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Complete Genomic Sequence of a Dengue Type 2 Virus from the French West Indies

H. Tolou,* P. Couissinier-Paris,* V. Mercier,* M-R. Pisano,* X. de Lamballerie,†
P. de Micco,† and J-P. Durand†

**Unité de Virologie Tropicale, Institut de Médecine Tropicale du Service de Santé des Armées, and †Laboratoire de Virologie Moléculaire, Tropicale et Transfusionnelle, Faculté de Médecine, Unité des Virus Emergents, Marseille, France*

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Severe forms of dengue fever, dengue haemorrhagic fever, and dengue shock syndrome, were not prominent in the Americas until the epidemic of Cuba in 1981. Since that time, they have spread to other countries in Central and South America, correlating with the spread of dengue type 2 viruses related to Southeast Asian strains. We report here the complete genomic sequence of a dengue type 2 virus isolated during the epidemic in La Martinique in 1998. This constitutes the first complete genetic characterization of a dengue virus strain from French West Indies, and also the first molecular identification in this region of a dengue 2 strain phylogenetically related to the emerging American type 2 dengue viruses.

Key Words: **Dengue virus; genotype 2; genome; sequencing; phylogeny.**

Dengue virus, a species in the *Flavivirus* genus, is a positive-strand virus transmitted by the *Aedes aegypti* mosquito and responsible for the most frequent arboviral disease in the world: more than 50 million people each year suffer dengue fever (DF) in tropical areas. Since 1953, severe forms of the disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), have been reported with increasing frequency (1). They are now threatening areas where they were previously unknown, particularly in the Americas. The mechanisms leading to these severe and sometimes lethal forms are not clearly understood. In particular, the respective contributions of host genetic susceptibility, antibody dependent enhancement of infection (2) or viral virulence (3), are still debated.

Recent observations done in Central and South America, however, are indicative of the possible existence of viral determinants of virulence. Although dengue has been endemic in these areas for a long time, the Cuban epidemic in 1981 is regarded as the first epidemic of DHF in the region. Dengue type 2 viruses isolated during this epidemic are genetically close to Southeast Asian strains (4). Since 1981, these dengue 2 strains have spread to several countries (Venezuela, Colombia, Brazil, Mexico), where DHF and DSS outbreaks are now reported, while native strains of dengue type 2 are more rarely isolated (5). Extension of the new strains is now threatening the entire area (Central and South America, Caribbean Islands).

In this paper, we report the complete genomic sequence determination of a dengue type 2 virus strain isolated from a human case who became infected in La Martinique, French West Indies, in 1998. This constitutes the first complete genetic characterization of a dengue virus strain from French West Indies, and also the first molecular identification in this region of a dengue 2 strain phylogenetically related to the emerging American type 2 dengue viruses.

MATERIALS AND METHODS

Viral strain. Dengue virus type 2 strain DEN2/H/IMTSSA-MART/98-703 (referred as strain D2-Mart in this article) was isolated from the serum of a 18-year-old girl exhibiting a classical dengue fever syndrome following a travel in La Martinique in September 1998.

Virus propagation. The patient serum was inoculated to C6/36 cells grown at 28°C in Leibowitz's L15 medium supplemented with 1% L-glutamin and 2% tryptose phosphate broth. Fetal bovine serum (5% final) was added one hour later. A dengue type 2 virus was identified at eleven days postinfection using a type 2-specific monoclonal antibody (provided by Dr. Karabatsos, Fort Collins). The virus was passaged once under identical culture conditions and the supernatant was used for RNA extraction.

RNA preparation and reverse transcription. RNA was extracted from 200 μl aliquots of cell culture supernatant using the High Pure Viral RNA isolation kit (Roche Molecular Biochemicals), according to manufacturer's instructions and eluted in 50 μl of RNase free water. Five microliters of this RNA solution were used for reverse transcription reactions using the SuperScript II reverse transcriptase (Gibco BRL) and specific primers designed from available sequences of dengue type 2 strains (Fig. 1).

