COMPARATIVE NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCE OF THE ENVELOPE GLYCOPROTEIN GENE AMONG THREE DENGUE VIRUS TYPE 2 STRAINS ISOLATED FROM PATIENTS WITH DIFFERENT DISEASE SEVERITIES IN MAHA SARAKHAM, NORTHEAST THAILAND

Sumlee Duangchanda[1,2], Mariko Tanaka[1], Kouichi Morita[1], Suntharee Rojanasuphot[2], and Akira Igarashi[1]

[1]Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852, Japan; [2]Virus Research Institute, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000, Thailand

Abstract. The nucleotide (nt) sequence of the envelope glycoprotein (E) gene of dengue virus type 2 was determined by the primer-extension dideoxy chain-termination method for 3 dengue virus type 2 (D2) strains which had been isolated from patients with dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), in Maha Sarakham, Northeast Thailand, in 1986-1987. Their nt sequences were essentially the same except for a single silent nt replacement in each DHF and DSS strain compared with DF strain. Therefore, these 3 strains possessed identical deduced amino acid (AA) sequences in their E protein. The result indicated that the primary structure of the E protein of D2 virus is not related to the clinical severity of the infected patients. Eleven nt replacements which resulted in 4 amino acid replacements were found to be unique to these 3 Northeast Thai strains. Sequence similarity showed that the 3 Northeast Thai strains were closest to the DSS isolate (H) followed by the DHF isolate (D) identified in Bangkok in 1980.

INTRODUCTION

Dengue viruses with 4 different serotypes (D1, D2, D3, D4) belong to the family Flaviviridae (Westaway *et al,* 1985), and are transmitted by mosquito bites, of which *Aedes aegypti* has been documented as the major vector species. Most of the dengue virus infected cases show acute febrile illness accompanied by joint-muscle pain and rash (DF), however, some patients manifest severe clinical symptom of hemorrhage (DHF) and shock (DSS) which is fatal if not properly treated (reviewed by Monath, 1986). Besides appearance of DHF/DSS, increasing numbers of patients and enlarged epidemic areas have currently made dengue virus infection a major health problem in many tropical countries including Thailand (Halstead, 1992). It has been proposed that immunopathological mechanisms play important roles in the development of DHF/DSS (Halstead, 1988). Particular attention has been paid to the immune enhancement of dengue virus growth in human monocytes by pre-existing non-neutralizing antibodies, which could be produced by the previous infection by heterologous dengue virus type in the case of secondary infection (Halstead, 1981), or could be conferred as maternal antibodies in the case of primary infection (Kliks *et al,* 1988). On the other hand, heterogeneity of epidemic dengue virus strains has been shown by serological tests and at the molecular level. However, it is not clear which genomic change may be related with the virulence of a particular strain. Recently, Morens *et al* (1991) reported that D2 strains isolated from severe cases multiplied better in human leukocyte cultures and their growth rate was elevated by enhancing antibodies compared with the strains isolated from mild cases. Because the E protein is the major antigenic component on the surface of dengue virion and D2 was shown to be the most frequently associated serotype with DHF/DSS in Thailand (Sangkawibha *et al,* 1980), we analyzed nt sequence of the E gene for three D2 strains which

*Correspondence: MS Sumlee Duangchanda, Virus Research Institute, Department of Medical Sciences, Ministry of Public Health, 88/7 Soi Bamrasnaradura Hospital, Amphur Muang, Nonthaburi 11000, Thailand.

had been isolated from DF, DHF and DSS cases in Maha Sarakham, Northeast Thailand. Although similar comparative sequence analysis has been reported for strains isolated in Bangkok Metropolitan area, none of the strains isolated in other parts of Thailand have so far been analyzed.

MATERIALS AND METHODS

Viruses

The D2 strains were isolated from patients hospitalized between 1986 and 1987, in Maha Sarakham Provincial Hospital, about 450 km northeast from Bangkok. The MK 42-86 strain was isolated from a DF case, MK 244-87 from a DHF case, and MK 116-87 from a DSS case, respectively. Each patient's clinical severity was classified according to WHO (1986) guidelines. These strains were isolated from patients' sera by inoculation into an *Aedes albopictus* cloned C6/36 cell line (Igarashi, 1978), determined for their serotypes by type-specific monoclonal antibodies (Henchal *et al,* 1982), and stored at -70°C at the Virus Research Institute, Department of Medical Sciences, Ministry of Public Health, Thailand. An aliquot of each strain was transferred to the Department of Virology, Institute of Tropical Medicine, Nagasaki University, Japan, and inoculated to C6/36 cells to prepare seed virus, which was aliquoted and stored at -70°C.

Preparation of virus RNA

Seed virus was first amplified by inoculation into stationary culture C6/36 cells in 500 ml bottles and infected culture fluid was harvested 6 days after incubation at 28°C. The amplified virus of 50 ml volume was then inoculated into spinner culture C6/36 cells of 1 liter volume and the infected culture fluid was collected 6 days after incubation at 28°C. Virions were concentrated by polyethylene glycol precipitation (6%) and purified by 30-50% sucrose gradient sedimentation (Srivastava *et al,* 1987). RNA was extracted from the purified virion with ISOGEN reagent (Molecular Research Center Inc) and resuspended in 40 µl distilled water.

Primers and sequence information

Six primers used in this study were synthesized by an Applied Biosystems DNA Synthesizer Model 392. Their sequences shown in Table 1 were selected according to the published sequences of D2 Jamaica and S2 strains. The purity of the product was examined by an ion exchange chromatography (Gen-pack, Waters). When more than 1% of incomplete oligomers were found, the product was discarded and the primer was newly synthesized. The sequence information on D2 strains was obtained from the following sources: Jamaica (Deubel *et al,* 1986); PR-159 (S1) (Hahn *et al,* 1988); New Guinea C and PUO-218 (Gruenber *et al,* 1988); M1, M2, and M3 (Samuel *et al,* 1989 a, b, c); 16681, D, F, G, H, a, b and c (Blok *et al,* 1989); TH-36 (Shiu *et al,* 1992).

