak (clade I), however there were no isolates available from the 1989 outbreak to compare with the 1999–2000 outbreak. If we assume that the DEN-1 virus present in 1988 was also the cause of the 1989 outbreak, it would indicate that there has been a large accumulation of susceptible individuals over the 10-year period between outbreaks.

In our study, the amino acid difference found in the E/NS1 region between clades I and II at position 722 was from a hydrophobic to a hydrophilic (both uncharged) amino acid (Ala $\Rightarrow$ Thr). Thr 722 was only present in clade II strains. The only amino acid difference in the C/prM region between clades I and II was found at position 100 (Arg $\Rightarrow$ Lys), but both amino acid residues are positively charged (hydrophilic), which may confer an esteric difference but not a charge difference. The impact of these changes on biological properties is not known.

Complete genome sequence analysis by Tolou et al. demonstrates the likelihood of recombination between different strains of DEN-1 virus. The E region is considered to be a hot spot for this event [31]. In our study, we sequenced one region outside (nucleotide positions 193–525) and one region inside (nucleotide positions 2133–2465) this possible recombination site. Our sequences group in the same two clades regardless of character sets used (C/prM or E/NS1 regions alone or combined), so, no recombination event occurred in any of our strains, at least in the regions analyzed.

The geographic distribution of viral isolates from Paraguay and Argentina are shown in Fig. 2. Isolates from Paraguay and from Argentinean provinces of Misiones and Formosa were found in both clades I and II, thus there were two subgroups of the virus circulating simultaneously in these adjacent geographic regions. The two isolates from Jujuy grouped with clade I, however there were only 6 other cases from this region and it is possible that these individuals had contracted the virus when travelling to the northeast. It remains to be determined if the two different clades we found circulating together are neutralized to the same extent by acute and convalescent patient sera. This would help in determining if epitope differences exist between these clades.

Other investigators have demonstrated the wide geographic distribution of one DEN-1 genotype by viral nucleotide sequence comparison [24]. The largest genotypic group (genotype I) consisted of DEN-1 viruses from three continents: The Americas, Africa and Southeast Asia. It has been suggested that in the recent past, possibly during the early 1960s with the advent of jet travel, a progenitor of these viruses was widely disseminated and established itself in numerous niches. After this initial event, multiple lineages were formed by divergent evolution [24].

Although the geographic movement of DEN-1 virus can not be unequivocally determined from the genetic relationship shown here, the molecular characterization combined with the epidemiological observations allowed us to make reasonable inferences. The virus isolated in Paraguay in 1988 was closely related to the viruses isolated from French Guiana in 1989, Brazil in 1990, Peru in 1997, and Argentina and Paraguay in 2000. All of the viruses circulating in the Americas analyzed in this study fell into the same genotype, and were found in several different countries. This observation suggests that the recent epidemics in Argentina and Paraguay were due either to the re-emergence of a previously circulating strain, or the virus was circulating in the area at a lower level between epidemics, rather than to the introduction of a new genotype.

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