

Molecular epidemiology of dengue virus type 3 in Venezuela

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During the past 40 years, dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS) have emerged in humans, with approximately 3 million cases reported and over 58 000 deaths. Dengue virus serotypes 1, 2 and 4 (DENV-1, -2 and -4) have been co-circulating in Venezuela for at least the past 10 years, causing minor or major outbreaks of dengue fever (DF) and DHF/DSS. The first recorded outbreak due to DENV-3 in Venezuela dates to 1964 and the virus then seems to have disappeared. However, DENV-3 re-appeared recently (in July, 2000) in Venezuela after 32 years of absence and produced a prolonged major outbreak, which, by the end of 2001, involved 83 180 cases of dengue, mostly DF (92%). Previous phylogenetic studies revealed that the DENV-3 circulating during the 1960s Latin American outbreak was a genotype V virus. To gain a better understanding of the nature of the current epidemic, the complete sequence was determined of the envelope (E) gene of 15 Venezuelan DENV-3 viruses isolated during 2000 and 2001 from patients presenting with different disease severity. Sequence data were used in phylogenetic comparisons with global samples of DENV-3. Analysis revealed that the strain circulating in Venezuela is closely related to isolates that were previously present in Panama and Nicaragua in 1994 and since then have spread through Central American countries and Mexico. This study also confirms previous reports showing that the DENV-3 strain currently circulating in the Americas is related to the strain that caused DHF epidemics in Sri Lanka and India in 1989–1991 (genotype III). Finally, no evidence of the re-emergence of the strain that circulated in Venezuela in the late 1960s and 1970s (genotype V) was found.

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INTRODUCTION

Dengue fever (DF) is caused by four antigenically related but genetically distinct viruses, namely dengue virus types 1, 2, 3 and 4 (DENV-1, -2, -3 and -4), which belong to the genus *Flavivirus*, family *Flaviviridae* (Heinz *et al.*, 2000). These viruses traditionally cause DF, a flu-like illness that is not life threatening, and is thought to have occurred as epidemics in tropical areas throughout the world since at least the 17th Century (Gubler, 1997). In the 1950s, an epidemic of dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS) emerged in Asia, and 30 years later it appeared for the first time in the Americas. DHF/DSS is a

life-threatening disease and in many tropical and sub-tropical countries, including Venezuela, it constitutes a major public health problem. Here, we attempt to follow the history of the most recent DENV outbreak in Venezuela, DENV-3.

An epidemic of DF appeared in Venezuela in 1964, which continued until 1967. Margarita Island in Nueva Esparta State was the first area to be affected and from there the outbreak spread westward. The total number of cases reported was 18 315 in 1964, 4047 in 1965, 7754 in 1966, 1330 in 1967 and 383 in 1968 (PAHO, 1979). During this time, 23 deaths were attributed to dengue, but no

investigations were carried out to confirm the occurrence of DHF/DSS as the cause of death. At the same time, there was an unusual increase in the incidence of rubella, particularly in 1965 when there were 27 857 cases reported, a significant difference from the number of cases notified for previous years. However, there were no case investigations. It is possible, therefore, that some of these cases were due to DENV (PAHO, 1979). An outbreak due to DENV-2 started in 1969 and lasted until the mid 1970s. The total number of cases reported was 3917 in 1969 and 405 in 1970 (PAHO, 1979). After 1971, the small number of cases reported indicated that DENV activity was low (PAHO, 1979), until 1977 when DENV-1 epidemics occurred in Jamaica and Cuba and 1 year later, in Venezuela and Puerto Rico (PAHO, 1979). In the succeeding 4 years, DENV-1 spread throughout the Caribbean Islands, Mexico, Texas, Central America and northern South America. In 1981, DENV-4 was introduced into the Americas but did not cause a major epidemic in Venezuela. Also in 1981, a new strain of DENV-2 was introduced into Cuba from Southeast Asia, causing the first epidemic of DHF in the Americas (Gubler & Trent, 1993; Kouri *et al.*, 1989; Lewis *et al.*, 1993; Rico-Hesse, 1990). A second epidemic of DHF occurred in Venezuela in 1989–1990 (PAHO, 1990) and although DENV-1, -2 and -4 were isolated during this epidemic period, DENV-2 virus was associated with most of the fatal cases (Gubler & Meltzer, 1999).