Synthesis of cDNA from viral RNA

Gubler and Hoffman's method (1983) was used with some modifications. The virion RNA, approximately 10 µg, was heat-denatured at 90°C for 3 minutes in annealing buffer (100 mM Tris-HCl, pH 7.5, 50 mM EDTA, 500 mM KCl) containing 100 pmoles antisense primer D2-2474C (Table 1), followed by quenching on ice for 30 minutes. Then, 500 mM Tris-HCl, pH 8.3, 80 mM $MgCl_2$, 15 mM DTT, 20 mM dNTPs, 60 U reverse transcriptase (Life Science Inc), and 145 U RNase inhibitor (Takara) were added. The mixture was incubated at 42°C for 1 hour for the 1st strand cDNA synthesis, and extracted by phenol-chloroform. The 2nd strand cDNA was synthesized by adding 20 mM BNAD, 20 mM dNTPs, 100 mM DTT, 8 U RNase H (Takara), 20 U *Escherichia coli* ligase (Takara) and 28 U DNA polymerase I (Takara) in 5X buffer containing 250 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 50 mM ammonium sulfate, 250 mM KCl, and 250 µg/ml BSA. The mixture was incubated at 12°C for 1 hour, followed by 22°C for 1 hour, extracted with phenol-chloroform, and kept at -80°C until use. The double-stranded cDNA was blunt-ended by 2 U of T4 DNA polymerase (Takara) mixed with 20 mM dNTPs in 10X buffer containing 100 mM NaCl, 100 mM $MgCl_2$, 100 µg/ml BSA and 500 mM Tris-HCl, pH 7.9, by incubation at 37°C for 15 minutes, and extracted with phenol-chloroform. The size of the cDNA product was estimated by 0.9% agarose gel electrophoresis followed by ethidium bromide staining.

Cloning and nt sequencing

The double-stranded and blunt-ended cDNA

Table 1

Nt sequence of the D2 primers.

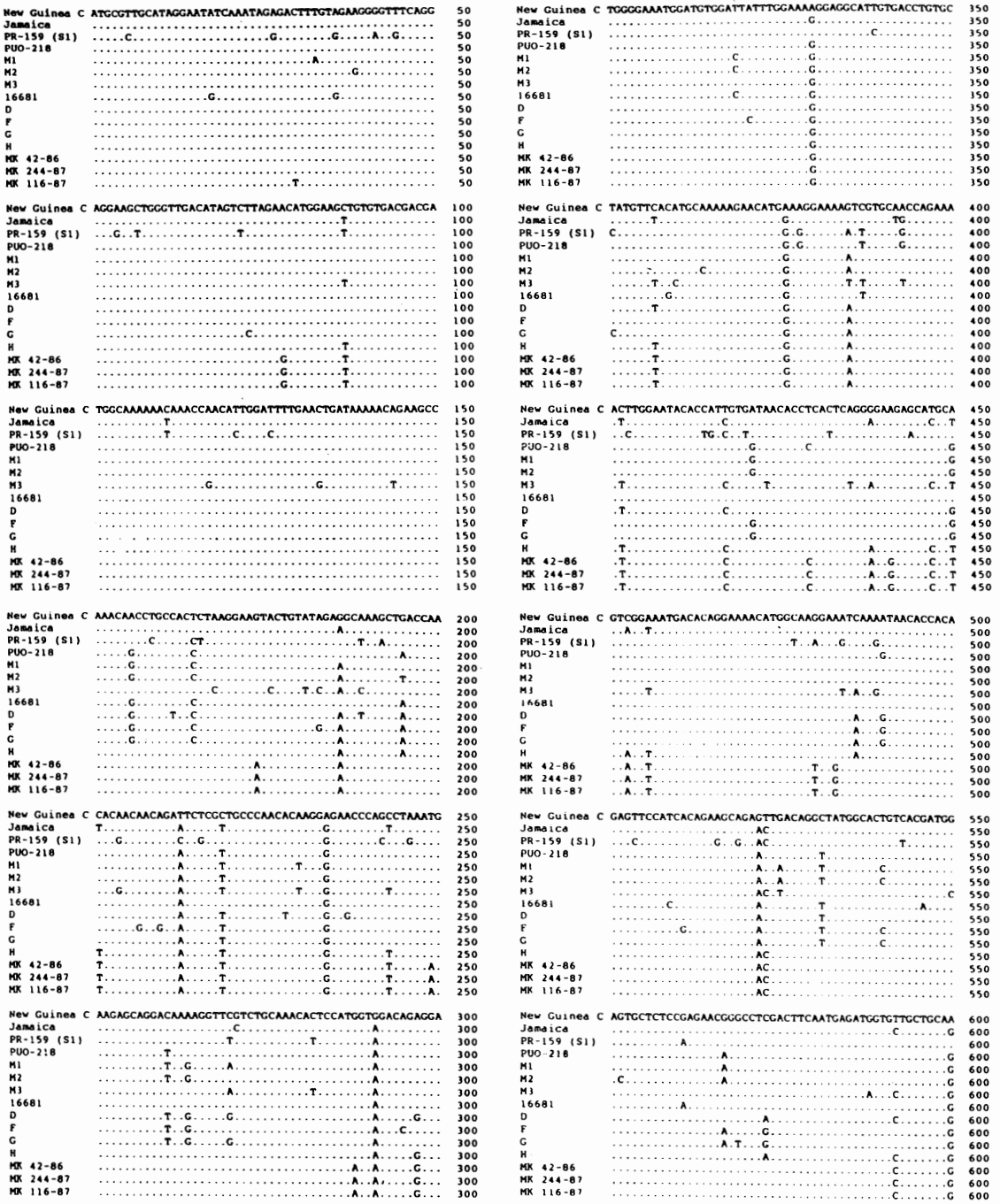
Code	Sequence (5′ − > 3′)	Position*
D2-810S	AACCTGGATCTTGAGACATC	829-810
D2-1120C	TAATCCACATCCATTTCC	1257-1120
D2-1535C	CCAAGCTTTGTCTTCCATCT	1555-1535
D2-1830C	TTCCTGTACACATAGAGTAT	1849-1830
D2-2143C	TGTGCACGTTATCTGTGATGAAGATCC	2163-2143
D2-2474C	TGTGCACGTTATCTGTGATGAAGATCC	2500-2474

