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Phylogenetic analysis of dengue-3 viruses prevalent in Guatemala during 1996–1998*

Brief Report

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Summary. Dengue is an acute viral disease transmitted by the *Aedes aegypti* mosquito which is present in most tropical urban areas of the world. There are four antigenically distinct serotypes, designated dengue-1 (DEN-1), dengue-2 (DEN-2), dengue-3 (DEN-3) and dengue-4 virus (DEN-4). In this study, we determined the serotypes of dengue viruses isolated in Guatemala in 1995–1998, and found that DEN-3 viruses appeared in 1995 and became predominant in the following three years. We then sequenced cDNAs from fifteen DEN-3 isolates recovered during 1996–1998. From the nucleic acid sequences and previously determined DEN-3 sequences, a phylogenetic tree was constructed using the neighbor joining method. The tree indicated that all fifteen isolates and other DEN-3 viruses isolated in Sri Lanka, India, Samoa and Mozambique formed subtype III. More than two decades ago, DEN-3 virus was prevalent in the Caribbean, but the isolates obtained at that time belonged to subtype IV. Therefore, we concluded that the 1996–1998 dengue epidemic in Guatemala was caused by DEN-3 strains, imported from a tropical area of Asia or Africa or from a Pacific island.

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Dengue is an acute viral disease transmitted by the *Aedes aegypti* mosquito which is present in most tropical urban areas of the world. A small proportion

*The DNA sequence data reported here have been deposited in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with accession numbers AB038465 to AB038479.

of those infected develop more severe forms of the disease, dengue hemorrhagic fever/dengue shock syndrome. There are four antigenically distinct serotypes, designated dengue-1 (DEN-1), dengue-2 (DEN-2), dengue-3 (DEN-3) and dengue-4 virus (DEN-4).

Owing to eradication campaigns carried out in the Americas around 1960, *Aedes aegypti* almost disappeared from the continent, and except for DEN-2 and DEN-4 epidemics in rather restricted areas, dengue had been absent in the Americas [4]. The first re-emergence of dengue in the Americas in 1977 has been reviewed previously [13], but briefly, a milestone in the re-emergence of dengue in the America was a dengue epidemic caused by a DEN-1 strain, possibly imported from Africa. This virus was first found in Jamaica, from where it spread throughout the Caribbean, South and Central America and Mexico during 1977–1980. In 1981, a DEN-4 strain probably imported from the Pacific islands emerged in the Americas, causing a series of outbreaks in the Caribbean, northern South America, Central America and Mexico.

In 1994, DEN-3 re-emerged in Central America after an absence of seventeen years [18]. This serotype was initially detected in Panama and Nicaragua and subsequently spread to other Central American countries and Mexico [19]. The question arose as to whether this DEN-3 epidemic was caused by endemic or imported viruses.

Genetic typing of dengue viruses should help to address this issue. According to phylogenetic analyses, DEN-3 viruses are grouped into four subtypes, I to IV [9, 10]. The nucleic acid sequence of the initial DEN-3 isolate obtained in 1994 in Panama was compared to the sequences of reference DEN-3 viruses, and it was found that this isolate belonged to subtype III [18]. Furthermore, two DEN-3 viruses isolated in Puerto Rico in 1998 were shown to belong to subtype III [19]. It was concluded that the recent DEN-3 epidemic in the Americas was caused by a DEN-3 strain, probably imported from Asia, Africa or the Pacific [18]. However, as the genetic analyses described above were performed on only a few isolates (a details of the analyses and data were not presented), further study is required to draw a definite conclusion.

Harris et al. [5] developed a new PCR-based typing method, restriction site-specific (RSS)-PCR. Using this method, 1996–1998 Nicaraguan, 1997 Guatemalan and 1998 El Salvador DEN-3 isolates were all typed as RSS-PCR type C [1, 5]. This type probably corresponds to phylogenetic subtype III [9, 10], because some DEN-3 subtype III viruses were grouped as type C [5]. However, since subtype IV remained to be analyzed by RSS-PCR [5], there was a need to further clarify the correspondence between phylogenetic subtypes and RSS-PCR types.

