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Diversity and composition of dengue virus type 2 in Venezuela

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SUMMARY

Dengue is a mosquito-borne disease caused by four closely related dengue virus (genus *Flavivirus*) serotypes (DENV-1–4). The clinical outcomes vary from mild febrile illness to life-threatening haemorrhagic manifestations. DENVs are endemic in the tropics and subtropics globally and currently no specific treatment or vaccines are available. In Venezuela, the American-Asian genotype of DENV-2 is the most prevalent and has been associated with severe disease outcomes. We aimed to follow-up the molecular epidemiology of DENV-2 in Venezuela to investigate if the evolution of the virus has remained the same throughout time or if the same dynamics documented in Brazil (hyperendemic co-circulation) also occurred. The results show that whereas the epidemiology of DENV in several endemic areas is characterized by serotype replacements through time, in Venezuela the American-Asian genotype DENV-2 has evolved into several genetic lineages and has remained in hyperendemic co-circulation with the other serotypes.

Key words: American-Asian genotype, dengue virus type 2, phylogeny, Venezuela.

INTRODUCTION

Dengue is a mosquito-borne viral disease caused by four closely related dengue virus serotypes (DENV-1–4) belonging to the genus *Flavivirus*. DENVs are positive-sense single-stranded enveloped RNA viruses that are transmitted by *Aedes* spp. mosquitoes [1]. The clinical outcomes are categorized according to the disease severity into dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. DENVs are endemic in more than 100 countries in the subtropics and tropics of the world causing an estimated 50–100 million infections annually; currently no specific treatment or vaccines are available [3].

Dengue is a significant public health problem in Latin America. During the last 30 years an almost fivefold increase in reported cases has been observed [4]. In this region, DENV-2 has been involved in epidemics for several decades [5–8]. DENV-2 viruses

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so far have been genetically categorized into six genotypes, one representing the isolates found in sylvatic cycles involving primates while the others are associated with human epidemics [9–12]. The DENV-2 genotype, originally found in the Americas, was designated as the American genotype. This genotype was prevalent in Latin America until the early 1980s when a novel genotype of Asiatic origin, known as American-Asian genotype [13] or subtype III [9] was introduced into the area [6]. Unlike the American genotype, the new American-Asian genotype was associated with severe disease including DHF and DSS [14, 15]. This genotype spread rapidly and became the predominant DENV-2 genotype in the area [6, 7, 9, 15, 16].

The highest numbers of DHF cases in the Latin American region are reported from Venezuela, where currently all four DENV types are co-circulating. In 2007, DHF incidence in Venezuela was 62·9/100000 in infants [4]. Previously DENV-2 was associated with severe disease and fatalities in Venezuela, and was shown to evolve locally [17–19]. DENV-2 is still circulating in Venezuela; however, the more recent virus genotype and lineage compositions are unknown. The aim of this study was to follow-up the molecular epidemiology of DENV-2 in Venezuela by analysing a set of previously uncharacterized isolates and the available database sequences, which overall encompass a time-frame spanning more than 15 years.

MATERIALS AND METHODS

Virus strains and epidemiological data

The materials and epidemiological information were collected at the Regional Laboratory of Aragua state, Maracay, which is a centre for DENV research in Venezuela. The laboratory performs routine serological diagnostics of dengue and DENV serotyping by reverse transcription–polymerase chain reaction (RT–PCR) [20] and collects epidemiological data including numbers of diagnosed cases, clinical disease severity classifications according to the WHO [2] and the proportions of different DENV serotypes detected in DENV by RT–PCR typing. From all the diagnosed cases, the early serum samples are additionally studied by virus isolation in *Aedes albopictus* C6/36 cells and serotyped using DENV-type specific monoclonal antibodies [21] in immunofluorescence assay. All DENV-2 isolates obtained during 1999–2005 were included in this study, comprising of 23 previously uncharacterized strains.

RT–PCR and sequencing

The viral RNA was extracted from supernatants of infected C6/36 cells by using QiaAmp Viral RNA Mini kit (Qiagen, USA) according to the manufacturer's instructions. The envelope gene regions were amplified by RT–PCR using Expand Reverse Transcriptase (Roche, USA) and recombinant *Taq* DNA polymerase (Fermentas, USA) and *Taq* Extender (Stratagene, USA) using previously described primers [19]. The PCR products were purified using ExoZAP-IT (USB Laboratories, USA) and directly sequenced using a set of DENV-2 envelope gene specific primers (Supplementary Table S1).

