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Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for *in situ* virus evolution and recombination

N. Y. Uzcategui,[1] D. Camacho,[3] G. Comach,[3] R. Cuello de Uzcategui,[4] E. C. Holmes[2] and E. A. Gould[1]

[1] Centre for Ecology and Hydrology (CEH) Oxford, Formerly the Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, UK

[2] Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

[3] Laboratorio Regional de Diagnóstico e Investigación del Dengue y otras enfermedades virales (Lardidev), Maracay, Estado Aragua, Venezuela

[4] Instituto Venezolano de Investigaciones Científicas – IVIC, Centro de Microbiología y Biología Celular, Carretera Panamericana Km 11, Altos de Pipe, Apartado Postal 21827, Caracas 1020A, Venezuela

Epidemic outbreaks of dengue fever (DF) were first recorded in Venezuela in 1978 and were followed by the emergence of dengue haemorrhagic fever (DHF) outbreaks in 1989. To gain a better understanding of the nature of these epidemics, the complete envelope (E) gene sequence of 34 Venezuelan dengue type 2 (DEN-2) viruses, isolated between 1997 and 2000 was determined. Of these isolates, 16 were from patients with DF and 17 were from patients diagnosed with DHF. There were no diagnostic sequence differences between them, suggesting that the E gene alone does not determine disease severity. These sequence data were also used in phylogenetic comparisons with a global sample of DEN-2 viruses, including strains collected previously from Venezuela. This analysis revealed that the ancestors of the Venezuelan viruses were Asian in origin, implying that a DEN-2 virus strain from this region was introduced into Venezuela and the wider Caribbean region during the late 1970s or the early 1980s. The phylogenetic trees further indicate that evolution of DEN-2 virus in Venezuela has occurred *in situ*, with differentiation into a number of distinct but co-circulating lineages, rather than the repeated introduction of new strains from other localities. By incorporating additional sequence data from the virus capsid, premembrane and membrane genes, evidence is provided that a single Venezuelan strain sequenced previously, designated Mara4, is a recombinant virus, incorporating genome sequence from Venezuelan and Asian parental viruses.

Introduction

Since its recognition as a disease in the eighteenth century (Rush, 1789), the incidence, distribution and severity of dengue (DEN) virus epidemics have increased almost continuously. This is due largely to the spread and proliferation of *Aedes aegypti* mosquitoes amongst dense urban human populations, the introduction and rapid dissemination of new virus strains resulting from increased human mobility and the lack of a continuous programme of mosquito control (Gubler, 1987; Henchal & Putnak, 1990; Zanotto *et al.*, 1996).

In many Asian and, more recently, Latin American countries, dengue fever (DF), a rarely fatal flu-like illness, is gradually being replaced by more severe forms of the disease – dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Cuba was the first country in Latin America to be affected by a major outbreak of DHF/DSS (Guzman *et al.*, 1984; Gubler, 1987), although Venezuela, Jamaica, Honduras, Curacao and Puerto Rico had previously reported suspected cases of DHF (Gubler, 1997). The Cuban DEN type 2 (DEN-2) virus epidemic of 1981, in which there were 10312 cases and 158 deaths due to DHF/DSS, followed 4 years after a DEN-1 virus epidemic. Subsequently, an extended outbreak of DHF/DSS occurred in Venezuela from October 1989 to April 1990

Author for correspondence: E. A. Gould.
Fax +44 1865 281696. e-mail eag@ceh.ac.uk

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with over 6000 DHF cases and 73 deaths (PAHO, 1990). This outbreak could not be attributed to a particular serotype of DEN virus, since DEN-1, DEN-2 and DEN-4 viruses were isolated from different patients. Nevertheless, DEN-2 virus appeared to be associated most frequently with fatal cases (PAHO, 1990; Gubler, 1997). Taking into account the recurrent epidemics that have occurred since 1989, Venezuela has contributed more than 50% of the DHF/DSS cases in Latin America. This raises the question as to whether or not Venezuelan DEN-2 viruses have specific genetic characteristics that increase their associated disease severity and also whether or not the virus evolves *in situ* in this country or is continually imported from other localities.

In an attempt to answer these questions, we conducted an extensive phylogenetic analysis of DEN-2 virus based on envelope (E) gene sequences. This analysis incorporated strains that reflect the global diversity of DEN-2 virus, including 34 newly sequenced strains from Venezuela. Not only do we show that DEN-2 virus has evolved within Venezuela since at least the early 1980s, but that recombination may also be a source of genetic diversity. Finally, those Venezuelan strains causing either DF or DHF/DSS do not have consistent differences in E gene sequence.