Standard phylogenetic analysis [9, 10] should be useful in characterizing the recent DEN-3 epidemic in Central America. Here, we carried out serotyping of dengue isolates obtained in Guatemala during 1995–1998, and found that the conversion of prevalent serotypes from DEN-1/DEN-4 to DEN-3 occurred in 1996. We then performed a phylogenetic analysis of fifteen DEN-3 isolates obtained in Guatemala in 1996–1998 to elucidate the origin of the Guatemalan DEN-3 strains isolated during this period.

Dengue viruses were isolated from hospitalized patients with dengue fever, using *Aedes albopictus* cell line (C6/36) [6, 7]. In brief, C6/36 cells in a 25-cm^2 flask were inoculated with 100 μl of patient sera, and were incubated at 33 °C in Eagle's minimal essential medium supplemented with 10% fetal calf serum. To determine the serotypes of dengue isolates, infected cultures showing cytopathology were examined by indirect immunoperoxidase staining, using monoclonal antibodies: D2-1F1-3 (DEN-1), 3H5-1-21 (DEN-2), D6-8A1-12 (DEN-3) and 1H10-6-7 (DEN-4) (Chemicon International Inc., CA). These monoclonal antibodies were provided by Centers for Disease Control and Prevention (Atlanta, GA).

Infected C6/36 cells showing maximum cytopathic effect were harvested and the resultant culture fluids were used to extract the viral RNA using a single-step RNA isolation kit, Isogen (Wako Pure Chemicals, Osaka, Japan). Three partially-overlapping segments in the genomic region from nucleotides (nt) 1 to 1,181 encompassing the C, C′, preM and M protein genes and part of the E protein gene were reverse-transcribed into cDNAs using Ready-to-go you-prime first-strand beads (Amersham Pharmacia Biotech, Piscaiaway, NJ) using the antisense primers shown in Table 1, and the resultant cDNAs were amplified by 35 cycles using Ready-to-go PCR beads (Amersham Pharmacia Biotech). The three amplified fragments were directly sequenced using the primers shown in Table 1, dRhodamine terminator cycle sequencing ready reaction kit and Model 310 genetic analyzer (Applied Biosystems, Foster, CA).

The computer analysis of the nucleic acid sequence data was performed using DNASIS software version 2.4 (Software Development, Tokyo, Japan). A phylogenetic tree was constructed using the neighbor joining (NJ) method [15]. An NJ

Table 1. Oligonucleotide primers used for RT-PCR and sequencing

Primer	Sequence	Position[a]	Orientation[b]
RT-PCR			
D3001	5′ AGTTGTTAGTCTACGTGGAC 3′	1–20	S
D3066	5′ AAGTCACCCATGTCGATGTA 3′	665–646	C
D3018	5′ GTGTCAACTGGATCACAGTT 3′	161–180	S
D3088	5′ GGGTCAAGGAAGTGCCTATG 3′	884–865	C
D3055	5′ TGTGCACACTCATAGCCATG 3′	534–553	S
D3118	5′ GTAAAATCGCTTCCCCTTGG 3′	1181–1162	C
Sequencing			
D3002	5′ GTTGTTAGTCTACGTGGACC 3′	2–21	S
D3049	5′ CCTCTTTCATTCTTCCCCAC 3′	495–476	C
D3021	5′ CTCAAGAGGATTGCTGAACG 3′	193–212	S
D3070	5′ CTCTTATCGCGTCTATGCTC 3′	708–689	C
D3058	5′ GAGAGATGTGTGATGACACG 3′	561–580	S
D3118	5′ GTAAAATCGCTTCCCCTTGG 3′	1181–1162	C

[a]Positions in the sequence of strain H-87 [11]

[b]S = sense orientation, C = antisense orientation

Table 2. Dengue serotypes prevalent during 1995–1998

Serotype	No. of isolates (%) in			
	1995	1996	1997	1998
DEN-1	29 (45)	12 (27)	6 (12)	2 (6)
DEN-2	6 (9)	3 (7)	6 (12)	0 (0)
DEN-3	1 (2)	29 (66)	36 (69)	30 (94)
DEN-4	28 (44)	0 (0)	4 (8)	0 (0)
Total	64	44	52	32

tree was constructed using CLUSTAL W [17]. Divergences were estimated by Kimura's two-parameter method [8]. The phylogenetic tree was visualized using the TREEVIEW 1.6 program [12]. To assess the confidence of branching patterns of the NJ tree, 100 bootstrap replicates were performed [3].