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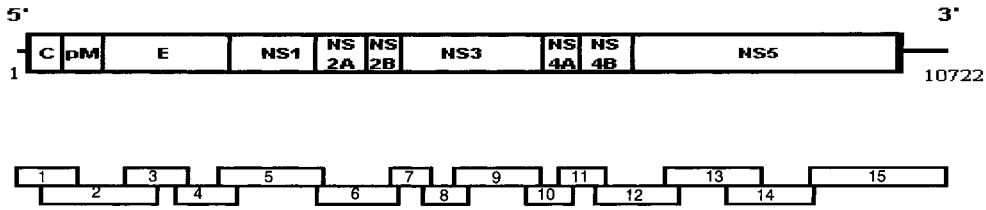

	Name	Position	Sequence (5'→3')	Name	Position	Sequence (5'→3')
1	D2/1/D	1-19	AGT TGT TAG TCT ACG TGG A	D2/644/R	644-616	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC
2	D2/279/D	279-293	AAC AGC AGG GAT ATC	D2/1668/R	1668-1649	ATG GCG ATT TTT GAA ACT CA
3	D2/1171/D	1171-1188	GAA CCC AGT CTA AAT GAA	D2/2023/R	2023-2006	TAT CTT TTT CTG TTA CGA
4	D2/1860/D	1860-1877	TGT GAA GGA AAT AGC AGA	D2/2459/R	2459-2440	AGT TCT TTG TTT TTC CAG CT
5	D2/2297/D	2297-2316	TTA TGA AAA TCC TCA TAG GA	D2/3506/R	3506-3996	AGT GAA AAG TTG TCA ATC TG
6	D2/3478/D	3478-3495	GGA CAT GGG CAG ATT GAC	D2/4518/R	4518-4501	TTG TTT CTT CAC TTC CCA
7	D2/4489/D	4489-4508	GCA TGG TAC CTG TGG GAA GT	D2/4947/R	4947-4927	TCT GTC GAC AAT TGG AGA TCC
8	D2/4916/D	4916-4933	CTG GAA CGT CAG GAT CTC	D2/5442/R	5442-5422	ACC CAT CTC TAC TCG AGT TGA
9	D2/5212/D	5212-5236	ATG GA GAA GCT CTT AGA GGA CTT C	D2/6375/R	6375-6358	CTT TCT TCC AGC TGC AAA
10	D2/6128/D	6128-6152	TAA TGA GAA GAG GGG ACT TGC CAG T	D2/6825/R	6825-6808	TGC CAT GGT TGC GGC CAC
11	D2/6533/D	6533-6550	AGA CAC TGC TTT TAC TGA	D2/7216/R	7216-7199	TGG TTG CTT TTG CTT GAA
12	D2/7109/D	7109-7126	CCA TTG GAT GCT ACT CAC	D2/7962/R	7962-7945	GTC AAC TCC ACT TTG CAG
13	D2/7775/D	7775-7799	ATA TGG TCA CAC CAG GGA AAG GT	D2/8915/R	8915-8898	GTT TCA CAC TTT CCT TCA
14	D2/8475/D	8475-8499	GTG GGC TTA CCA TGG CAG CTA TGA A	D2/9413/R	9413-9394	ATG TTG GTG AAA GTA TT
15	D2/9391/D	9391-9415	GGC CTT AAT ACT TTC ACC AAC ATG G	D2/10723/R	10723-10706	AGA ACC TGT TGA TTC AAC

FIG. 1. Schematic representation of the RT-PCR strategy for the amplification and sequencing of the D2-Mart RNA. Top: genome organization. Coding regions are shown as blocks. Middle: overlapping PCR fragments. Bottom: list of primers used for RT and PCR reactions. All the primers were designed from the sequence of the DEN-2 Jamaica or New Guinea C strains. Reverse primers (R) were used both for reverse-transcription and PCR. D, direct sense primers. Primers on the same line have been paired for PCR. Position is given relative to the published sequence of strain Jamaica.

PCR amplification and sequencing. Fifteen overlapping PCR fragments spanning the whole genome were produced using a combination of primers (Fig. 1). PCR amplifications were achieved in a 50 μl reaction volume under standard conditions, using 5 μl of cDNA solution, the Ampli-Taq DNA-polymerase FS (Applied Biosystems) and 35 cycles of denaturation, annealing and polymerization (30 s at 94°C; 30 s at 48 to 52°C; 2 min at 72°C, respectively).

PCR products were purified from agarose preparative gels, using the QIAquick Gel Extraction kit (Qiagen) and directly sequenced using either the primers used for PCR amplification or internal primers (list available on request), the DNA-sequencing kit and an ABI Prism A310 sequence analyzer (both from Applied Biosystems), in 176 independent reactions. Each position of the genome was read three times at least. The complete sequence was deposited in GenBank and assigned the Accession No. AF208496.

Sequence analysis. Full-length sequences of the following dengue type 2 strains were retrieved from databases (GenBank accession numbers are in parentheses): Jamaica 1409 [1983] (M20558); New Guinea NGC [1944] (AF038403); Mexico 0131 [1992] (AF100469); Peru IQT1797 [1995] (AF100467); Thailand 16681 [1964] (U87411); and Thailand K0010 [1994] (AF100460).