DENV-3 re-appeared in the Latin American region in 1994 after an absence of 17 years (CDC, 1995). The virus was detected initially in Panama, causing a small outbreak of classic DF. It then dispersed to Nicaragua, where it was associated with a countrywide epidemic of DF/DHF, although DENV-1 was also present. The virus seems to have continued spreading northwards to Mexico and in 1995 this introduction coincided with an increased number of DHF cases; however, only DENV-1 and particularly DENV-2 were associated with DHF (Balmaseda *et al.*, 1999; CDC, 1995; Guzman *et al.*, 1996; Pinheiro & Corber, 1997; Usuku *et al.*, 2001).

Hence, 32 years after its apparent disappearance from Venezuela, DENV-3 re-appeared and by the end of 2000, had caused most of the 21 101 cases of dengue reported officially; the attack rate was 87 per 100 000 inhabitants and the DHF:DF ratio was 1:10, which means that only 10% of cases were associated with DHF (Dirección de Epidemiología y Análisis Estratégico, 2000). By the end of the year 2001, 83 180 cases were reported, with an attack rate of 337·7 per 100 000 inhabitants and a DHF:DF ratio of 13:1 (only 8% of DHF) (Dirección de Epidemiología y Análisis Estratégico, 2001). This is the largest epidemic caused by DENV in Venezuela since the 1989 DENV-2 epidemic.

To investigate the spread of DENV-3 in Venezuela in more detail, we undertook a molecular epidemiological investigation. In particular, we undertook phylogenetic analyses to determine whether or not the DENV-3 strain circulating currently in Aragua State, Venezuela, could be assigned to

genotype V (previously subtype III) (Lanciotti *et al.*, 1994), as reported in neighbouring countries (Pinheiro & Corber, 1997; Usuku *et al.*, 2001). This phylogenetic analysis, the largest undertaken on DENV-3 to date, also enabled us to study the evolution and dispersal of this virus since its re-introduction into the Americas in 1994.

METHODS

Viruses. Virus samples were kindly provided by G. Comach [Regional Laboratory for Diagnosis and Research of Dengue and other Viral Diseases (LARDIDEV), Aragua State, Venezuela] and R. Shope (University of Houston, Texas, USA). The characteristics of the DENV-3 isolates determined are shown in Table 1.

Viral RNA extraction, RT-PCR and sequencing. Viral RNA was extracted from 200 µl of supernatant from virus-infected cells using a commercial kit (RNAagents Total RNA Isolation system, Promega). Reverse transcription (RT) was performed using 10 µl extracted RNA, 1 µl primer D3-RT (Table 2) and 10 µl RT reaction mixture. Initial denaturation of the 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 MgCl₂, 0·1 M DTT, 0·5 µl RNaseOUT recombinant ribonuclease inhibitor (40 U µl⁻¹) and 0·5 µl Superscript II (200 U µl⁻¹) (Gibco-BRL). RT reactions were incubated at 42 °C for 1 h and at 65 °C for 10 min.

Nucleotides from position 716 in the pre-membrane (prM) region to 2013 in the non-structural 1 (NS1) region of the DENV-3 genome encoding the prM/M, envelope (E) and NS1 genes were amplified using PCR. A 5 µl sample of cDNA from the RT reaction was then used in a PCR amplification using 35 cycles of denaturation at 94 °C (40 s), annealing at 61 °C (1 min) and extension at 72 °C (40 s). Final extension was continued for 7 min at 72 °C. DENV primers used for amplification and/or sequencing were designed on the basis of published DENV sequences and are presented in Table 2. Amplified