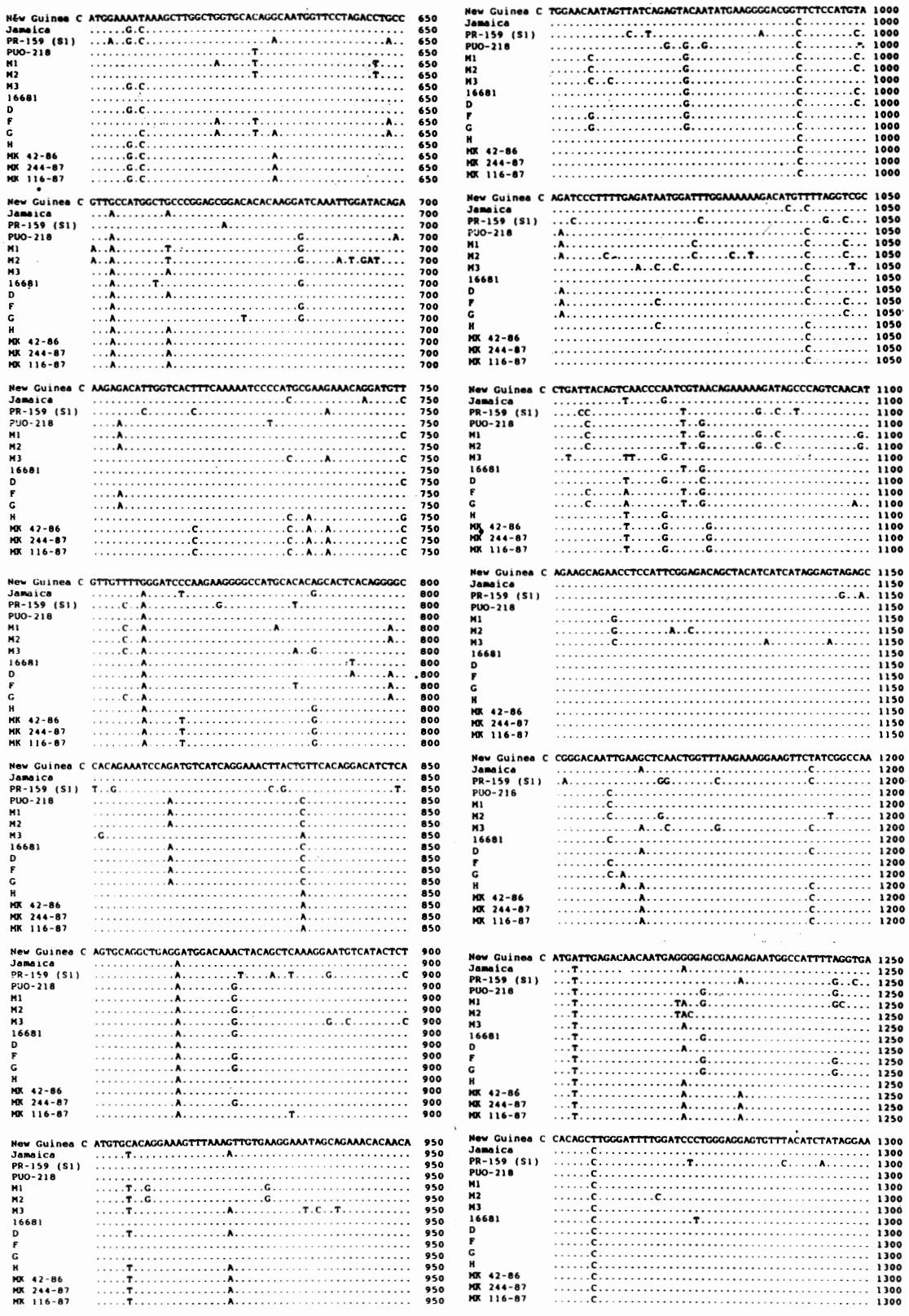
*Position of nt in entire D2 genome sequence

fragment was ligated to the pUC18-SmaI/BAP (Pharmacia) using DNA ligation kit (Takara). Resulting recombinant plasmid was used to transform *E. coli* strain JM 109 cells (Hanahan, 1983), and transformant colonies were screened by boiling methods (Sambrook *et al,* 1989). The nt sequence of the inserted cDNA was determined by the primer-extension dideoxy chain-termination method (Sanger *et al,* 1977) using the Taq Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc). Briefly, a mixture of 3.2 pmoles of each D2 primer (Table 1), 1 μg of cDNA template, 4 μl of 5X Terminator Ammonium Cycle Sequencing (TACS) buffer, each 1 μl fluorescent-labeled dideoxynucleotides (G, A, T, C), Dye-Deoxy Terminators and 4 U of Taq polymerase was prepared and overlaid with one drop of mineral oil. The reaction tubes were placed in a thermal cycler (Techne Ltd) preheated at 90°C, and subjected to a total 25 thermal cyclings (96°C 15 seconds, 50°C 1 second, and 60°C 4 minutes). Then, the excess Dye-Deoxy terminator was removed from the reaction mixture by filtration through a Quick Spin Column (Boehringer, Germany), and the specimen was dried in vacuum. The dried reaction product was dissolved in 6 μl each of a 5 : 1 mixture of deionized formamide and 50 mM EDTA, pH 8.0, heated at 90°C for 2 minutes before loading onto an Applied Biosystems DNA Sequencer model 373, according to the instruction manual. The obtained nt sequence was analyzed by DNASIS version 4.0 software (Hitachi Co, 1991) and converted into AA sequence.

RESULTS

Fig 1 shows the nt sequence of the E protein gene determined for MK 42-86 (DF strain), MK 244-87 (DHF strain), and MK 116-87 (DSS strain), in comparison with the nt sequence of New Guinea C strain as a reference. The 3 strains isolated in Mahasarakham, Northeast Thailand, possessed essentially identical nt sequences to each other, except that a single nt replacement was found for each DHF (A − > G at 873) and DSS (G − > T at 882) strain compared with the DF strain. Since both replacements were silent mutations, those deduced AA sequence of E protein was exactly identical for these 3 Northeast Thai strains, as shown in Fig 2. When the nt sequences of these 3 Northeast Thai strains were compared with the published sequences of other D2 strains, there were 11 unique nt replacements. Most of them (8/11) were transitions and resulted in 2 AA replacements (M − > I at 96 and T − > A at 359), and the remaining 7 transitional replacements were silent mutations (Table 2). On the other hand, two transversion replacements (T − > A at 249 and G − > T at 480) resulted in two AA replacements (N − > L at 83 amd K − > N at 160). Moreover, 5 other nt replacements, one of which resulted in an AA replacement (V − > A at 491), were shared by these 3 Northeast Thai strains and either one or both of the 2 other Thai strains isolated from DHF (D strain) or DSS (H strain) cases in Bangkok in 1980, as shown in Table 3 (Blok *et al,* 1989). Overall numbers of different nt and AA residues in the E protein gene sequence among the published D2 strains are summarized in Table 4. The results showed that the 3 Northeast Thai strains which were analyzed in this study possessed closest similarity to the H strain followed by the D strain and Jamaica strain. Sequence comparison also revealed that among 3 Malaysian