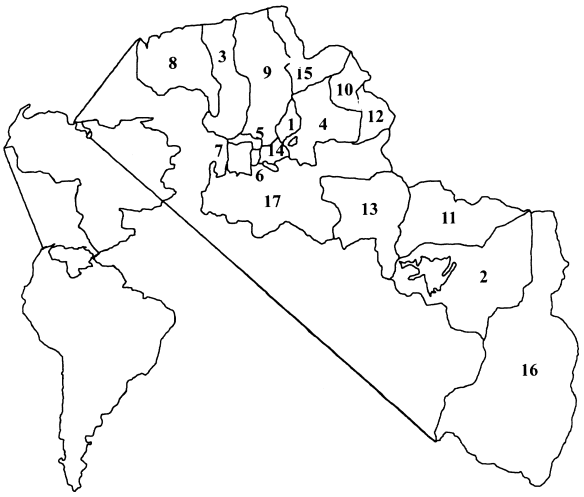

Fig. 1. Map of the geographical locations within Aragua State, Venezuela where the DEN-2 viruses were isolated. Kindly provided by G. Comach (LARDIDEV/BIOMED/CORPOSALUD). Locations of the municipalities from where the samples were isolated in Aragua State are Bolívar (1), Camatagua (2), Girardot (3), J. F. Ribas (4), F. L. Alcántara (5), J. A. Lamas (6), Libertador (7), M. B. Iragorry (8), S. Mariño (9), J. R. Revenga (10), San Casimiro (11), Santos Michelena (12), San Sebastián (13), Sucre (14), Tovar (15), Urdaneta (16), Zamora (17).

Methods

■ **Viruses.** Virus samples were provided by G. Comach from the Regional Laboratory for Diagnosis and Research of Dengue and other virus diseases (Lardidev) in Aragua State, Venezuela. Each sample was isolated by a single passage in C6/36 cells. Fig. 1 illustrates the geographical location of each virus sample in Aragua State, Venezuela. Table 1 shows the corresponding clinical diagnosis. The Mara3 virus isolate was kindly provided by R. Shope (University of Texas, USA).

■ **Virus identification.** Samples isolated from and identified in Venezuela were propagated by a single passage in C6/36 (*A. albopictus*) cells. Virus infection was confirmed according to serotype by indirect immunofluorescence assays (IFA) using type-specific monoclonal antibodies (MAbs) (Henchal *et al.*, 1983, 1985). Four type-specific MAb preparations were used: 15F3 (DEN-1), 3H5 (DEN-2), 5D4 (DEN-3) and 1H10 (DEN-4) (Table 2). After growth for 7 days at 28 °C, virus-infected supernatant media were collected, clarified by centrifugation and stored at −70 °C until use.

■ **Antigenicity.** Vero cells on 13 mm sterile glass coverslips were infected with 52 different flaviviruses to test the specificity of each MAb. Since the titre of each virus was different, the time of incubation was adjusted to obtain between 10 and 60% of infected cells. Indirect IFA were performed on the coverslips using each of the type-specific MAbs as the primary antibody. Goat anti-mouse polyvalent antiserum conjugated to fluorescein-isothiocyanate (Sigma) was used as the secondary antibody.

■ **RNA viral extraction and RT–PCR.** Viral RNA was extracted from 200 µl of supernatant medium of virus-infected cells using the RNAgents Total RNA Isolation system (Promega). Reverse transcription (RT) was performed using 10 µl of extracted RNA, 1 µl of primer D2-NS1-9 (Table 3) and 10 µl of the RT reaction mixture. Initial denaturation of viral RNA was carried out at 95 °C for 2 min. The RT reaction mixture contained 250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$, 0·1 M DTT, 0·5 µl of RNaseOUT Recombinant Ribonuclease Inhibitor (40 U/µl) and 0·5 µl of SuperScript II (200 U/µl) (Gibco-BRL). RT reactions were incubated at 42 °C for 1 h and 65 °C for 10 min.

Nucleotides from position 1 in the 5′ untranslated region (UTR) region to 2555 in the DEN-2 virus genome encoding the capsid (C), premembrane (prM)/membrane (M), E and nonstructural protein 1 (NS1) genes were amplified using PCR. A sample of 5 µl of the cDNA from the RT reaction was then used for PCR amplification by 35 cycles of denaturation at 94 °C (40 s), annealing at 61 °C (1 min) and extension at 72 °C (40 s). A final extension step was carried out at 72 °C for 7 min. The primers used for amplification and/or sequencing were designed on the basis of published DEN virus sequences and are presented in Table 3. Amplified cDNA products of DEN-2 virus were directly sequenced after purification using the Nucleospin DNA purification system (Clontech).