Table 1. Description of the DENV-3 isolates investigated in this study

No.	Strain	Pathology	Country	Year
1	Venezuela-5990	DF	Venezuela	2000
2	Venezuela-6007	DF	Venezuela	2000
3	Venezuela-6218	DHF	Venezuela	2000
4	Venezuela-6315	DF	Venezuela	2000
5	Venezuela-6318	DF	Venezuela	2000
6	Venezuela-6397	DF	Venezuela	2000
7	Venezuela-6411	DF	Venezuela	2000
8	Venezuela-6456	DF	Venezuela	2000
9	Venezuela-6666	DF	Venezuela	2001
10	Venezuela-6667	DF	Venezuela	2001
11	Venezuela-6668	DF	Venezuela	2001
12	Venezuela-6722	DF	Venezuela	2001
13	Venezuela-7110	DHF	Venezuela	2001
14	Venezuela-7812	DHF	Venezuela	2001
15	Venezuela-7984	DHF	Venezuela	2001
16	Puerto Rico/63	DF	Puerto Rico	1963
17	Puerto Rico/77 (1339)	DF	Puerto Rico	1977
18	Mexico/95 (6097)	DF	Mexico	1995

Table 2. Primers used for RT-PCR and sequencing reactions

Reaction	Genome region (nt position)	Sequence (5'→3')
RT	D3-RT (2728–2744)	CCACAACCCATGGAGC
PCR (E gene)	D3fE (717–737)	CGTTAGCTCCCCATGTCGGCA
PCR (E gene)	D3rE (2594–2663)	ACCAGAATGGAGAACCTCTT
Sequencing (E gene)	D3-Seq1S (912–930)	GCTGGTTACCCCATCCAT
Sequencing (E gene)	D3-Seq2S (1185–1202)	AGGAGCAGGACCAGAACT
Sequencing (E gene)	D3-Seq3S (1455–1473)	CTGAATATGGAACCCTCGG
Sequencing (E gene)	D3-Seq5S (2000–2017)	GTGACCAAGAAGGAGGAG
Sequencing (E gene)	D3-Seq2C (2165–2181)	GCCATCTTGGGAGACAC
Sequencing (E gene)	D3-Seq6C (1088–1104)	CAGCTGGCGACCCTAAG
Sequencing (E gene)	D3rI (2539–2558)	GGCAACAGCCATTGCAGGCG

cDNA products of DENV-3 were sequenced directly after purification using a DNA purification system (Nucleospin, Clontech).

Double-stranded sequencing of the prM/M and E gene was performed on an ABI sequencer using the manufacturer's protocol (Applied Biosystems). Briefly, for each sequencing reaction, 2 µl of the purified PCR product was mixed with 1 pmol primer, 5 µl 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 1 min 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 15 E gene sequences was determined from the Venezuelan DENV-3 isolates. Of these, 11 isolates were obtained from sera of patients presenting with DF and four isolates were from patients who had three of the four World Health Organization (WHO) criteria for DHF/DSS (PAHO, 1997), namely fever, haemorrhagic manifestations and thrombocytopenia. Since not all of the WHO criteria were met, these cases cannot be defined specifically as DHF cases; nevertheless, they required hospitalization and were severely ill. One virus isolate from the 1995 Mexican DENV-3 outbreak and two from the 1960s Puerto Rican outbreak were also determined in this study and included in a phylogenetic analysis along with published E gene sequences from global isolates of DENV-3 deposited in GenBank. This resulted in a final data set of 68 sequences of 1482 bp in length. Using a single DENV-2 sequence (Tonga/74) as an outgroup, the sequences assigned to genotype V (as represented by Puerto Rico/63), were found to be the most divergent and were, therefore, used to root the phylogenetic tree (results not shown).

Phylogenetic trees were estimated using the maximum-likelihood (ML) method. To make these trees as accurate as possible, we employed the most complex model of nucleotide substitution available (Rodriguez *et al.*, 1990); this allowed each type of nucleotide change to occur at a different rate (general time-reversible model; $A \leftrightarrow C = 1.775$, $A \leftrightarrow G = 15.673$, $A \leftrightarrow U = 1.914$, $C \leftrightarrow G = 0.753$, $C \leftrightarrow U = 46.859$, $G \leftrightarrow U = 1$). A proportion of 0.610 sites were defined as invariant and utilized a Γ distribution of among-site rate variation (α shape parameter) of 5.541. The estimated base composition was $A = 0.312$, $C = 0.205$, $G = 0.269$ and $U = 0.214$. The starting tree in this analysis was found using the neighbour-joining (NJ) method and was followed by successive rounds of tree-bisection-reconnection branch-swapping, identifying the ML substitution parameters at each stage until the tree of highest likelihood was found. To determine the support for different nodes on the tree, we conducted a bootstrap analysis using 1000 replicate NJ trees constructed under the substitution model defined above. All these analyses were undertaken using PAUP* (Swofford, 2000).