New Guinea C ATGCGTTGCATAGGAATATCAAATAGAGACTTTGTAGAAGGGGTTTCAGG 50
Jamaica 50
PR-159 (S1) 50
PUO-218 50
M1 50
M2 50
M3 50
16681 50
D 50
F 50
G 50
H 50
MK 42-86 50
MK 244-87 50
MK 116-87 50
New Guinea C AGGAAGCTGGGTTGACATAGTCTTAGAACATGGAAGCTGTGTGACGACGA 100
Jamaica 100
PR-159 (S1) 100
PUO-218 100
M1 100
M2 100
M3 100
16681 100
D 100
F 100
G 100
H 100
MK 42-86 100
MK 244-87 100
MK 116-87 100
New Guinea C TGGCAAAAAACAAACCAACATTGGATTTTGAACTGATAAAAACAGAAGCC 150
Jamaica 150
PR-159 (S1) 150
PUO-218 150
M1 150
M2 150
M3 150
16681 150
D 150
F 150
G 150
H 150
MK 42-86 150
MK 244-87 150
MK 116-87 150
New Guinea C AAACAACCTGCCACTCTAAGGAAGTACTGTATAGAGGCAAAGCTGACCAA 200
Jamaica 200
PR-159 (S1) 200
PUO-218 200
M1 200
M2 200
M3 200
16681 200
D 200
F 200
G 200
H 200
MK 42-86 200
MK 244-87 200
MK 116-87 200
New Guinea C CACAACAACAGATTCTCGCTGCCCAACACAAGGAGAACCCAGCCTAAATG 250
Jamaica 250
PR-159 (S1) 250
PUO-218 250
M1 250
M2 250
M3 250
16681 250
D 250
F 250
G 250
H 250
MK 42-86 250
MK 244-87 250
MK 116-87 250
New Guinea C AAGAGCAGGACAAAAGGTTCGTCTGCAAACACTCCATGGTGGACAGAGGA 300
Jamaica 300
PR-159 (S1) 300
PUO-218 300
M1 300
M2 300
M3 300
16681 300
D 300
F 300
G 300
H 300
MK 42-86 300
MK 244-87 300
MK 116-87 300
New Guinea C TGGGGAAATGGATGTGGATTATTTGGAAAAGGAGGCATTGTGACCTGTGC 350
Jamaica 350
PR-159 (S1) 350
PUO-218 350
M1 350
M2 350
M3 350
16681 350
D 350
F 350
G 350
H 350
MK 42-86 350
MK 244-87 350
MK 116-87 350
New Guinea C TATGTTCACATGCAAAAAGAACATGAAAGGAAAAGTCGTGCAACCAGAAA 400
Jamaica 400
PR-159 (S1) 400
PUO-218 400
M1 400
M2 400
M3 400
16681 400
D 400
F 400
G 400
H 400
MK 42-86 400
MK 244-87 400
MK 116-87 400
New Guinea C ACTTGGAATACACCATTGTGATAACACCTCACTCAGGGGAAGAGCATGCA 450
Jamaica 450
PR-159 (S1) 450
PUO-218 450
M1 450
M2 450
M3 450
16681 450
D 450
F 450
G 450
H 450
MK 42-86 450
MK 244-87 450
MK 116-87 450
New Guinea C GTCGGAAATGACACAGGAAAACATGGCAAGGAAATCAAAATAACACCACA 500
Jamaica 500
PR-159 (S1) 500
PUO-218 500
M1 500
M2 500
M3 500
16681 500
D 500
F 500
G 500
H 500
MK 42-86 500
MK 244-87 500
MK 116-87 500
New Guinea C GAGTTCCATCACAGAAGCAGAGTTGACAGGCTATGGCACTGTCACGATGG 550
Jamaica 550
PR-159 (S1) 550
PUO-218 550
M1 550
M2 550
M3 550
16681 550
D 550
F 550
G 550
H 550
MK 42-86 550
MK 244-87 550
MK 116-87 550
New Guinea C AGTGCTCTCCGAGAACGGGCCTCGACTTCAATGAGATGGTGTTGCTGCAA 600
Jamaica 600
PR-159 (S1) 600
PUO-218 600
M1 600
M2 600
M3 600
16681 600
D 600
F 600
G 600
H 600
MK 42-86 600
MK 244-87 600
MK 116-87 600


