Am. J. Trop. Med. Hyg., 60(6), 1999, pp. 904–909

TYPE-3 DENGUE VIRUSES RESPONSIBLE FOR THE DENGUE EPIDEMIC IN MALAYSIA DURING 1993–1994

NOBUYOSHI KOBAYASHI, RAVINDRAN THAYAN, CHIE SUGIMOTO, KAZUMASA ODA, ZAINAH SAAT, BALASUBRAMANIAM VIJAYAMALAR, MANGALAM SINNIAH, AND AKIRA IGARASHI

Division of Microbiology, Yokohama City Institute of Health, Yokohama, Japan; Division of Virology, Institute for Medical Research, Kuala Lumpur, Malaysia; Department of Viral Infection, Institute of Medical Science, University of Tokyo, Tokyo, Japan; Japan International Cooperation Agency, Tokyo, Japan; Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

Abstract. To characterize the dengue epidemic that recently occurred in Malaysia, we sequenced cDNAs from nine 1993–1994 dengue virus type-3 (DEN-3) isolates in Malaysia (DEN-3 was the most common type in Malaysia during this period). Nucleic acid sequences (720 nucleotides in length) from the nine isolates, encompassing the precursor of membrane protein (preM) and membrane (M) protein genes and part of the envelope (E) protein gene were aligned with various reference DEN-3 sequences to generate a neighbor-joining phylogenetic tree. According to the constructed tree, the nine Malaysian isolates were grouped into subtype II, which comprises Thai isolates from 1962 to 1987. Five earlier DEN-3 virus Malaysian isolates from 1974 to 1981 belonged to subtype I. The present data indicate that the recent dengue epidemic in Malaysia was due to the introduction of DEN-3 viruses previously endemic to Thailand.

Dengue viruses (members of the family Flaviviridae) are the causative agents of dengue fever (DF) and dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) in humans. In endemic areas, dengue viruses are maintained by the transmission cycle involving *Aedes* mosquitoes and humans.[1] Dengue viruses are classified into four antigenically distinct serotypes.

Recently, a phylogenetic method based on nucleic acid sequence data has been used for molecular epidemiology of dengue viruses.[2–5] The genomes of dengue viruses are linear, plus-stranded ssRNAs of about 11 kilobases in length. The 5′ end of the genome encodes structural proteins, including capsid proteins (C, C′), the precursor of membrane protein (preM), a membrane protein (M), and an envelope protein (E). Sequences within this genome region have been targeted for amplification by reverse transcriptase–polymerase chain reaction (RT-PCR) followed by direct sequencing of the amplified fragments. A phylogenetic tree constructed from the obtained sequence data has allowed the intra-serotypic subdivision of dengue viruses.

In Malaysia, the first outbreak of DHF was documented in 1962, and was followed by major outbreaks of DF and DHF in 1974, 1978, 1982, and 1990.[6] During interepidemic periods, significant numbers of DF and DHF cases have been reported.[6] The 1990 epidemic in Malaysia was quite extensive and lasted for several years (1990–1994). During the early period of this epidemic (1990–1991), type 2 dengue was the most common serotype, but type 3 dengue (DEN-3) was predominantly detected during the recent period (1992–1994). Using a phylogenetic method, we attempted to elucidate the origin of DEN-3 viruses recovered in Malaysia in 1993 and 1994.

MATERIALS AND METHODS

Viruses. The origins of the nine DEN-3 viruses sequenced in this study are shown in Table 1. They were isolated from the sera of hospitalized patients with DF or DHF, using an *Aedes albopictus* cell line (C6/36).[7,8] The serotyping of the isolates was performed by indirect immunoperoxidase staining using monoclonal antibodies against individual serotypes.

Extraction of viral RNA. Infected C6/36 cells were harvested 5–7 days postinfection, and the recovered culture fluids were used to extract the viral RNA using a single-step RNA isolation kit (Isogen™; Wako Pure Chemicals, Osaka, Japan).

Reverse transcriptase–polymerase chain reaction. The oligonucleotides used as primers are shown in Table 2. They were synthesized by Nippon Flour Mills (Atsugi, Japan). A genomic region from nucleotides 95 to 1,144 that encodes the C, C′, preM, and M proteins and part of the E protein was amplified by the RT-PCR essentially as described previously.[9–11] A reaction mixture of 100 μl contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 5 mM dithiothreitol, 38 units of the human placental ribonuclease inhibitor (Takara Shuzo, Ohtsu, Japan), 200 μM each of four dNTPs, 50 pM of each primer, 8 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and five units of *Taq* DNA polymerase (Promega). The reaction was performed in a thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) programmed to incubate for 10 min at 53°C and then to proceed with 35 cycles of the following profile: 92°C for 1 min, 53°C for 1.5 min, and 72°C for 1 min.

cDNA sequencing. The amplified fragments were purified using low temperature–melting agarose and a Geneclean II kit (Bio 101, La Jolla, CA) and sequenced using a Taq dye terminator-cycle sequencing kit (Applied Biosystems, Foster, CA, USA) and a Model 373A DNA sequencer (Applied Biosystems).

Nucleic acid and protein sequence analysis. The computer analysis of nucleic acid sequence data was performed using DNASIS Software version 2.4 (Software Development, Tokyo, Japan).