Double-stranded sequencing of the C, prM/M, E and NS1 genes was performed on an ABI sequencer using the manufacturer's protocol (Applied Biosystems). Briefly, for each sequencing reaction, 2 µl of the purified PCR products was mixed with 1 pmol of primer, 5 µl of water and a reaction mixture containing the four dye-labelled dideoxynucleotide terminators. Cycle sequencing was then performed as follows: 25 cycles at 96 °C for 30 s, 50 °C for 60 s and 60 °C for 4 min. The sequencing reaction was purified by precipitation with 75% isopropanol and DNA was then dried using a vacuum centrifuge before sequencing.

■ **Phylogenetic analysis.** A total of 34 E gene sequences was obtained from the Venezuelan DEN-2 virus isolates. These were combined with all available published E gene sequences from global isolates of DEN-2 viruses deposited in GenBank. Prior to analysis, all DEN-2 virus sequences thought to be recombinants were removed, as were isolates that were very closely related to others in the data set and hence which added little evolutionary information. Thus, the final data set used in our phylogenetic analysis comprised 96 sequences of 1485 bp in length. Four of these sequences were used to root the phylogenetic tree: P8–1407 from a sentinel monkey of Malaysian origin, DAKArA578 isolated from *A. taylori sensu lato* in the Ivory Coast, the sylvatic PM33974 strain from Guinea and the sylvatic DAKHD10674 strain from Senegal (Wang *et al.*, 2000).

Table 1. Clinical data of patients in relation to each virus isolated

DF, DHFI, DHFII and DHFIII indicate the World Health Organization classification for DEN virus pathogenesis.

Sample	Pathogenesis	County	Date
1390	DHFI	Girardot	October 1997
1432	DHFI	Mariño	October 1997
1557	DHFIII	Girardot	October 1997
1657	DHFII	Mario B. Iragorry	October 1997
1675	DHFI	Libertador	October 1997
1681	DF	Girardot	October 1997
1688	DF	Mariño	November 1997
1689	DF	Mario B. Iragorry	November 1997
1693	DHFII	Mariño	October 1997
1701	DF	Mariño	November 1997
1809	DHFI	Mario B. Iragorry	November 1997
1811	DF	Mariño	November 1997
1910	DF	Mariño	November 1997
1913	DHFII	Girardot	November 1997
1996	DF	Mariño	December 1997
2213	DHFII	Girardot	February 1998
2217	DF	Mario B. Iragorry	February 1998
2252	DF	Libertador	February 1998
2303	DHFII	Girardot	March 1998
2396	DHFII	Girardot	April 1998
2891	DF	Girardot	July 1998
2893	DHFIII	Girardot	July 1998
2957	DF	Zamora	July 1998
2995	DF	Mario B. Iragorry	August 1998
3146	DHFII	Girardot	August 1998
4256	DF*	Mario B. Iragorry	April 1999
4341	DF	Pot. Nutrias, Barinas	April 1999
4924	DHFII	José Félix Rivas	October 1999
5207	DHFII	Girardot	February 2000
5942	DHFII	Lamas	September 2000
5952	DHFI	Lamas	October 2000
6045	DF	Santos Michelena	October 2000
6123	DF	Girardot	October 2000
Mara3	DF	–	1990

* DF with spontaneous bleeding (petechiae) but without thrombocytopenia.

Table 2. Characteristics of MAbs

MAbs used for IFA, as described by Henchal *et al.* (1983, 1985), haemagglutination inhibition tests (HAI) and plaque-reduction neutralization tests (PRNT).

Clone designation	DEN virus reactivity	Isotype	Serological tests
15F3	DEN-1	IgG 1	IFA
3H5	DEN-2	IgG 1	IFA, HAI, PRNT
5D4	DEN-3	IgG 1	IFA
1H10	DEN-4	IgG 3	IFA, HAI