Table 2 shows the serotypes of dengue viruses which were isolated from patients with dengue fever in Guatemala during 1995–1998 (patients with hemorrhagic fever/dengue shock syndrome were rarely detected in Guatemala during this period). In 1995, two serotypes, DEN-1 and DEN-4, were predominant, but it should be noted that a single DEN-3 isolate was recovered. The latter became the predominant serotype in the following three years. DEN-3 accounted for more than 90% of the dengue viruses isolated in 1998. Thus, DEN-3 viruses rapidly expanded in Guatemala after appearing in 1995.

Four, five and six isolates obtained in 1996, 1997 and 1998, respectively (Table 3), were used to produce cDNAs by RT-PCR. These isolates were selected

Table 3. Description of the fifteen DEN-3 isolates analyzed in this study

Strain	Code	Year	Symptom	Passage history	Accession no.
G3051	GUATE96-1	1996	DF	1	AB038465
G3064	GUATE96-2	1996	DF	1	AB038466
G3087	GUATE96-3	1996	DF	1	AB038467
G3406	GUATE96-4	1996	DF	1	AB038468
G0235	GUATE97-1	1997	DF	2	AB038469
G0703	GUATE97-2	1997	DF	1	AB038470
G1969	GUATE97-3	1997	DF	2	AB038471
G2830	GUATE97-4	1997	DF	1	AB038472
G2877	GUATE97-5	1997	DF	2	AB038473
G0018	GUATE98-1	1998	DF	2	AB038474
G0029	GUATE98-2	1998	DF	2	AB038475
G0038	GUATE98-3	1998	DF	2	AB038476
G0114	GUATE98-4	1998	DF	2	AB038477
G1161	GUATE98-5	1998	DF	2	AB038478
G1551	GUATE98-6	1998	DF	2	AB038479

Table 4. Variations in the nucleotide sequences among the fifteen DEN-3 isolates[a]

Isolate	Position[b]																
	141	162	183	231	249	290	297	344	360	402	423	462	534	555	573	639	651
Consensus	U	C	A	C	C	A	A	U	A	A	A	C	U	A	U	C	G
GUATE96-1	–[c]	–	–	–	–	–	–	–	G	G	–	–	–	G	–	–	–
GUATE96-2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE96-3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE96-4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE97-1	C	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–
GUATE97-2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–
GUATE97-3	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	A
GUATE97-4	–	–	–	–	–	–	–	–	–	–	–	U	–	–	–	–	–
GUATE97-5	–	–	G	–	–	–	G	C	–	–	–	–	–	–	–	–	–
GUATE98-1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE98-2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE98-3	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–
GUATE98-4	–	–	–	U	–	–	–	–	–	–	–	–	–	–	–	A	–
GUATE98-5	–	U	–	–	U	–	–	–	–	–	–	–	–	–	–	–	A
GUATE98-6	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–

[a]A 1,050-nucleotide sequence that encompasses the complete C, C′, preM and M protein genes and part of the E protein gene was compared among the fifteen DEN-3 isolates obtained from the sera of Guatemalan patients with dengue fever between 1996–1998. Nucleotides shown are those at positions where differences were detected

[b]Nucleotides were numbered from the 5′ end of the genomic RNA

[c]Indicates a nucleotide identical with those found in the consensus sequence

so that they included those obtained in various geographic regions and in various times. However, no recovery of the single isolate in 1995 for the production of cDNA was successful. For each isolate, we sequenced a 1,050-nucleotide genomic region (position 95-1,144) that encompasses the complete C, C′, preM and M protein genes and part of the E protein gene. In total, 34 nucleotide changes were detected at 34 positions (Table 4).

The features of the nucleic acid and amino acid sequences of the fifteen isolates can be summarized as follows. (i) The nucleic acid sequences differed by 0.1–1.1%, indicating that the fifteen isolates are closely related. (ii) Twenty-eight of the 34 nucleotide changes were silent, and six caused amino acid changes (Table 5). These amino acid changes occurred between amino acids with similar properties (Table 5).