RESULTS

Nucleotide sequence determination and phylogenetic analysis of Venezuelan DENV-3 isolates

RNA extracted from each DENV-3 isolate, grown in C6/36 cells, was subjected to RT-PCR using the primers and protocols described in Methods. PCR products were purified and sequenced directly. The sequence obtained for each virus represents the results of four separate determinations. These sequences have been deposited in GenBank (accession numbers AY146761–AY146778).

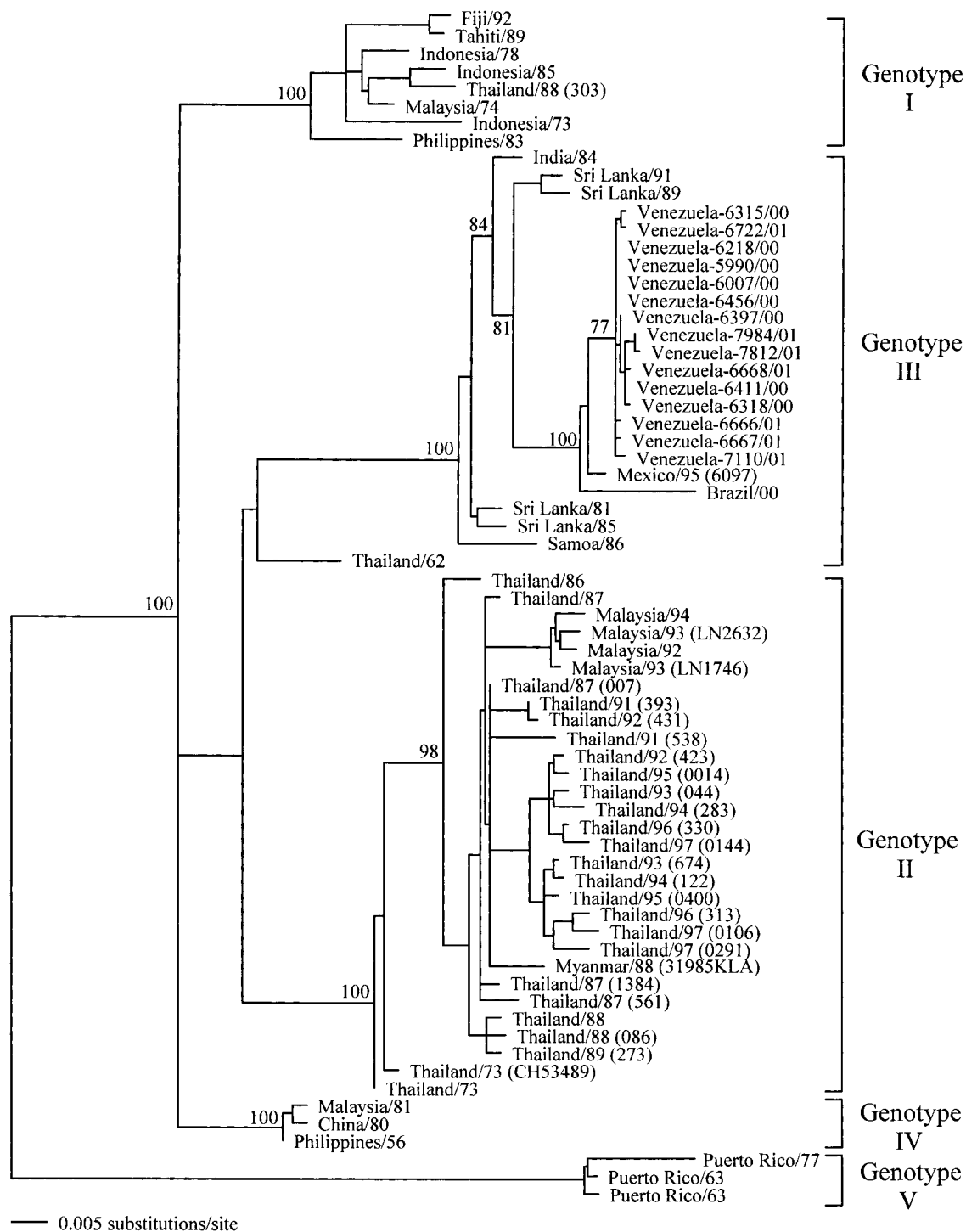
The E gene sequences of the 15 Venezuelan isolates, all obtained between 2000 and 2001 in Aragua State, two Puerto Rican strains from the 1960s and 1970s epidemic and one Mexican strain [Mexico/95 (6097)] from the 1995 outbreak, were compared with the sequences of DENV-3 viruses isolated worldwide. A ML tree of all 68 sequences is presented in Fig. 1. This tree reveals that the 15 Venezuelan isolates fall into one distinct group with 100 % bootstrap support. This group was most closely related to the Mexican isolate sampled from the 1995 epidemic (100 % bootstrap support) and a Brazilian isolate sampled in 2000. These Latin American strains then grouped with various strains sampled from Sri Lanka, India and Samoa, together forming genotype III (100 % bootstrap support). Hence, the viruses isolated in Aragua State, Venezuela, seem to be derived ultimately from viruses circulating in the Americas since 1994; therefore, it is highly likely that there has been *in situ* evolution of DENV-3 in the Americas and that the DENV-3 epidemics in this region were caused by the same virus strain. In turn, these American isolates seem ultimately to be derived from Indo/Asian countries. Nevertheless, branch lengths on the phylogenetic tree indicate that recent American viruses have accumulated a relatively large number of genetic differences from the Indian/Sri Lankan viruses.