Table 3. Primers used for RT–PCR and sequencing

Reaction	Genome region (position)	Sequence 5′ → 3′
RT	D2-NS1-9 (2624–2640)	GGAAACAAATAACACCA
PCR (E gene)	D2-prM-6 (764–785)	CATGGATGTCATCAGAAGGGGC
PCR (E gene)	D2-NS1-6 (2534–2555)	CCCCTTCAAAACTAGCTTCAGC
PCR/sequencing (E gene)	D2-prM-7 (864–886)	CACCATAGGAACGACACATTTCC
PCR/sequencing (E gene)	D2-NS1-8 (2454–2476)	AGAACTGAAATGTGGCAGTGGAA
PCR/sequencing (C gene)	E19 (1481–1499)	CGATGGAGTGCTCTCCGAG
PCR/sequencing (C gene)	D2-UTR (1–22)	AGTAGTTAGTCTACGTGGACCG
Sequencing (E gene)	D2-Seq1 (1153–1170)	CGTTGCCCAACACAAGGG
Sequencing (E gene)	D2-Seq2 (1490–1507)	GCTCTCCGAGAACAGGCC
Sequencing (E gene)	D2-Seq3 (1777–1796)	GGACATCTCAAGTCCAGGC
Sequencing (E gene)	D2-Seq4 (2041–2057)	GCAGAACCTCCATTCGG
Sequencing (E gene)	D2-NS1-4 (2449–2428)	GGTTGCGTTGTGAGCTGGAAAA
Sequencing (E gene)	E11 (2260–2280)	GCAATCTATGGAGCTGCCTTC
Sequencing (C gene)	D2-C (355–370)	GAGATTGGAAGGATGC
Sequencing (C gene)	D2-prM (571–588)	TGTGAAGACACAATCACG
Sequencing (C gene)	D2-E (1270–1292)	GGCATTGTGACCTGTGCTATGTT

The first task in the phylogenetic analysis was to determine which model of nucleotide substitution best described DEN-2 virus sequence evolution. This was undertaken using Modeltest 3.0 (Posada & Crandall, 1998), which compares 56 models of DNA substitution. Accordingly, the most complex general time-reversible value was the best fit to the data (relative substitution rates of A↔C = 1·156, A↔G = 7·867, A↔T = 1·636, C↔G = 0·989, C↔T = 21·958 and G↔T = 1), with a proportion of 0·436 sites being invariable and a Γ distribution of among-site rate variation (α shape parameter) of 1·249. Finally, the estimated base composition was A = 0·346, C = 0·218, G = 0·239 and T = 0·197. A maximum likelihood phylogenetic tree using these settings was then reconstructed using the PAUP* package (Swofford, 2000). The starting tree was found using the neighbour-joining method. This was followed by successive rounds of tree bisection–reconnection branch swapping, identifying the maximum likelihood substitution parameters at each stage, until the tree of highest likelihood was found.

To determine whether or not recombination had occurred among any of the Venezuelan DEN-2 virus sequences, we conducted a preliminary analysis of 23 complete DEN-2 virus genomes, including those determined previously by Leitmeyer *et al.* (1999). First, neighbour-joining trees (estimated using HKY85 substitution model) were constructed for 500 bp regions along the whole genome alignment. These trees were then inspected visually to determine if any isolate changed phylogenetic position, as might be expected under recombination. Next, to determine the approximate location of the recombination breakpoints, plots of pairwise sequence diversity were constructed between the putative recombinants and their closest parental sequences as identified in the phylogenetic analysis. The percentage pairwise divergence between query and comparison sequences was estimated using the program DIVERT (Gao *et al.*, 1998), in which a window of 200 bp was moved along the alignments in 10 bp increments.

To confirm the existence of the putative recombinants, an additional 1203 bp representing the C, prM and M was determined from five of our Venezuelan isolates – Mara3, Ven1432, Ven1701, Ven1910 and Ven1996. The optimal recombination breakpoints were then determined using the maximum likelihood method (program LARD) (Holmes *et al.*, 1999). In this analysis, the sequence alignments for the recombinant and its closest parental sequences were divided in two at every possible breakpoint and the branch lengths for each optimized. The two likelihood values were then combined to obtain the likelihood score for that breakpoint position. The breakpoint position with the highest likelihood score was then identified. This likelihood value was compared to that obtained under a model of no recombination using a likelihood ratio test in which significance was evaluated using a null distribution of ratios generated by comparing 200 Monte Carlo simulated sequences subjected to the same breakpoint analysis as the reference data, but with no recombination. Finally, maximum likelihood phylogenetic trees (substitution parameters as above), with 1000 bootstrap neighbour-joining replications, were then reconstructed for the sequence regions on either side of the breakpoint.

Results

Confirmation of identity of DEN-2 virus isolates using serotype-specific DEN virus MAbs

Each of the four serotype-specific antibodies and a pan-specific flavivirus antibody MAb 813 (Gould *et al.*, 1985) was tested for reactivity in indirect IFA with 59 flaviviruses representing the three major groups, i.e. tick-borne, mosquito-borne and no-known vector viruses. The results (not shown) confirmed the specificity of the MAbs and showed that each isolate, obtained from Venezuela between 1997 and 2000 and identified previously by IFA and RT–PCR, was DEN-2 virus.