We next analyzed the phylogenetic relationships among the fifteen Guatemalan and previously reported DEN-3 isolates. The nucleic acid sequences of the C and C′ protein genes have not been reported for most DEN-3 viruses. However, the sequences of the preM and M protein genes and part of the E protein gene of

Table 4 (*continued*)

Isolate	Position																
	7144	7763	7800	8004	8008	8220	8255	8288	8773	8944	9110	9448	9775	10000	10002	10029	10035
Consensus	G	G	U	U	A	C	C	C	U	U	G	G	U	G	G	G	U
GUATE96-1	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	A	–
GUATE96-2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–
GUATE96-3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–
GUATE96-4	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE97-1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE97-2	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE97-3	–	–	A	–	G	–	–	–	–	–	–	–	–	–	–	–	–
GUATE97-4	–	–	–	–	–	–	U	U	–	–	–	–	–	–	A	–	A
GUATE97-5	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE98-1	A	–	A	C	–	U	–	–	–	–	–	–	–	–	–	–	–
GUATE98-2	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE98-3	–	–	–	–	–	–	–	–	–	C	U	–	C	–	–	A	–
GUATE98-4	–	U	–	–	–	–	–	–	–	–	–	A	–	A	A	–	–
GUATE98-5	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GUATE98-6	–	–	–	–	–	–	–	–	–	C	–	–	C	–	–	A	–

thirty-two DEN-3 isolates obtained from various geographic regions and at various times were available [9, 10]. Thus, we aligned the 720-nucleotide sequences encompassing the preM and M protein genes and part of the E protein gene from the fifteen Guatemalan and thirty-two previously established isolates [9, 10]. From the aligned sequences, we constructed an phylogenetic tree using the NJ method [15] (Fig. 1), using a DEN-2 isolate (Jamaica) [2] as the outgroup.

Consistent with previous phylogenetic analyses using the parsimony [10] and NJ method [9], the DEN-3 isolates analyzed diverged into four clusters, designated as subtypes I, II, III and IV (Fig. 1). The fifteen Guatemalan isolates, together with four Sri Lanka, one Indian, one Samoan and one Mozambique isolate, formed subtype III. The fifteen Guatemalan isolates clustered together with a relatively high bootstrap confidence level (87%). These findings suggested that the DEN-3 viruses prevalent in Guatemala during 1996–1998 were transmitted from Nicaragua or Panama where this subtype was recovered in 1994 (Panama) [18].

During 1981–1991, DEN-3, subtype III was frequently detected in Sri Lanka [10], suggesting that it was endemic in this region. The subtype III virus was also isolated in India in 1984, in Mozambique in 1985 and in Samoa in 1986 [10]. The strain detected in Panama in 1994 was probably from these tropical regions. After being isolated in Nicaragua and Panama, DEN-3, subtype III rapidly spread

Table 5. Variations in the amino acid sequences among the fifteen DEN-3 isolates[a]

Isolate	Position[b]					
	97	115	255	270	304	334
Consensus	K	F	A	I	V	A
GUATE96-1	–[c]	–	–	–	–	–
GUATE96-2	–	–	–	–	–	–
GUATE96-3	–	–	–	–	–	–
GUATE96-4	–	–	–	–	–	–
GUATE97-1	–	–	–	–	–	–
GUATE97-2	–	–	–	–	–	–
GUATE97-3	–	–	–	V	–	–
GUATE97-4	–	–	–	–	–	–
GUATE97-5	–	S	–	–	–	–
GUATE98-1	–	–	–	–	–	–
GUATE98-2	–	–	–	–	–	–
GUATE98-3	R	–	–	–	L	–
GUATE98-4	–	–	S	–	–	T
GUATE98-5	–	–	–	–	–	–
GUATE98-6	R	–	–	–	–	–