It is also significant that the Latin American isolates associated with recent DENV-3 activity were assigned to a different virus genotype (III) than those sampled from Puerto Rico during the 1963 and 1977 outbreaks and

denoted here as genotype V [these viruses were classified originally as genotype IV but a wider sampling of DENV-3 isolates has led to a re-classification of genetic diversity in this virus (Wittke *et al.*, 2002) and the five distinct genotypes are clearly visible in the ML tree presented here]. Hence, although our sample size is small, the analysis presented here suggests that previous to 1977 DENV-3 genotype V viruses circulated in Venezuela and that a new strain closely related to Indo/Asian genotype III is now present.

Determinants of disease severity in Venezuelan DENV-3 isolates

Our sample of 15 Venezuelan sequences was collected from patients suffering from either DF or DF with haemorrhagic manifestations. 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 in DENV-3-infected patients, it cannot be attributed to the E gene.

DISCUSSION

Re-introduction of DENV-3 into the Americas

In 1994, DENV-3 re-appeared in the American region, causing a small outbreak associated with classic DF in Panama. The virus seems to have spread northwards, since outbreaks were reported in Nicaragua and later in Mexico (CDC, 1995; Guzman *et al.*, 1996). In Nicaragua in 1994, the introduction of DENV-3 was associated with a country-wide epidemic of DF/DHF, but DENV-1 was also present. The introduction of DENV-3 into Mexico in 1995 coincided with an increased number of DHF cases; however, only DENV-1 and in particular, DENV-2 were associated with DHF (Pinheiro & Corber, 1997). This virus strain of DENV-3 has remained circulating in Central America, outbreaks being reported in Guatemala and El Salvador (PAHO, 2000; Pinheiro & Corber, 1997). At 7 years after its introduction into the Americas through Panama, DENV-3 spread southwards into Venezuela and then Brazil, causing major outbreaks (CDC, 1995; Figueiredo, 2000; Nogueira *et al.*, 2001; PAHO, 2000; Rocco *et al.*, 2001). Our phylogenetic analysis shows, in accordance with the phylogenetic studies of DENV-3 strains as reported in neighbouring countries (Pinheiro & Corber, 1997; Usuku *et al.*, 2001), that this virus is genetically distinct from the DENV-3 strain that occurred previously in the Americas (genotype V) and has been shown to belong to the same genotype (genotype III) as the virus that caused the 1989–1991 DHF epidemics in Sri Lanka and India (Gubler, 1998; Lanciotti *et al.*, 1994).