Nucleotide sequence determination and phylogenetic analysis of Venezuelan DEN-2 virus isolates

RNA extracted from each DEN-2 virus isolate grown in C6/36 cells was subjected to RT–PCR using the primers and protocols described in Methods. The PCR products were purified using the Nucleospin DNA purification system and sequenced directly. The consensus sequence obtained for each virus presents the results of four separate determinations. These sequences have been deposited in GenBank (acces-

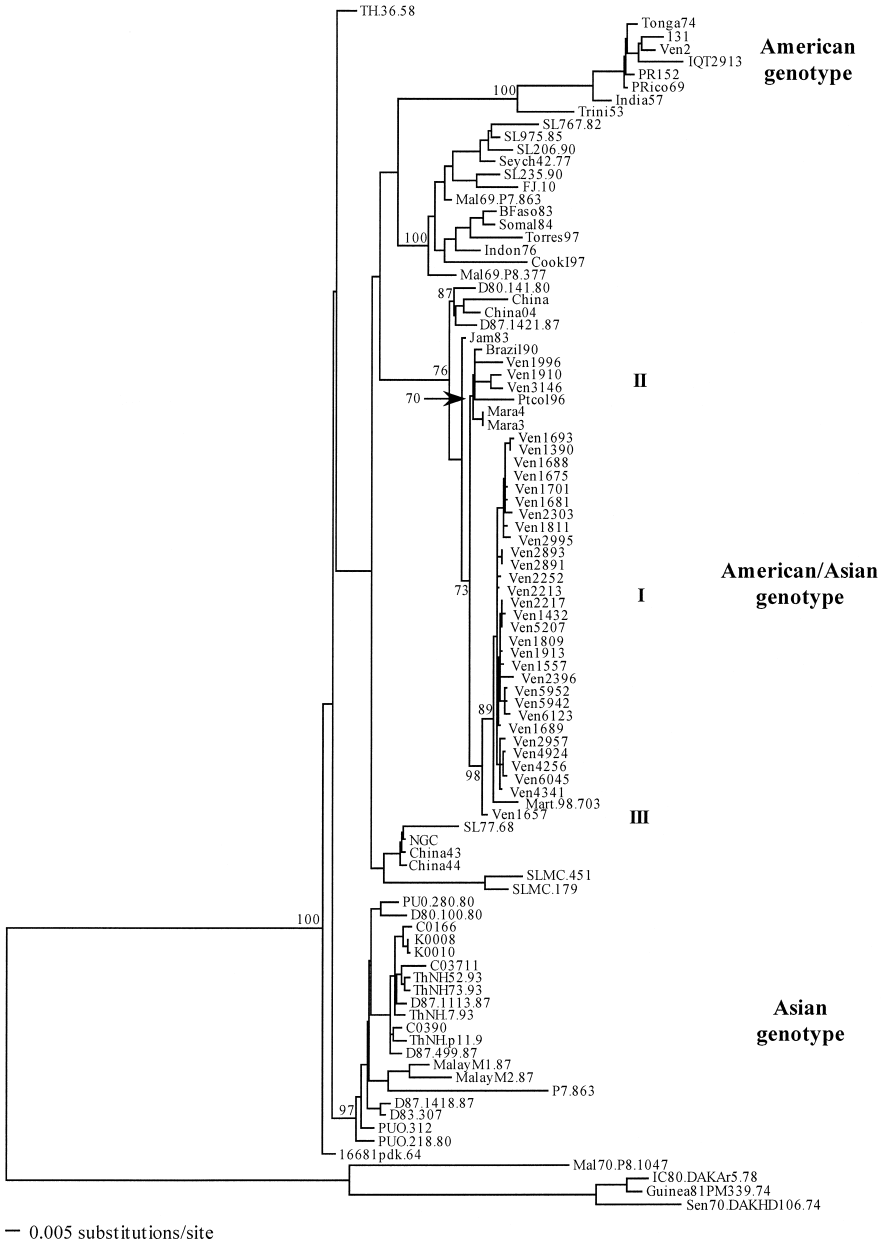

Fig. 2. Maximum likelihood tree showing the phylogenetic relationships among the E gene sequences of 96 strains of DEN-2 virus. The tree is rooted using four non-human strains, P8-1407, DAKArA578, PM33974 and DAKHD10674 (Wang *et al.*, 2000). Horizontal branch lengths are drawn to scale. Neighbour-joining bootstrap values (1000 replications) are shown for key nodes. Roman numerals denote the different lineages of DEN-2 virus circulating in Venezuela.

sion nos AF360860–AF360863, AF363069–AF363092, AY044442 and AF398106–AF398114).