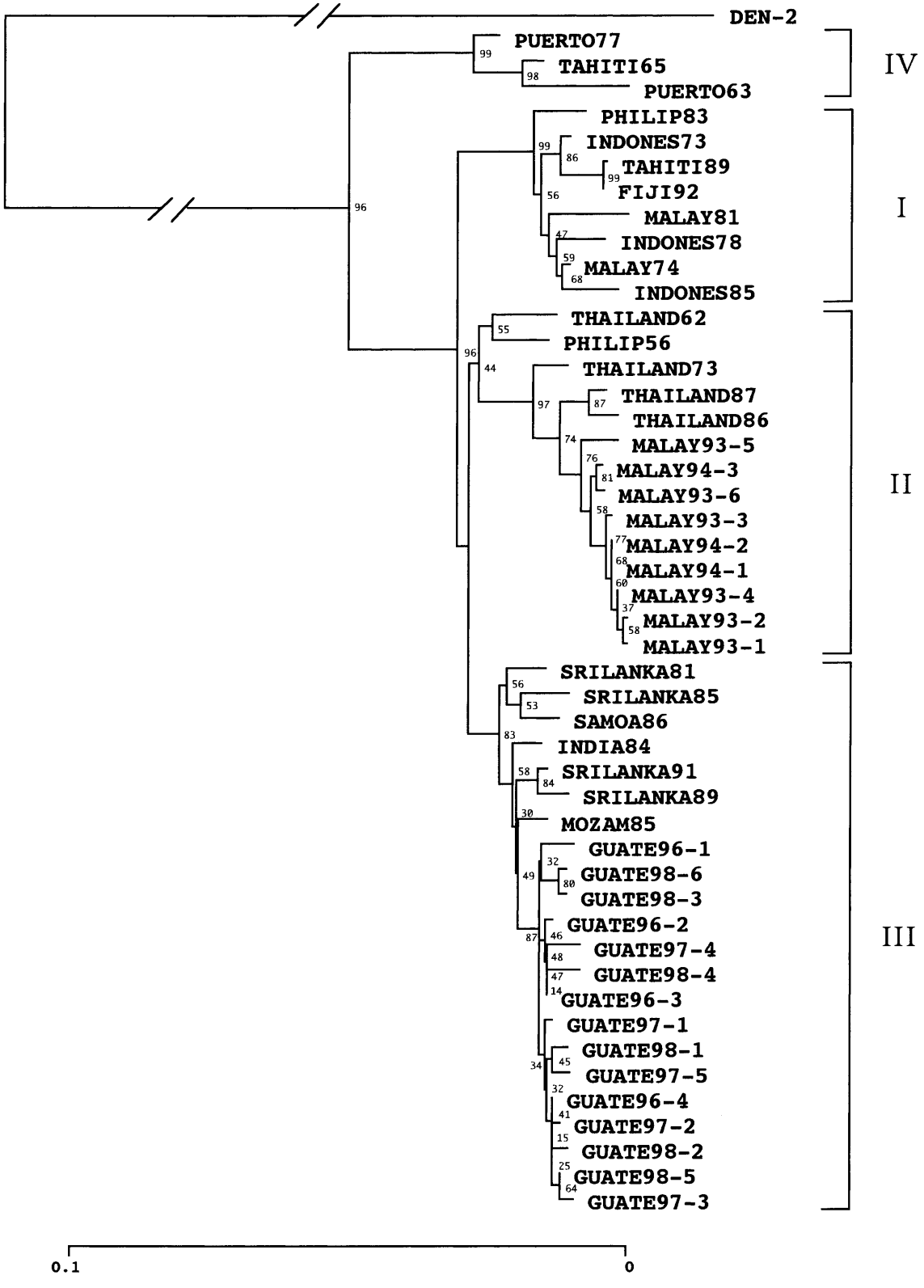
[a]The deduced amino acid sequences of C, C′, preM and M protein and part of the E protein from the fifteen DEN-3 isolates. Amino acids shown are those at positions where differences were detected

[b]Amino acid residues are numbered from the C protein region of the genomic RNA

[c]Indicates an amino acid identical with those found in the consensus sequence

to other Central American (Guatemala and El Salvador) and Caribbean countries (Puerto Rico) [5, 18, this study]. In Guatemala, we found that the change in the most prevalent dengue virus serotype from DEN-1/-4 to DEN-3 occurred in 1996, two years after the appearance of this subtype in Panama [18]. The rapid expansion of DEN-3 virus in Central America probably has two explanations. First, although eradication campaigns remarkably controlled the mosquito vector from 1960s, *Aedes aegypti* regained most of its pre-1930 domain within three decades [4]. Second, most Central Americans had not been infected and therefore did not have neutralizing antibody to DEN-3 [16].

In summary, we presented phylogenetic evidence that the recent DEN-3 epidemics in Central America were caused by imported viruses. The finding of this study, including sequence data for fifteen DEN-3 strains isolated in Guatemala, should be useful for molecular-epidemiological analysis of DEN-3 virus infections in tropical regions of the world.