Phylogenetic analysis

The sequence analysis was performed for 122 DENV-2 envelope gene sequences including the 23 DENV-2 strains sequenced in this study and 55 Venezuelan DENV-2 viruses for which the complete envelope gene sequence was available in the GenBank database (accessed in February 2012). Additionally, sequences from neighbouring countries sharing high identity percentages with the Venezuelan isolates and a global selection of DENV-2 sequences representing the epidemic DENV-2 genotypes were included (Supplementary Table S2). The sequences were aligned using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and the phylogenetic analysis was performed using the Bayesian approach implemented in BEAST [22]. The HKY-G-I model of nucleotide substitution was used, convergence of parameters was assessed using Tracer v. 1.4 (http://beast.bio.ed.ac.uk/Tracer) and each run was continued until the effective sampling size of all parameters was >200. The available complete genome sequences of Venezuelan isolates ($n=31$) were further examined for the presence of recombination events using SimPlot (http://sray.med.som.jhmi.edu/SCRoftware/simplot/) and RDP (http://darwin.uvigo.es/rdp/rdp.html).

RESULTS

DENV-2 isolates studied here originated from the period 1999–2005 (Table 1). During this period, the only year with no isolates was 2002. The sequence analysis of Venezuelan DENV-2 envelope gene

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Table 1. *DENV-2 isolates sequenced in this study*

Isolate designation in phylogenetic tree	Strain name	Month, year	Grading	Sex	Age (yr)	Platelet count ($\times 1000/mm^3$)	GenBank accession no.
L4286_Venezuela_99	LARD4286	Apr. 1999	DF	F	33	n.d.	HM210477
L5075_Venezuela_99	LARD5075	Nov. 1999	DF	F	19	71	HM210463
L5124_Venezuela_99	LARD5124	Dec. 1999	DHF	M	87	49	HM210476
L5109_Venezuela_99	LARD5109	Dec. 1999	DF	M	30	120	HM210478
L5690_Venezuela_00	LARD5690	Aug. 2000	DF	M	8	138	HM210461
L5993_Venezuela_00	LARD5993	Oct. 2000	DF	M	4	198	HM210479
L8139_Venezuela_01	LARD8139	Jan. 2001	DF	F	4	102·5	HM210464
L8336_Venezuela_01	LARD8336	Feb. 2001	DF	F	9	202	HM210466
L8249_Venezuela_01	LARD8249	Feb. 2001	DF	F	56	83	HM210470
L8399_Venezuela_01	LARD8399	Feb. 2001	DF	M	13	274	HM210471
L9638_Venezuela_01	LARD9638	June 2001	DHF	F	6	23	HM210462
L12926_Venezuela_01	LARD12926	Aug. 2001	DF	F	7	156	HM210469
L13618_Venezuela_01	LARD13618	Sept. 2001	DF	F	23	84	HM210472
L19094_Venezuela_03	LARD19094	Jan. 2003	DF	M	29	115	HM210480
L21994_Venezuela_03	LARD21994	Dec. 2003	DF	F	9	n.d.	HM210467
L22000_Venezuela_03	LARD22000	Dec. 2003	DF	F	12	90	HM210483
L26062_Venezuela_05	LARD26062	Apr. 2005	DHF	M	4	135	HM210473
L26085_Venezuela_05	LARD26085	Apr. 2005	DF	M	6	133	HM210481
L26688_Venezuela_05	LARD26688	July 2005	DHF	M	31	74	HM210475
L26853_Venezuela_05	LARD26853	July 2005	DF	F	15	56	HM210465
L27057_Venezuela_05	LARD27057	Aug. 2005	DF	F	6	131	HM210468
L28105_Venezuela_05	LARD28105	Sept. 2005	DF	M	21	143	HM210474
L27791_Venezuela_05	LARD27791	Sept. 2005	DF	M	11	234	HM210482

DF, Dengue fever; DHF, dengue haemorrhagic fever; n.d., no data.