The E gene sequences of the 34 Venezuelan isolates, all sampled between 1997 and 2000 in Aragua State, were compared with the sequences of DEN-2 viruses isolated worldwide. A maximum likelihood tree of all 96 sequences is presented in Fig. 2 and shows that the Venezuelan isolates fall into two distinct groups. Of the Venezuelan isolates, 29 grouped closely together (89% bootstrap support) along with one other strain – Mart98.703 isolated from Martinique, French West Indies (Tolou *et al.*, 2000) (group I in Fig. 2). Three other Venezuelan isolates, Ven1910, Ven1996 and Ven3146 were more distant and, instead, formed a sister group (70% bootstrap support) with four viruses: Mara3 and Mara4, two DHF-associated viruses both isolated from Maracay, Aragua State, Venezuela in 1990 (Mara3 sequenced here; Mara4 from Leitmeyer *et al.*, 1999), Brazil90 from Brazil (Lewis *et al.*, 1993) and Ptcol96 from Colombia (Romero-Vivas *et al.*, 2000) (group II in Fig. 2). Thus, these two groups contain only Latin American isolates of DEN-2 virus. One other Venezuelan

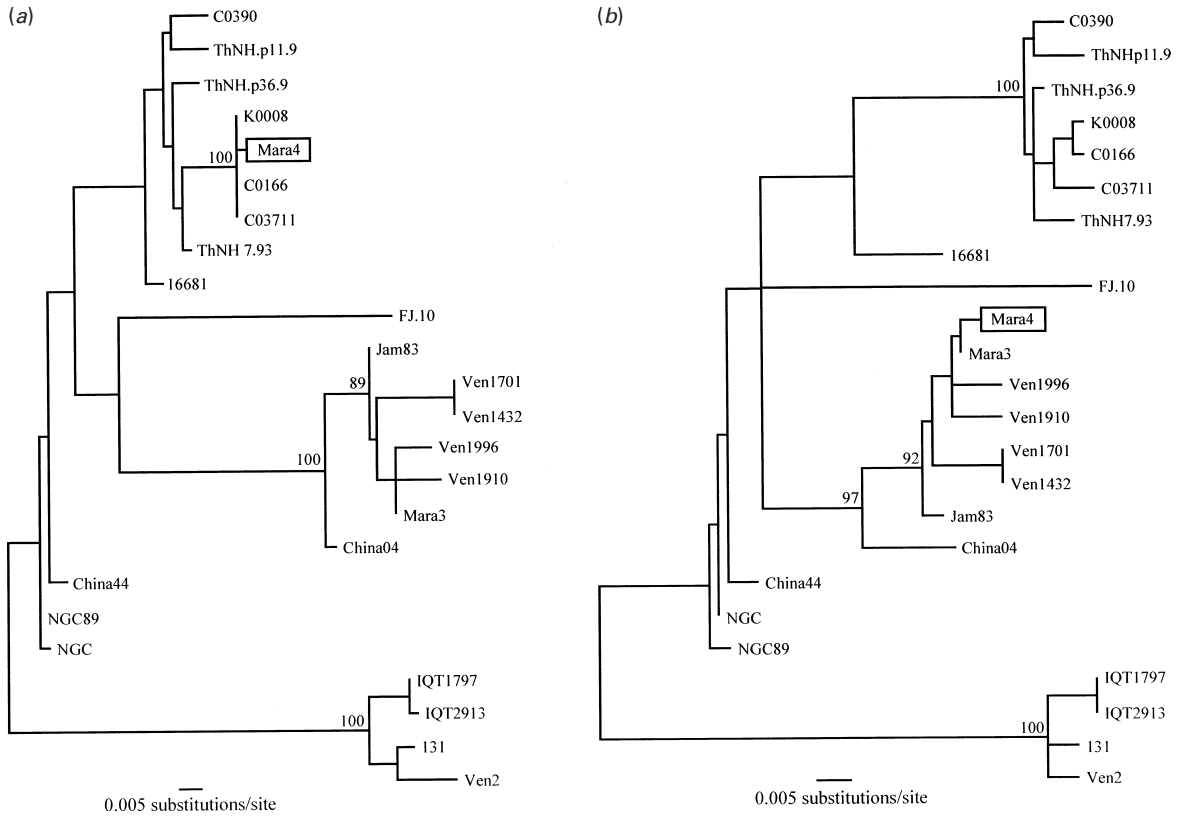

Fig. 3. Maximum likelihood trees showing the phylogenetic relationships between C–prM–M sequences from 24 strains of DEN-2 virus. Tree (*a*) was estimated from the nucleotide region 1–508, while tree (*b*) was estimated from the nucleotide region 509–1203. The recombinant Mara4 sequence, which moves position among the two trees, is boxed. The tree is rooted using four American genotype viruses for purposes of clarity only. Horizontal branch lengths are drawn to scale. Neighbour-joining bootstrap values (1000 replications) are shown for key nodes.