The alignments of complete nucleotide and amino acid sequences were generated using the ClustalW software (6). The percentage of identity between pairwise sequences was calculated. A phylogram based on the alignment of full-length nucleotide sequences was constructed with the help of the MEGA program (7), using the Jukes Cantor algorithm for genetic distance determination and the Neighbor Joining method for tree drawing. The robustness of the resulting groupings was tested by 500 bootstrap replications.

RESULTS AND DISCUSSION

The dengue virus strain the genome of which was characterized in this study was isolated from an infected woman during the epidemic of 1998 in La Martinique. Its complete nucleotide sequence was determined, with the exception of the 21 nucleotide long fragments at both 5′ and 3′ termini of the viral RNA molecule which correspond to the most external primers used for PCR amplification (and were supposed to be identical to those of the closely related Jamaica 1409 strain). This sequence is 10,722 nucleotide long and belongs to the genotype 2 of dengue viruses. It is very close to that of the strain Jamaica 1409, with a 98.3% identity at the nucleotide level (Table 1). Differences between the two strains include a one base deletion at position 10,449 for the strain D2-Mart. The length of the unique ORF (10,173 nucleotides) and the positions and sequences of the different proteolytic cleavage sites on the polyprotein are conserved. The 29 amino-acid changes are distributed along the coding sequence without any "hot spot."

Comparison of genetic distances at both the nucleotide and amino acids levels shows that strains D2-Mart and Jamaica 1409 are more closely related to dengue 2 strains originating from Southeast Asia (represented in this study by strains from Thailand and New Guinea) than to "native" dengue 2 strains isolated in the Americas (represented by strains from Mexico and Peru). This is fully confirmed by the analysis of an evolutionary tree (Fig. 2), which identifies with a 100% bootstrap confidence level the existence of a common ancestor for Asian strains and strains D2-Mart and

TABLE 1

Sequence Identity between Full-Length Dengue Type 2 Virus Sequences (Percentages)

	D2-Mart	Jamaica 1409	New Guinea NGC	Thailand 16681	Thailand K0010	Mexico 0131	Peru IQT1797
D2-Mart	—	98.3	94.6	93.6	92.6	89.7	89.8
Jamaica 1409	**99.1**	—	95.4	94.4	93.2	90.1	90.1
New Guinea NGC	**97.9**	**98.4**	—	96.9	95.4	91.3	91.3
Thailand 16681	**97.4**	**97.8**	**98.8**	—	96.7	90.6	90.6
Thailand K0010	**97.2**	**97.6**	**98.3**	**98.6**	—	89.1	90.0
Mexico 0131	**96.6**	**96.8**	**97.4**	**96.9**	**96.8**	—	97.8
Peru IQT1797	**96.5**	**96.7**	**97.4**	**96.9**	**96.8**	**99.2**	—

Note. Upper-right values (normal characters): nucleotides; lower-left values (bold characters): amino acids.

Jamaica 1409. Such analysis also indicates that the two latter strains share a common ancestor (100% bootstrap confidence level). This point is important to notice since it highly suggests that these two Asian-like dengue 2 strains do not correspond to iterative introductions of Asian strains in the Americas, but are the evolutionary products of a unique imported strain.

Motifs specific for Asian-like dengue 2 viruses associated with severe clinical forms have been recently identified by Leitmeyer and colleagues, in both coding and non-coding regions of the RNA (8). All these motifs are present in the sequence of strain D2-Mart. At positions where Southeast Asian and American native genotypes differ, all amino acids of strain D2-Mart are those of the Southeast Asian strains (Table 2). In non-coding regions, the differences include nucleotide changes at positions 69 and 77 (T → A and G → A, respectively, in Southeast Asian strains compared to native American ones), and a 8-nucleotide deletion downstream the stop codon (10,276–10,283), specific to the native American strains. Although the significance of these differences is not currently understood, several of them are thought to play a role in the ability of the virus to infect humans and to promote severe disease (8, 9). Accordingly, the introduction of Asian-like dengue viruses in La Martinique might represent a determining factor in the appearance of DHF and DSS in this region. However, to date DHF and DSS are still