sequences including the 23 DENV-2 strains sequenced in this study suggested that the Venezuelan isolates fell into six genetic lineages exclusively within the American-Asian genotype (Fig. 1) in 1991–2008. Lineage I included strains from several countries in the Caribbean islands, Paraguay, Brazil, Colombia and the Venezuelan strains from the late 1990s (1990–1998). This lineage was separated from the rest of the Venezuelan strains by two clusters, one consisting solely of Brazilian strains (2001–2006) and the other of strains from the late 1990s from Martinique, Cuba and Puerto Rico, and Brazil 2008. Lineage II was temporally clustered, including exclusively strains after 2000, such as the most recent available Venezuelan sequences from 2003 to 2008 in addition to some Colombian isolates from 2004, 2005 and 2007. One of the Venezuelan isolates sequenced in this study from 1999 was not grouped with any of the other strains in the analysis (L5109), and thus it was designated as a lineage of its own (lineage III). However, support for this branch was not very high (0·66 posterior probability). The rest of the sequences were divided into three main clusters, designated as lineages IV, V and VI. Lineage IV consisted of Venezuelan isolates from 2003 to 2007, lineage V included strains from 1996 to 2001, and lineage VI strains from 1998 to 2001. In the previous study consisting of Venezuelan DENV-2 E isolates obtained between 1997 and 2000, three genetic lineages were separated, including two main groups and one strain that was not clearly associated with either of the main lineages [19]. These groups are now implemented within lineages I, V and VI in the current tree. Most of the available sequence data of Venezuelan DENV-2 consists of E-gene sequences. No evidence of recombination could be detected in the strains included in this analysis. The rarity of recombination was also confirmed by analysis of the available complete genome sequences of Venezuelan isolates ($n=31$) that showed no evidence of recombination besides the previously described recombination in the preM gene of MARA4 strain [19] (data not shown).

The Venezuelan DENV-2 sequences determined in this study had envelope protein amino acid changes located in domains II and III, including the amino acid signature described for the American-Asian genotype [7] defined by E91:I, E129:V,

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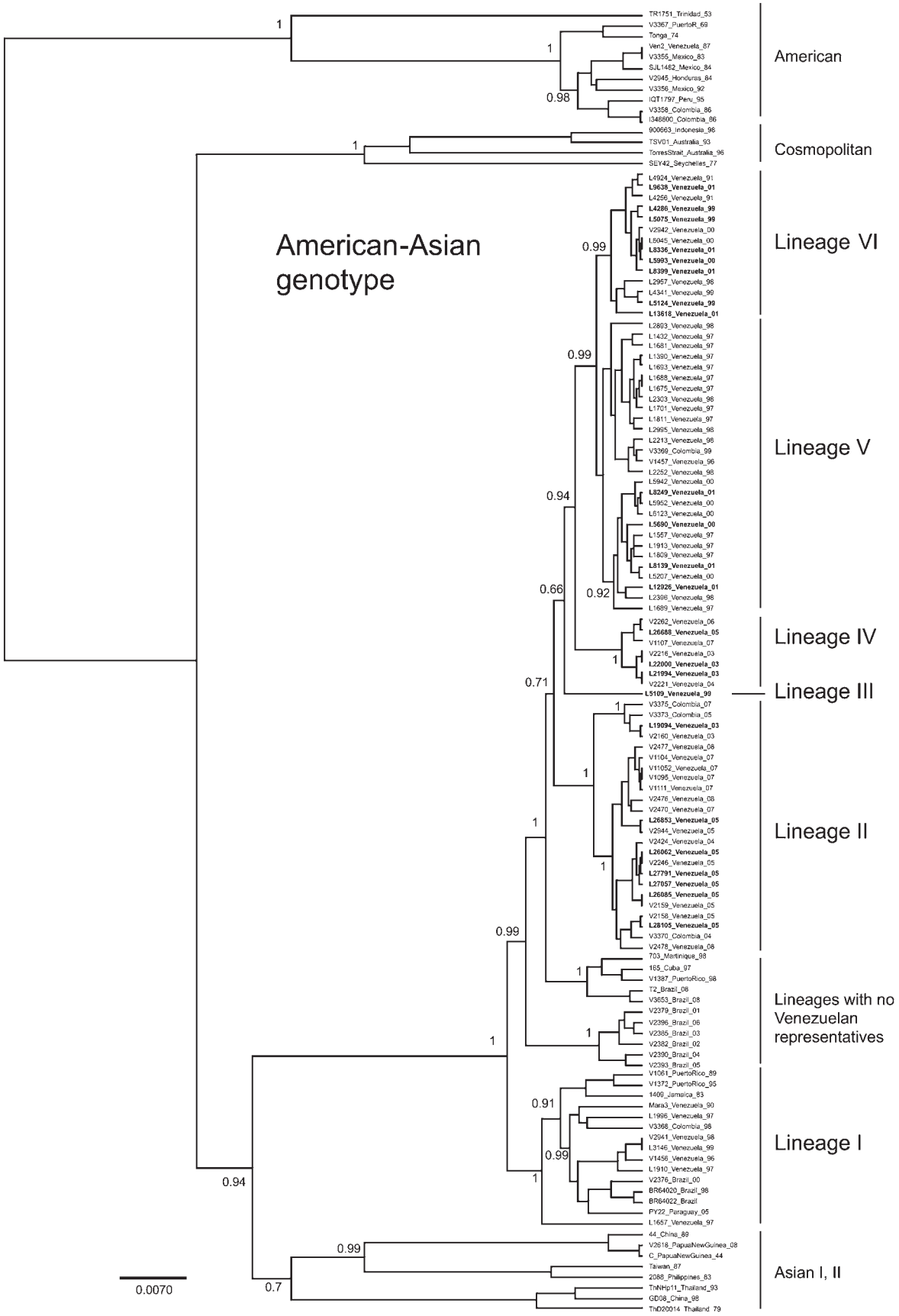