isolate, Ven1657, was separated slightly from the main group of viruses, suggesting that it also represents a phylogenetically distinct strain (group III in Fig. 2). More distantly related to all these groups was Jam83, isolated from Jamaica in 1983 (Deubel *et al*., 1988) and three Asian isolates, D80.141 and D87.1421, both isolated in Thailand in the 1980s (Blok *et al*., 1989; Sittisombut *et al*., 1997), and a Chinese isolate, China04 (whole genome unpublished, accession no. AF119661; variant E gene sequence from the same isolate described in Yang *et al*., 1994). Hence, the viruses we document in Venezuela seem to be ultimately derived from isolates circulating in various Asian countries. Finally, this entire grouping received high bootstrap support (100%) and was connected by a long branch to the remaining isolates of DEN-2 virus. Such separation suggests that this virus group constitutes a separate genotype of DEN-2 virus (Rico-Hesse, 1990) containing both Asian and American strains that are phylogenetically distinct from the main Asian and American genotypes.

Determinants of disease severity in Venezuelan DEN-2 viruses

Our sample set of 34 Venezuelan sequences was collected from patients suffering from either DF or DHF. Significantly, there were no consistent differences between the E gene sequences associated with these differing clinical outcomes nor did they form phylogenetically distinct groups, indicating that if disease severity does have a genetic basis, it cannot be due to the E gene alone. However, it is notable that all of the 34 Venezuelan isolates described in this paper possess an asparagine (N) amino acid at position 390 in the E gene (E-390), which was highlighted previously as a probable genetic determinant of DHF in Asiatic strains (Leitmeyer *et al*., 1999). In contrast, all of the American genotype viruses have an aspartic acid residue (D) at E-390, which may be partially responsible for the reduction in associated disease severity in these viruses. If this disease correlation is correct, then all of the DEN-2 viruses determined here have the potential to cause DHF.

Recombination in Venezuelan DEN-2 virus isolates

A preliminary analysis based on 23 complete genome sequences, including 12 determined by Leitmeyer *et al*. (1999), demonstrated that the Venezuelan isolate Mara4 dramatically moved phylogenetic position among gene regions. Specifically, in sequence positions approximately 501–10176 (i.e. in most of the viral genome), Mara4 groups with the Jam83

and China04 isolates in the mixed American/Asian genotype, as demonstrated previously by Leitmeyer *et al*. (1999). However, from genome positions approximately 1–500, Mara4 clearly clusters with the main group of Asian viruses, being related very closely to various Thai strains of DEN-2 virus (phylogenetic trees and diversity plots are available from the authors on request).

To study this putative recombination event in more detail, we obtained the genome sequence covering the putative breakpoint from five Venezuelan isolates (accession nos AF360860–AF360863), three of which, Mara3, Ven1910 and Ven1996, appeared to be related closely to Mara4 in the E gene phylogeny. We also used a maximum likelihood method to determine whether or not the change in topology with respect to Mara4 is more than would be expected by chance alone and, if this is the case, to locate the breakpoint with more precision. This analysis revealed that two phylogenetic trees are needed to explain the evolution of the Mara4 isolate, as expected from recombination ($P < 0{\cdot}005$). The optimal breakpoint under this analysis was located at nucleotide position 508 within the prM gene. The affect of this recombination event can be seen clearly in the very different phylogenetic trees constructed either side of the breakpoint at position 508 (Fig. 3): on the 5′ side, Mara4 groups closely with other Venezuelan isolates of DEN-2 virus, most notably Mara3, which was isolated from the same hospital on the same day as Mara4 (R. Rico-Hesse, personal communication), while on the 3′ side, Mara4 clusters tightly with various Thai strains of DEN-2 virus and is very distant from Mara3. These disparate phylogenetic positions were supported by very high levels of bootstrap support in both cases. Significantly, none of the other Venezuelan isolates moved position in this manner, indicating that only Mara4 is a recombinant virus.

Discussion

DHF due to DEN-2 virus is now a major problem in Venezuela. Previously, it was suggested that the appearance of DHF was linked to the introduction of a South East Asian strain of DEN-2 virus into the Caribbean region (Rico-Hesse *et al*., 1997), as indigenous Latin American strains of DEN-2 virus do not naturally induce DHF. This was supported by studies in Peru that demonstrated that no cases of DHF occurred in a population suffering high secondary infection rates with DEN-2 virus, perhaps because only the American genotype of DEN-2 virus was present (Watts *et al*., 1999). More recent work has identified the nucleotide substitutions that distinguish the American genotype and, hence, which may be central to the determination of disease severity (Leitmeyer *et al*., 1999). For example, the E-390 substitution identifies an amino acid that may be a primary determinant of DHF: all American genotype DEN-2 viruses, which are not associated with DHF, possess a D residue at position E-390, while all Asian strains, which have the capacity to induce DHF, have an N residue in this position.