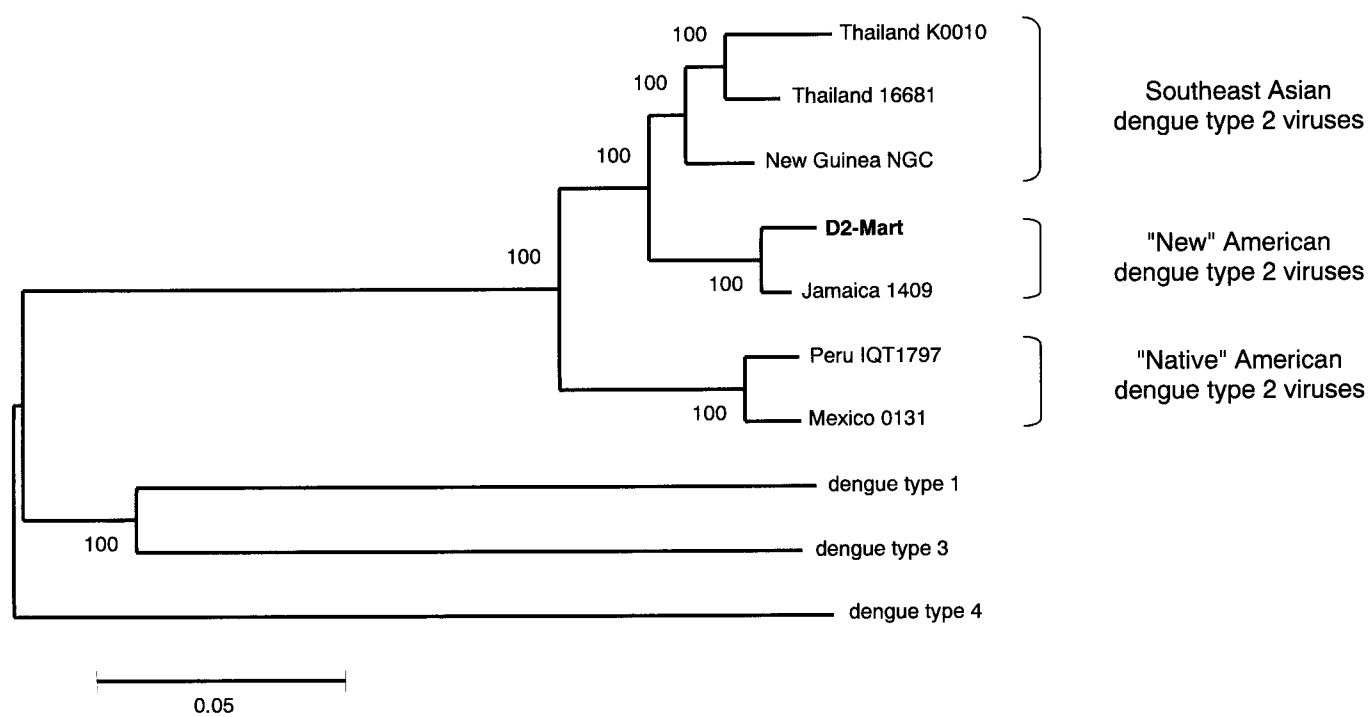

FIG. 2. Phylogenetic analysis of dengue-2 complete nucleotide sequences using MEGA software and the Neighbor Joining method. Bootstrap resampling values are indicated at the forks. GenBank Accession Numbers for dengue type 1 (Singapore S275/90), type 3 (H87), and type 4 (Dominica 814669) sequences are M87512, M93130, and M17255, respectively.

TABLE 2

Amino Acid Changes Differentiating Southeast Asian and American Genotypes (Analyzed in (8)), in Comparison with Strain Martinique

		Amino acids		
Gene	Position on the gene	Southeast Asian strains	D2-Mart	American strains
prM	28	E	E	K
	31	V	V	T
Envelop	390	N	N	D
NS1	128	S	S	L
NS3	567	I	I	T
NS4B	17	S	S	H
NS5	271	I	I	T
	645	N	N	D
	676	S	S	R/K
	800	K	K	S
	819	Q	Q	L

rarely reported in Martinique, although dengue fever epidemics occur annually, suggesting that the viral markers are probably not the only determinants required for severe dengue forms.

Concerning the evolution of dengue virus strains, an important point must be highlighted: there is very little genetic distance between the two Asian-like strains D2-Mart and Jamaica 1409, although these viruses have been isolated at a 15-year interval. This is consistent with the idea that stable topotypes of dengue virus exist, as reported for yellow fever virus (10, 11), and more generally that rapid evolution of flaviviruses is essentially driven by environmental parameters and not by a steady biological clock. Finally, it has been pinpointed that direct sequencing of dengue viruses from patient plasma might be important to avoid selection of variants not relevant to the pathogenesis of the disease (8). However, limited passaging on mosquito cells (one passage in our observation) did not seem to have significant effect, since all mutations differentiating American and Southeast Asian strains could be identified in the strain D2-Mart.

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