Fig. 1. Phylogenetic tree of 1485-bp E-gene sequences of DENV-2. The strains sequenced in this study are indicated in bold. A Bayesian maximum clade credibility tree is shown with mean branch lengths (substitutions per site), and Bayesian posterior probabilities given at the key nodes for the main clades.

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E131:Q, E390:N and E485:V. A total of 12 additional amino-acid substitutions were observed, some of them exclusively found in Venezuelan strains (Supplementary Table S3). In DENV-2 strains from Puerto Rico, the typical American-Asian genotype envelope gene domain I substitutions were associated with additional changes in the domain III, in positions E491 or E359 [16]. Six of our Venezuelan isolates had similarly additional change in E359, from threonine to isoleucine.

The results of RT–PCR typing of DENV serotypes circulating in 1997–2008 suggest that the serotype composition had varied from year to year (Supplementary Fig. S1). Moreover, a change from three circulating serotypes (DENV-1, −2, −4) to the co-circulation of all four DENV serotypes had occurred since 2000. Within this time-frame the years 1997 and 1999 had low numbers of DENV diagnoses, and these were the only years that DENV-2 was the predominant serotype. After 1999, DENV-3 and DENV-1 were detected more often than DENV-2, which was also the case during the peak years of 2001 and 2007.

DISCUSSION

The new sequence data obtained in this study combined with the existing database sequences of DENV-2 provide a long-term insight to the molecular epidemiology of DENV-2 in Venezuela, and demonstrate a wide local diversification of strains belonging to the American- Asian genotype. In this study no other genotypes of DENV-2 were detected, which is in line with previous studies [19, 23] suggesting that since the introduction of the American-Asian genotype in the 1980s DENV-2 has evolved locally in Venezuela.

Since the previous comprehensive study of Venezuelan DENV-2 in 1997–2000 [19], the genetic diversification of DENV-2 has continued, and instead of three lineages, the strains are now separated into six separate lineages. The Venezuelan DENV-2 strains were only partly temporally clustered, suggesting co-circulation of variable strains. The E gene sequences determined in this study, and their comparisons to database sequences suggest that the Venezuelan DENV-2 strains had some unique amino acid changes, that may serve as identifiers for strains of Venezuelan origin. However, as no associations with disease severity could be made with any of the defined amino acid changes (or described phylogenetic lineages), further studies will be required to elucidate their possible biological significance.

The available information on the circulating DENV serotypes in 1997–2008 was based on results of RT–PCR typing of DENV that were not available for all diagnosed patients. However, the available data of RT–PCR-positive patients suggest that co-circulation of all four DENV serotypes had occurred since 2000, the time when DENV-2 also seemed to have fallen from the position of a 'leading' serotype. The lowered numbers of DENV-2 could be explained by the previous massive DENV-2 epidemics that probably left the majority of the population immune to this serotype, and additionally, the co-circulation of the other serotypes could have impaired DENV-2 transmission through short-term cross-protection [24]. However, DENV-2 was detected each year, demonstrating that it was not completely replaced by the other serotypes.

In addition to Venezuela, the local evolution and survival of the American-Asian genotype DENV-2 in co-circulation with the other serotypes was also observed in Puerto Rico and Brazil [25–27]. In Venezuela, DENV-2 has not been the major cause of DENV epidemics for a number of years, but it may have the capability to re-emerge, as previously seen in Puerto Rico [25].

The maintenance and re-emergence of a certain DENV serotype or genotype seems to be a more rare observation than the introduction and replacement pattern observed in many endemic countries [28–30]. Currently the epidemiological and ecological factors behind these different patterns are poorly understood. The American-Asian genotype DENV-2 has been experimentally shown to have relatively high fitness in both humans and mosquitoes [31, 32], which may explain its survival in competition with other circulating DENVs. While epidemiological studies often concentrate on the detection and study of DENV from clinically ill patients, enlightening information could be obtained by monitoring and studying asymptomatic individuals and mosquitoes. The detailed understanding of DENV endemicity, including maintenance, is important for successful prevention and control strategies in the affected areas, such as Venezuela.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268812002324.

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DECLARATION OF INTEREST

None.

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