We have compared the E gene sequence of 34 strains of DEN-2 virus isolated from Aragua State, Venezuela between 1997 and 2000 when DHF was common. Of the 34 strains, 17 were from DHF patients. Significantly, there were no consistent differences in the E gene between Venezuelan DEN-2 virus isolates from either DF or DHF cases. This is in agreement with previous studies of Asian DEN-2 viruses (Blok *et al*., 1991; Mangada & Igarashi, 1998). Furthermore, the Venezuelan strains sequenced here all possess the typical Asian N residue at position E-390. Not only does this hint at the Asian ancestry of these Venezuelan strains, but it also suggests that *all* have the potential to cause DHF, with the differences in disease expression being a function of the underlying host or environmental factors. Furthermore, all of our Venezuelan isolates contained the two other amino acid substitutions in the structural proteins that are typical of Asian genotype DEN-2 viruses as described by Leitmeyer *et al*. (1999) – the glutamate at position prM-28 and valine at position prM-31.

Our phylogenetic analysis of the newly sequenced Venezuelan isolates demonstrated that they were descendants of an Asian DEN-2 virus strain which was presumably introduced into Latin America during the late 1970s or early 1980s, the earliest representative obtained to date being Jam83. Following this introduction, the virus obviously went through a period of *in situ* evolution within Venezuela, diversifying into a number of distinct phylogenetic groups with some migration to and from other Caribbean countries, such as Martinique and Colombia. Because of the close phylogenetic relationship between two of these viruses, Ven1910 and Ven1996, obtained from Mariño County (Aragua State) in 1997, and the Mara3 and Mara4 isolates, sampled from Maracay (also Aragua State) during the earlier 1989–1993 epidemic, it seems likely that this particular virus group has been responsible for DEN virus cases for at least 8 years. Furthermore, our Venezuelan isolates are clearly distinct from those determined by Leitmeyer *et al*. (1999) which fall into the American genotype. Of particular relevance here is the Ven2 virus which was also isolated from Maracay in 1987. Ven2 is related closely to the virus that caused the 1993–1997 DF outbreak in Peru, represented by strain IQT2913 in Fig. 2, and which is thought to constitute a low virulence strain of DEN-2 virus. Given that none of our Venezuelan DEN-2 virus strains fall into the American genotype, it is tempting to speculate that the putative low virulence strains, which, as shown by Ven2, must have existed in Venezuela in the past, have been replaced selectively by strains that have the potential to cause DHF. Overall, our results demonstrate that DEN-2 viruses have circulated for extended time periods within Venezuela and nearby countries, that different strains can co-circulate during an epidemic and that *in situ* evolution of DEN-2 virus has occurred during the epidemics in Venezuela.

Comparison of trees based on full-length genome sequences, as well as more detailed analysis of the C–M gene

region, also suggested that one of the Venezuelan DEN-2 virus isolates may be a recombinant. Specifically, the phylogenetic position of the Mara4 isolate varied according to the particular gene region used in the analysis, with a recombination breakpoint most likely occurring at position 508 in the prM gene. Such large-scale phylogenetic movement can only be explained by recombination or laboratory contamination, for example, if an artificial hybrid is sequenced (Tolou *et al.*, 2001; Worobey *et al.*, 1999). If recombination is the cause of this mosaic genome, then it must be that the parental Asian and American viruses have circulated in the same locality in order for co-infection to take place, again demonstrating the widespread movement of DEN viruses. These results also confirm the importance of determining the sequences of entire viral genomes, as recombination will act to produce new phenotypes, most notably by mixing virus-encoded determinants of disease severity.

One of many possible consequences of these observations is that once introduced into Latin America from Asia, DEN-2 viruses may become established, either by continually circulating in the large South American human population or perhaps by adapting to the local suburban or rural ecology. The data presented herein imply that some of the viruses studied represent lineages that have been circulating in Venezuela for 10 or more years. This is not without precedent since it has been long recognized that yellow fever virus was introduced into Latin America from Africa and is now established in the natural environment. Field studies to determine whether or not DEN-2 virus can establish a sylvatic cycle that would act as a natural reservoir for introduced viruses clearly need to be undertaken.

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