New Guinea C ATGGAAAATAAAGCTTGGCTGGTGCACAGGCAATGGTTCCTAGACCTGCC 650
Jamaica ......G.C..... 650
PR-159 (S1) ...A..G.C.....A.....A.. 650
PUO-218 .....T..... 650
M1 .....A....T.....T.... 650
M2 .....T.....T.... 650
M3 ......G.C..... 650
16681 650
D ......G.C..... 650
F .....A....T.....A.. 650
G .....C.....A....T..A.....A.. 650
H ......G.C..... 650
MK 42-86 ......G.C.....A..... 650
MK 244-87 ......G.C.....A..... 650
MK 116-87 ......G.C.....A..... 650
New Guinea C GTTGCCATGGCTGCCCGGAGCGGACACACAAGGATCAAATTGGATACAGA 700
Jamaica ...A.....A..... 700
PR-159 (S1) .....A..... 700
PUO-218 ...A.....G.....A. 700
M1 A..A.....T.....G..... 700
M2 A..A.....T.....G.....A.T.GAT.... 700
M3 ...A.....A..... 700
16681 ...A.....T.....G..... 700
D ...A.....A..... 700
F ...A.....G..... 700
G ...A.....T.....G..... 700
H ...A.....A..... 700
MK 42-86 ...A.....A..... 700
MK 244-87 ...A.....A..... 700
MK 116-87 ...A.....A..... 700
New Guinea C AAGAGACATTGGTCACTTTCAAAAATCCCCATGCGAAGAAACAGGATGTT 750
Jamaica .....C.....A.....C 750
PR-159 (S1) .....C.....C.....A..... 750
PUO-218 ....A.....T..... 750
M1 ....A.....C 750
M2 ....A..... 750
M3 .....C.....A.....C 750
16681 750
D .....C 750
F ....A..... 750
G ....A..... 750
H .....C..A.....G 750
MK 42-86 .....C.....C..A..A.....C 750
MK 244-87 .....C.....C..A..A.....C 750
MK 116-87 .....C.....C..A..A.....C 750
New Guinea C GTTGTTTTGGGATCCCAAGAAGGGGCCATGCACACAGCACTCACAGGGGC 800
Jamaica .....A.....T.....G..... 800
PR-159 (S1) .....C..A.....G.....T..... 800
PUO-218 .....A..... 800
M1 .....C..A.....A.....A.. 800
M2 .....C..A.....A.. 800
M3 .....C..A.....A..G..... 800
16681 .....A.....T..... 800
D .....A.....A.....A.. 800
F .....A.....T.....A.. 800
G .....C..A.....A.. 800
H .....A.....G..... 800
MK 42-86 .....A.....T.....G..... 800
MK 244-87 .....A.....T.....G..... 800
MK 116-87 .....A.....T.....G..... 800
New Guinea C CACAGAAATCCAGATGTCATCAGGAAACTTACTGTTCACAGGACATCTCA 850
Jamaica 850
PR-159 (S1) T..G.....C.G.....T. 850
PUO-218 .....A.....C..... 850
M1 .....A.....C..... 850
M2 .....A.....C..... 850
M3 .G.....A..... 850
16681 .....A.....C..... 850
D .....A.....C..... 850
F .....A.....C..... 850
G .....A.....C..... 850
H .....A..... 850
MK 42-86 .....A..... 850
MK 244-87 .....A..... 850
MK 116-87 .....A..... 850
New Guinea C AGTGCAGGCTGAGGATGGACAAACTACAGCTCAAAGGAATGTCATACTCT 900
Jamaica .....A..... 900
PR-159 (S1) .....A.....T....A..T.....G.....C 900
PUO-218 .....A.....G..... 900
M1 .....A.....G..... 900
M2 .....A.....G..... 900
M3 .....A.....G.....G..C.....C 900
16681 .....A.....G..... 900
D .....A..... 900
F .....A.....G..... 900
G .....A.....G..... 900
H .....A..... 900
MK 42-86 .....A..... 900
MK 244-87 .....A.....G..... 900
MK 116-87 .....A.....T..... 900
New Guinea C ATGTGCACAGGAAAGTTTAAAGTTGTGAAGGAAATAGCAGAAACACAACA 950
Jamaica .....T.....A..... 950
PR-159 (S1) 950
PUO-218 950
M1 .....T..G.....G..... 950
M2 .....T..G.....G..... 950
M3 .....T.....A.....T.C..T..... 950
16681 950
D .....T.....A..... 950
F 950
G 950
H .....T.....A..... 950
MK 42-86 .....T.....A..... 950
MK 244-87 .....T.....A..... 950
MK 116-87 .....T.....A..... 950
New Guinea C TGGAACAATAGTTATCAGAGTACAATATGAAGGGGACGGTTCTCCATGTA 1000
Jamaica .....C..... 1000
PR-159 (S1) .....C..T.....A.....C.....C. 1000
PUO-218 .....G..G..G.....C.....C. 1000
M1 .....C.....G.....C.....C. 1000
M2 .....C.....G.....C.....C. 1000
M3 .....C..C.....G.....C..... 1000
16681 .....G.....C.....C. 1000
D .....G.....C.....C. 1000
F .....G.....G.....C..... 1000
G .....G.....G.....C..... 1000
H .....C..... 1000
MK 42-86 .....C..... 1000
MK 244-87 .....C..... 1000
MK 116-87 .....C..... 1000
New Guinea C AGATCCCTTTTGAGATAATGGATTTGGAAAAAAGACATGTTTTAGGTCGC 1050
Jamaica .....C..C..... 1050
PR-159 (S1) ...C.....C.....G..C... 1050
PUO-218 .A.....C..... 1050
M1 .A.....C.....C.....C... 1050
M2 .A.....C.....C..T.....C.....C... 1050
M3 .....A..C..C.....C.....T.. 1050
16681 .....C..... 1050
D .A.....C..... 1050
F .A.....C.....C.....C... 1050
G .A.....C... 1050
H .....C.....C..... 1050
MK 42-86 .....C..... 1050
MK 244-87 .....C..... 1050
MK 116-87 .....C..... 1050
New Guinea C CTGATTACAGTCAACCCAATCGTAACAGAAAAAGATAGCCCAGTCAACAT 1100
Jamaica .....T.....G..... 1100
PR-159 (S1) ....CC.....T.....G..C..T..... 1100
PUO-218 .....C.....T..G..... 1100
M1 .....C.....T..G.....G..C.....G. 1100
M2 .....C.....T..G.....G..C.....G. 1100
M3 ..T.....TT....G..... 1100
16681 .....T..G..... 1100
D .....T.....G.....C..... 1100
F .....C.....A.....T..G..... 1100
G .....C.....A.....T..G.....A.. 1100
H .....T.....G..... 1100
MK 42-86 .....T.....G.....G..... 1100
MK 244-87 .....T.....G.....G..... 1100
MK 116-87 .....T.....G.....G..... 1100
New Guinea C AGAAGCAGAACCTCCATTCGGAGACAGCTACATCATCATAGGAGTAGAGC 1150
Jamaica 1150
PR-159 (S1) .....G..A. 1150
PUO-218 1150
M1 .....G..... 1150
M2 .....G.....A..C..... 1150
M3 .....C.....A.....A..... 1150
16681 1150
D 1150
F 1150
G 1150
H 1150
MK 42-86 1150
MK 244-87 1150
MK 116-87 1150
New Guinea C CGGGACAATTGAAGCTCAACTGGTTTAAGAAAGGAAGTTCTATCGGCCAA 1200
Jamaica .....A.....C..... 1200
PR-159 (S1) .A.....GG.....C.....C..... 1200
PUO-218 .....C..... 1200
M1 .....C..... 1200
M2 .....C.....G.....T..... 1200
M3 .....A...C.....G.....C..... 1200
16681 .....C..... 1200
D .....A.....C..... 1200
F .....C..... 1200
G .....C.A..... 1200
H .....A..A.....C..... 1200
MK 42-86 .....A.....C..... 1200
MK 244-87 .....A.....C..... 1200
MK 116-87 .....A.....C..... 1200
New Guinea C ATGATTGAGACAACAATGAGGGGAGCGAAGAGAATGGCCATTTTAGGTGA 1250
Jamaica ...T..... .....A..... 1250
PR-159 (S1) ...T.....A.....G..C.. 1250
PUO-218 ...T.....G.....G..... 1250
M1 ...T.....TA..G.....GC.... 1250
M2 ...T.....TAC..... 1250
M3 ...T.....A..... 1250
16681 ...T.....G..... 1250
D ...T.....A..... 1250
F ...T.....G.....G..... 1250
G ...T.....G.....G..... 1250
H ...T.....A..... 1250
MK 42-86 ...T.....A.....A..... 1250
MK 244-87 ...T.....A.....A..... 1250
MK 116-87 ...T.....A.....A..... 1250
New Guinea C CACAGCTTGGGATTTTGGATCCCTGGGAGGAGTGTTTACATCTATAGGAA 1300
Jamaica .....C..... 1300
PR-159 (S1) .....C.....T.....C.....A..... 1300
PUO-218 .....C..... 1300
M1 .....C..... 1300
M2 .....C.....C..... 1300
M3 .....C..... 1300
16681 .....C.....T..... 1300
D .....C..... 1300
F .....C..... 1300
G .....C..... 1300
H .....C..... 1300
MK 42-86 .....C..... 1300
MK 244-87 .....C..... 1300
MK 116-87 .....C..... 1300