In this study, we document the appearance of a new strain of DENV-3 in Venezuela. Whilst there is currently no evidence, it is possible that earlier strains have adapted to forest or rural environments, rather than suffering extinction, as occurred previously after the introduction of *Yellow fever virus*. Indeed, phylogenetic studies show that the same

strain can be found circulating several years after it was identified originally in the same location but in a different outbreak, as occurred in the 1998 DENV-2 epidemic in Venezuela (Uzcategui *et al.*, 2001). Investigations that take the rural and forest environment into account are, therefore, required to resolve this issue.

The introduction of an Asiatic DHF-associated DENV strain into the Americas, on this occasion of the DENV-3 genotype, did not cause a particularly large number of DHF/DSS cases (<1%). In fact, in the Americas, DHF/DSS cases have been associated mostly with DENV-1 and DENV-2 serotypes. The association of this particular DENV-3 strain with DHF/DSS in Asia rather than Latin America probably reflects the different immunological backgrounds of the Asian, compared with the Latin American, communities. For example, it is likely that a higher proportion of individuals in Asia compared with Latin America have partial cross-reactive immunity due to one of the other DENV serotypes. This would effectively increase the likelihood of antibody-dependent enhancement (Halstead, 1989) of DENV-3 infection in Asia. Another explanation for this observation is that DENV-3 might have evolved lower virulence following its introduction into the Americas. However, there were no consistent differences between the E gene sequences from patients suffering from DF or DF with haemorrhagic manifestations and disease severity cannot, therefore, be related to a genetic difference in the E gene alone. A similar observation was made for the Venezuelan DENV-2 isolates (Uzcategui *et al.*, 2001). Finally, it is also possible that host factors play some role in determining clinical outcome [for example, related to HLA haplotype (Loke *et al.*, 2001)], if individuals of Latin American origin are less susceptible to DHF/DSS following infection with this particular genotype of DENV-3.

As well as the appearance of a new strain of DENV-3 for Venezuela, the analysis presented here also shows *in situ* evolution of DENV-3 following its introduction into the Americas. Isolates from Central America diverged as the

Fig. 1. ML phylogenetic tree showing the evolutionary relationships among the E gene sequences of 68 strains of DENV-3. The tree is rooted using strains from Puerto Rico collected during the 1960s and 1970s (genotype V), which have been shown to represent an appropriate outgroup. Horizontal branch lengths are drawn to scale. NJ bootstrap values (1000 replications) are shown for key nodes. Roman numerals denote the different genotypes of DENV-3. GenBank accession numbers of the other DENV-3 strains used in this analysis are as follows: Brazil/2000 (AY038605); China/80 (AF317645); Fiji/92 (L11422); India/84 (L11424); Indonesia/73 (L11425); Indonesia/78 (L11426); Indonesia/85 (L11428); Malaysia/74 (L11429); Malaysia/81 (L11427); Malaysia/92 (AF147457); Malaysia/93 – LN1746 (AF147458); Malaysia/93 – LN2632 (AF147459); Malaysia/94 (AF147460); Myanmar/88 (31985KLA) (AY145712); Philippines/56 (L11423); Philippines/83 (L11432); Puerto Rico/63 (L11433); Samoa/86 (L11435); Sri Lanka/81 (L11431); Sri Lanka/85 (L11436); Sri Lanka/89 (L11437); Sri Lanka/91 (L11438); Tahiti/89 (L11619); Thailand/62 (L11440); Thailand/73 (L11620); Thailand/73 – CH53489 (M86733); Thailand/86 (L11441); Thailand/87 (L11442); Thailand/87 (007) (AY135419); Thailand/87 (1384) (AF533079); Thailand/88 (AF349753); Thailand/88 (086) (AY145713); Thailand/88 (303) (AY145714); Thailand/89 (273) (AY145715); Thailand/91 (393) (AY145716); Thailand/91 (538) (AY145717); Thailand/92 (431) (AY145719); Thailand/92 (423) (AY145718); Thailand/93 (044) (AY145720); Thailand/93 (674) (AY145721); Thailand/94 (122) (AY145722); Thailand/94 (283) (AY145723); Thailand/95 (0014) (AY145724); Thailand/95 (0400) (AY145725); Thailand/96 (313) (AY145726); Thailand/96 (330) (AY145727); Thailand/97 (0106) (AY145728); Thailand/97 (0144) (AY145729); and Thailand/97 (0291) (AY145730).