DEN-2
PUERTO77
TAHITI65
PUERTO63
PHILIP83
INDONES73
TAHITI89
FIJI92
MALAY81
INDONES78
MALAY74
INDONES85
THAILAND62
PHILIP56
THAILAND73
THAILAND87
THAILAND86
MALAY93-5
MALAY94-3
MALAY93-6
MALAY93-3
MALAY94-2
MALAY94-1
MALAY93-4
MALAY93-2
MALAY93-1
SRILANKA81
SRILANKA85
SAMOA86
INDIA84
SRILANKA91
SRILANKA89
MOZAM85
GUATE96-1
GUATE98-6
GUATE98-3
GUATE96-2
GUATE97-4
GUATE98-4
GUATE96-3
GUATE97-1
GUATE98-1
GUATE97-5
GUATE96-4
GUATE97-2
GUATE98-2
GUATE98-5
GUATE97-3
IV
I
II
III
0.1
0

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References

1. Balmaseda A, Sandoval E, Perez L, Gutierrez CM, Harris E (1999) Application of molecular typing techniques in the 1998 dengue epidemic in Nicaragua. Am J Trop Med Hyg 61: 893–897
2. Deubel V, Kinney RM, Trent DW (1988) Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome. Virology 165: 234–244
3. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791
4. Halstead SB (1993) Global epidemiology of dengue: health systems in disarray. Trop Med 35: 137–146
5. Harris E, Sandoval E, Xet-Mull AM, Johnson M, Riley LW (1999) Rapid subtyping of dengue viruses by restriction site specific (RSS)-PCR. Virology 253: 86–95
6. Igarashi A (1978) Isolation of a Singh's Aedes albopictus cell clone sensitive to Dengue and Chikungunya viruses. J Gen Virol 40: 531–544
7. Igarashi A, Fujita N, Okuno Y, Oda T, Funahara Y, Shirahata A, Ikeuchi H, Hotta S, Wiharta AS, Sumarmo (1982) Isolation of dengue viruses from patients with dengue haemorrhagic fever (DHF) and those with fever of unknown origin (FUO) in Jakarta, Indonesia, in the year of 1981 and 1982. ICMR Ann (Kobe University) 2: 7–17
8. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111–120
9. Kobayashi N, Thayan R, Sugimoto C, Oda K, Saat Z, Vijayamalar B, Sinniah M, Igarashi A (1999) Type-3 dengue viruses responsible for the dengue epidemic in Malaysia during 1993–1994. Am J Trop Med Hyg 60: 904–909
10. Lanciotti RS, Lewis JG, Gubler DJ, Trent DW (1994) Molecular evolution and epidemiology of dengue-3 viruses. J Gen Virol 75: 65–75
11. Osatomi K, Sumiyoshi H (1990) Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology 176: 643–647
12. Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comp Appl Biosci 12: 357–358
13. Pan American Health Organization (1997) Re-emergence of Dengue in the Americas. Bull Pan Am Health Organ 18: 1–6

Fig. 1. Phylogenetic relationships among the fifteen Guatemalan and previously established DEN-3 isolates. Alignment of preM and M protein gene sequences and part of the E protein gene sequence determined in this study and previous studies [9, 10] generated 720-nucleotide sequences. An NJ tree was constructed from the sequences using the CLUSTAL W program [17]. The tree was rooted using a DEN-2 (Jamaica) [2] as the outgroup. The phylogenetic tree was visualized using TREEVIEW 1.6 [12]. The numbers at the nodes in the NJ tree indicate the bootstrap confidence levels that were obtained by 100 replicates [3]. The code for each sequence is shown in Table 3 and [9]. Subtypes are indicated to the right of the tree

14. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, Boshell J, de Mesa MT, Nogueira RM, da Rosa AT (1997) Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230: 244–251
15. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425
16. Scherer WF, Dickerman RW, Ordonez JV (1977) Serologic surveys for antibodies to western, eastern, California, and St. Louis encephalitis and dengue 3 arboviruses in Middle America, 1961–1975. Bull Pan Am Health Organ 11: 212–223
17. Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680
18. US Centers for Disease Control and Prevention (1995) Dengue type 3 infection-Nicaragua and Panama, October–November 1994. MMWR 44: 21–24
19. US Centers for Disease Control and Prevention (1998) Dengue outbreak associated with multiple serotypes – Puerto Rico, 1998. MMWR 47: 952–956

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