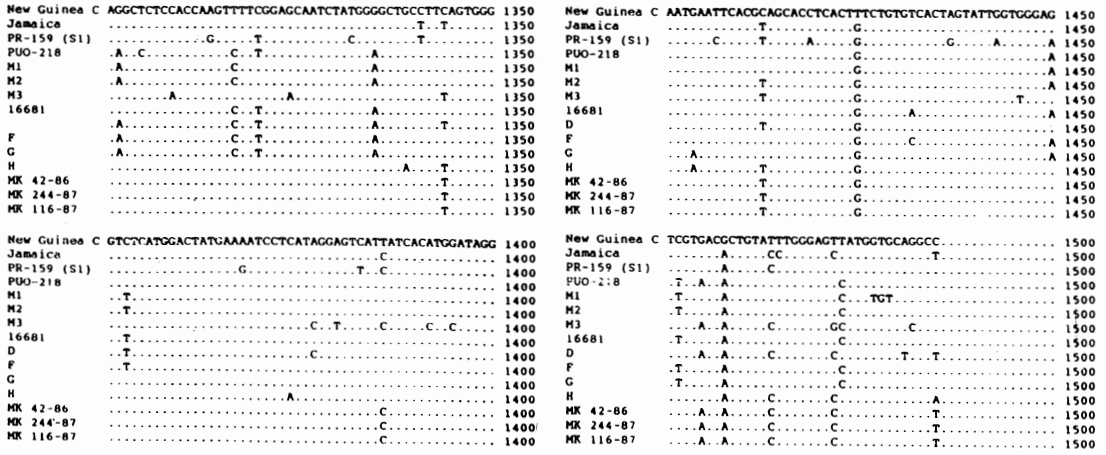


Fig 1—Nt sequence of the E protein gene of D2 strains. Abbriviation of strains and sources of the sequence informations was shown in the Materials and Methods.

strains, the M3 isolated from a DF possessed the highest similarity to the 3 Northeast Thai strains.

DISCUSSION

Heterogeniety of the epidemic D2 strains at the molecular level has been analyzed by oligonucleotide fingerprinting (Trent *et al,* 1983, 1989), cDNA-RNA hybridization (Blok *et al,* 1984), antigen signature analysis (Monath *et al,* 1986), hybridization using synthetic deoxyoligonucleotides (Kerschner *et al,* 1986), restriction enzyme mapping (Walker *et al,* 1988), and more recently by nt sequencing (Blok *et al,* 1989; Rico-Hesse, 1990). These studies demonstrated that D2 strains could be classified into several geographical types (or topotypes). However, it is still not clear whether any particular molecular changes are associated with the pathogenicity of DHF/DSS. Comparative sequence analysis of the E protein gene of several D2 strains by Blok *et al* (1989) indicated that certain AA replacements, such as I at 308 and A at 491, were observed in 2 DHF/DSS strains (D and H), while V was found at these positions in remaining 2 DHF/DSS and 6 DF strains. In our analysis, all 3 Northeast Thai strains possessed I at 308 and A at 491, indicating that these AA replacements may be characteristic of certain local strains but not of DHF/DSS strains. The fact that a Malaysian DF strain, M3 and jamaica strains, also possessed I at 308 supports this possibility. Therefore, it is difficult to imagine that a certain AA replacement in the E protein is related with severe or mild clinical manifestations of the D2 virus-infected patients. If the clinical severity of the D2 infection depends on the growth capacity and the enhanceability of each viral strain in human leukocyte culture, (Morens *et al,* 1991), such biological characteristics may be determined by viral gene sequences other than that of the E protein gene. All 3 Northeast Thai D2 strains which were analyzed in our study had conserved potential glycosylation sites (NTT at 67-69; NDT at 153-155), as well as conserved type-specific hypervariable domain (GADTQGSN at 223-230; Shiu *et al,* 1992). Among 4 AA replacements unique to the Northeast Thai strains, 2 mutations (N − > K at 83; M − > I at 96) were present in the R1 region (AA 1-122) which was postulated by Nowak and Wengler (1987) for West Nile flavivirus. While the remaining mutations (K − > N at 160; T − > A at 359) exist in L1 and L2 loops, respectively, the implication of these AA replacements for the antigenicities or biological characteristics of D2 strains is unknown. However, they may elicit strain-specific antibody responses which could be observed only in certain epidemic regions like Northeast Thailand. Another AA mutation (V − > A at 491) was shared by these 3 Northeast and 2 other Bangkok strains isolated in Thailand, but may not exert so much effect on the antigenicities, because it is present in the transmembrane domain.