epidemic spread northwards to Mexico, as represented by isolate Mexico/95 (6097), and later southwards into Venezuela and Brazil, the latter documented by isolate Brazil/00. A similar epidemic and *in situ* evolutionary behaviour was observed with the DENV-2 strains in the 1998–1999 epidemic in Venezuela (Uzcatogui *et al.*, 2001). In addition, we observed considerable evolutionary change between the DENV-3 strains that circulated in the 1960s (Puerto Rico/63) epidemic and later in the 1970s (Puerto Rico/77) epidemic. During these 14 years of presumed epidemic silence, a high number of nucleotide substitutions must have occurred to explain the evolutionary change of DENV-3.

Re-introduction of DENV-3 into Venezuela

DENV-3 cases from Venezuela were reported first in the central region of the country, which is an industrial area. Since the 1989 epidemic, all dengue outbreaks have commenced in this region, particularly Aragua State, and then spread throughout the country (G. Comach, D. Camacho, M. Salcedo, M. Cabello de Quintana, M. Jimenez & G. Sierra, unpublished data).

It is difficult to determine the precise route of importation of DENV into the Americas because available data are limited. If we consider the first Venezuelan DENV-2 lineages to diverge, before the 1989 epidemic, there appear to have been exchanges between Caribbean and Asiatic/Pacific countries, since these isolates grouped closest to strains from India and Tonga within the 'American' genotype of DENV-2. This also appears to be true of viruses from the 1989 epidemic, since they grouped closest to Chinese strains, as part of the 'American/Asian' genotype (Twiddy *et al.*, 2002). Clearly, the same pattern of Asian-to-Caribbean transfer applies to the American DENV-3 isolates from the current epidemic, which are most closely related to DENV-3 strains from India and Sri Lanka.

A characteristic of dengue in Venezuela is that the first outbreaks were reported in neighbouring countries, specifically in Central America and the Caribbean Islands, and then the epidemic spread northwards to Mexico and southern United States and then southwards into South America. Therefore, the introduction of DENV strains into Venezuela is more likely the result of the spread of a virus circulating in Central America or the Caribbean islands and not to a direct introduction or importation of Asiatic strains, a notion supported by the phylogenetic analysis presented here. If this is true, then containment of the first outbreaks when introduced into Central America and the Caribbean islands should prevent subsequent outbreaks in neighbouring countries. However, it has to be borne in mind that the number of samples analysed and their geographical distribution is limited and therefore, a larger number of isolates should be studied to confirm this pattern of dispersal.

Since the 1990s, DENV serotypes have been recovered

constantly from the Venezuelan population. In particular, DENV-2 and DENV-4 have been circulating constantly at a low rate, since one or two cases have been reported every month. During the DENV-3 epidemic in Venezuela, this hyperendemicity was also present. In Aragua State, for example, between September 2000 and August 2001, 665 cases were diagnosed, of which 69·4 % was due to DENV-3, 15·9 % was due to DENV-2, 12·3 % was due to DENV-4 and 2·4 % was due to DENV-1 (G. Comach, D. Camacho, M. Salcedo, M. Cabello de Quintana, M. Jimenez & G. Sierra, unpublished data). Under such circumstances, one can assume that the population would exhibit a wide range of serological responses to DENV, which would probably increase the likelihood of DHF/DSS. However, this was not the case with the DENV-3 epidemic in Venezuela, indicating that either only a small proportion of the population has cross-reactive immunity to other DENV serotypes or the strains circulating currently induce DHF with only very low frequency.

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