ACKNOWLEDGEMENTS

The first author was supported by the Japan

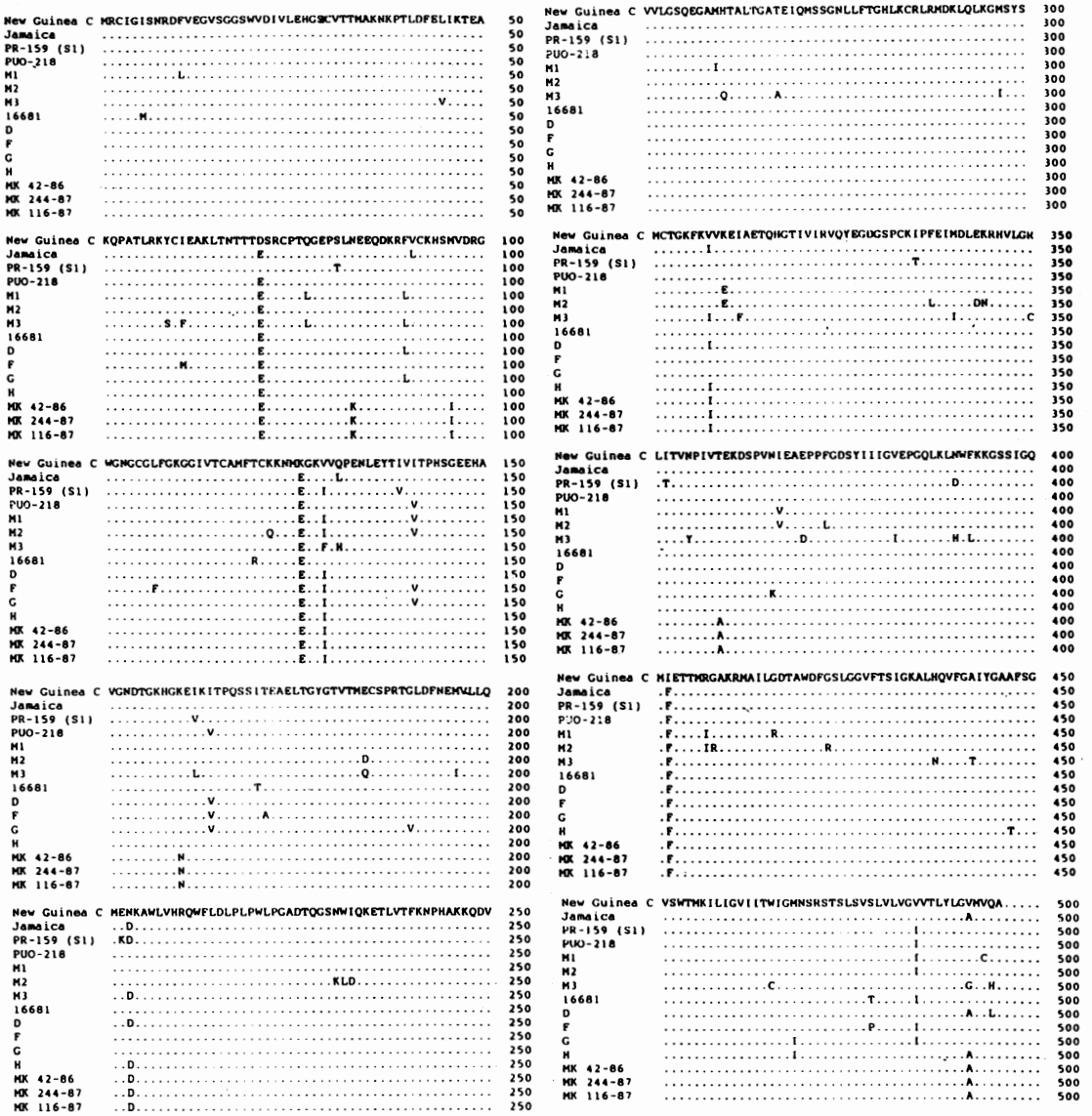


Fig 2—AA sequences of the E proteins of D2 strains. AA sequences were deduced from the nt sequences in Fig 1.

International Cooperation Agency (JICA) for her travel and stay in Japan under the Project for the Promotion of Medical Cooperation at the National Institute of Health of Thailand, in the fiscal year 1992. A part of this study was supported by the Special Research Grant for the Institute of Tropical Medicine, Nagasaki University, in the fiscal year 1992.

REFERENCES

Blok J, Henchal EA, Gorman MB. Comparison of dengue viruses and some other flaviviruses by cDNA-RNA hybridization analysis and detection of a close relationship between dengue virus serotype 2 and Edge Hill virus. *J Gen Virol* 1984; 65 : 2173-81.

Table 2

Nt and AA replacements unique to 3 northeast Thai D2 strains.

Nt	Position*	AA	Position*
A->G	79	-	
G->A	174	-	
T->A	249**	N->K	83
G->A	288	M->I	96
T->C	429	-	
A->G	441	-	
C->A	453**	-	
G->T	480**	K->N	160
A->G	483	-	
C->T	765	-	
A->G	1,075	T->A	359

* Position in the E protein gene (nt), or E protein (AA)
** Transversion, other replacements are transitions

Table 3

Nt and AA replacements unique to 3 northeast and 2 Bangkok D2 strains.

Nt	Position*	AA	Position*	Bangkok D2 strains
C->T	201	-		H
A->G	297	-		D, H
G->A	735	-		H
T->A**	1472	V->A	491	D, H
C->T	1485			D

* Position in the E protein gene (nt), or E protein (AA)
** Transversion, other replacements are transitions

Blok J, Samuel S, Gibbs AJ, Vitarana UT. Variation of the nucleotide and encoded amino acid sequences of the envelope gene from eight dengue-2 viruses. *Arch Virol* 1989; 105 : 39-53.

Deubel V, Kinney RM, Trent DW. Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue 2 virus, Jamaica genotype. *Virology* 1986; 155 : 365-77.

Gruenberg A, Woo WS, Biedrzycka A, Wright PJ. Partial nucleotide sequence and deduced amino acid sequences of the structural proteins of dengue virus type 2, New Guinea C and PUO-218 strains. *J Gen Virol* 1988; 69 : 1391-8.

Gubler U, Hoffmann BJ. A simple and very efficient method for generating cDNA libraries. *Gene* 1983; 25 : 263.

Halstead SB. The pathogenesis of dengue: molecular epidemiology of infectious disease. *Am J Epidemiol* 1981; 114 : 632-48.

Halstead SB. Pathogenesis of dengue; challenge to molecular biology. *Science* 1988; 239 : 476-81.

Halstead SB. The XXth century dengue pandemic: need for surveillance and research. *World Health Stat Q* 1992; 45 : 292-8.

Hahn YS, Galler R, Hunkapillar T, Dalrymple JM, Strauss JH, Strauss EG. Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. *Virology* 1988; 162 : 167-80.

Hanahan D. Studies on translation of *E. coli* with plasmid. *J Mol Biol* 1983; 166 : 557-80.

Henchal EA, Gentry MK, McCown JM, Brandt WE. Dengue virus specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *Am J Trop Med Hyg*

Table 4
Number of different nt in E protein gene and AA in E protein among different D2 strains.

Strain	New Guinea C	Jamaica	PR-159 (S1)	PUO-218	M1	M2	M3	16681	D	F	G	H	MK 42-86	MK 244-87	MK 116-87
New Guinea C	-	59	121	57	79	86	107	49	71	69	69	61	69	70	71
Jamaica	8	-	133	79	94	100	72	76	53	86	90	24	27	28	29
PR-159 (S1)	12	14	-	137	154	160	164	130	146	147	140	141	135	136	135
PUO-218	6	8	12	-	48	57	115	34	61	32	34	80	84	83	87
M1	15	17	19	11	-	31	121	56	71	43	44	94	99	98	101
M2	20	22	24	16	17	-	135	63	78	55	57	99	106	105	108
M3	31	27	34	30	35	43	-	111	86	120	119	70	76	75	78
16681	8	10	14	6	15	20	33	-	62	44	49	76	83	82	85
D	10	6	14	10	15	22	26	12	-	60	60	46	55	56	57
F	11	13	15	7	15	19	34	10	11	-	22	82	94	93	96
G	11	13	15	7	12	19	33	11	9	8	-	80	94	93	96
H	9	5	12	9	16	21	28	11	5	12	10	-	26	27	28
MK 42-86	11	7	15	11	18	23	30	13	7	14	14	6	-	1	2
MK 244-87	11	7	15	11	18	23	30	13	7	14	14	6	0	-	3
MK 116-87	11	7	15	11	18	23	30	13	7	14	14	6	0	0	-

Numbers above the diagonal represent the nt, and those below the diagonal the AA, respectively. Abbreviation of the strains: see Materials and Methods in the text.

1982; 31 : 830-6.

Igarashi A. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. *J Gen Virol* 1978; 40 : 531-44.

Kerschner JH, Vorndam V, Monath TP, Trent DW. Genetic and epidemiological studies of dengue type 2 viruses by hybridization using synthetic deoxyoligonucleotides as probes. *J Gen Virol* 1986; 69 : 2645-61.

Kliks SC, Nimmannitya S, Nisalak A, Burke DS. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. *Am J Trop Med Hyg* 1988; 38 : 411-9.

Morens DM, Marchettes NJ, Chu MC, Halstead SB. Growth of dengue type 2 virus isolates in human peripheral blood leukocytes correlates with severe and mild dengue disease. *Am J Trop Med Hyg* 1991; 45 : 644-51.

Monath TP. Pathology of flaviviruses. In: S Schlesinger, MA Schlesinger, eds. The Togaviridae and Flaviviridae. New York: Plenum Press 1986: pp 375-440.

Monath TP, Wands JR, Hill IJ, *et al.* Geographic classification of dengue-2 virus strains by antigen signature analysis. *Virology* 1986; 154 : 313-24.

Nowak T, Wengler G. Analysis of disulfides present in the membrane proteins of the West Nile flavivirus. *Virology* 1987; 156 : 127-37.

Rico-Hesse R. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology* 1990; 174 : 479-93.

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. In: C Nolan, ed. A laboratory manual, 2nd ed. New York : Cold Spring Harbor Laboratory Press, 1989.

Samuel S, Koh CL, Blok J, Pang T, Lam SK. Nucleotide sequence of the envelope protein gene of a Malaysian dengue-2 virus isolated from a patient with dengue haemorrhagic fever. *Nucleic Acid Res* 1989a; 17 : 8875.

Samuel S, Koh CL, Blok J, Pang T, Lam SK. Nucleotide sequence of the envelope protein gene of a Malaysian dengue-2 virus isolated from a patient with dengue fever. *Nucleic Acid Res* 1989b; 17 : 8887.

Samuel S, Koh CL, Blok J, Pang T, Lam SK. Nucleotide sequence of the envelope protein gene of a Malaysian dengue-2 virus isolated from a patient with dengue shock syndrome. *Nucleic Acid Res* 1989c; 17 : 8888.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhabitors. *Proc Natl Acad Sci* USA 1977; 74 : 5463-7.

Sangkawibha N, Rojanasuphot S, Ahandrik S, *et al.* Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreask. *Am J Epidemiol* 1980; 120 : 653-69.

Shiu SYW, Jiang WR, Porterfield JS, Gould EA. Envelope protein sequences of dengue virus isolates TH-36 and TH-Sman, and identification of a type-specific genetic marker for dengue and tick-borne flaviviruses. *J Gen Virol* 1992; 73 : 207-12.

Srivastava AK, Aira Y, Mori C, Kobayashi Y, Igarashi A. Antigenicity of Japanese encephalitis virus envelope glycoprotein V3 (E) and its cyanogen bromide cleaved fragments examined by monoclonal antibodies and western blotting. *Arch Virol* 1987; 96 : 97-107.

Trent DW, Grant JA, Rosen L, Monath TP. Genetic variation among dengue 2 viruses of different geographic origin. *Virology* 1983; 128 : 271-84.

Trent DW, Grant JA, Monath TP, Monske CL, Corina M, Fox GE. Genetic variation and microevolution of dengue 2 virus in southeast Asia. *Virology* 1989; 172 : 523-35.

Walker DJ, Henchal EA, Blok J, *et al.* Variation in dengue type 2 viruses isolated in Bangkok during 1980. *J Gen Virol* 1988; 69 : 591-602.

Westaway EG, Brinton MA, Gaidamovich SYA, *et al.* Flaviviridae. *Intervirology* 1985; 24 : 183-92.

World Health Organization. Dengue haemorrhagic fever: diagnosis, treatment and control. Geneva, WHO, 1986.