Phylogenetic analysis. A neighbor-joining phylogenetic tree[12] was constructed using the CLUSTAL W program.[13] Divergences were estimated by the two-parameter method.[14] A phylogenetic tree was visualized using the TREEVIEW 1.4 program.[15] The bootstrap test was applied to estimate the confidence of the branching patterns of the neighbor-joining tree.[16]

RESULTS

Variation in the nucleic acid and deduced amino acid sequences. We isolated 22 dengue viruses in various sites

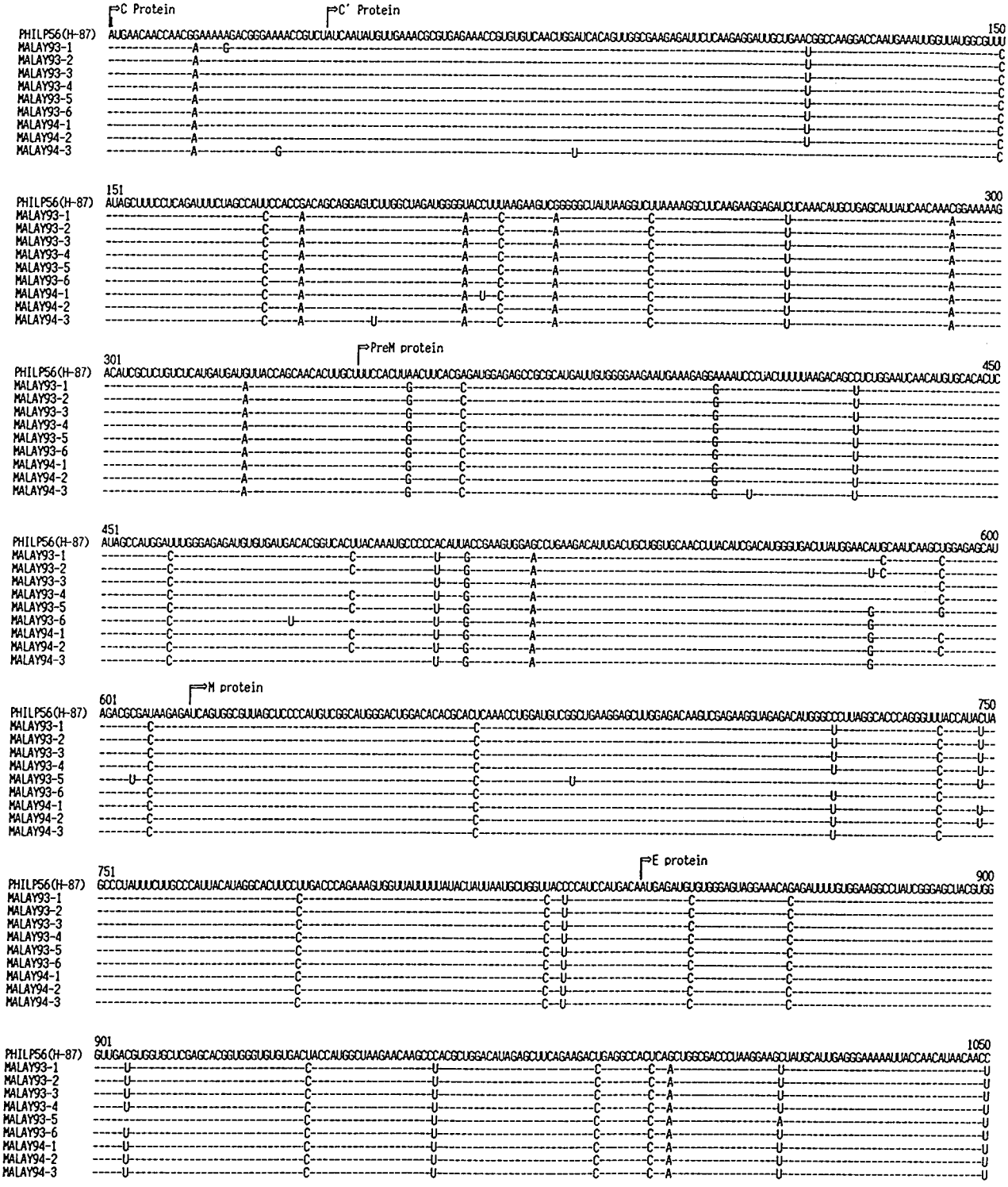

FIGURE 1. Nucleotide sequences of the partial dengue type 3 virus genome RNA. Nucleotide residues are numbered from the 5′ end of the genomic RNA. C = capsid; preM = premembrane; M = membrane; E = envelope.

of Malaysia in 1993 and 1994. All were determined to be type 3 by serologic testing. Of these DEN-3 virus isolates, the nine shown in Table 1 were used to generate cDNAs by the RT-PCR (see Materials and Methods). For each isolate, we sequenced a 1,050-nucleotide genomic region (positions 95–1,144) that encompasses the complete C, C′, preM, and M protein genes and part of the E protein gene. The 1,050-nucleotide sequences of the nine Malaysian isolates and that of a Philippine DEN-3 isolate, H-87, for which the entire nucleic acid sequence was determined,[17] are shown in Figure

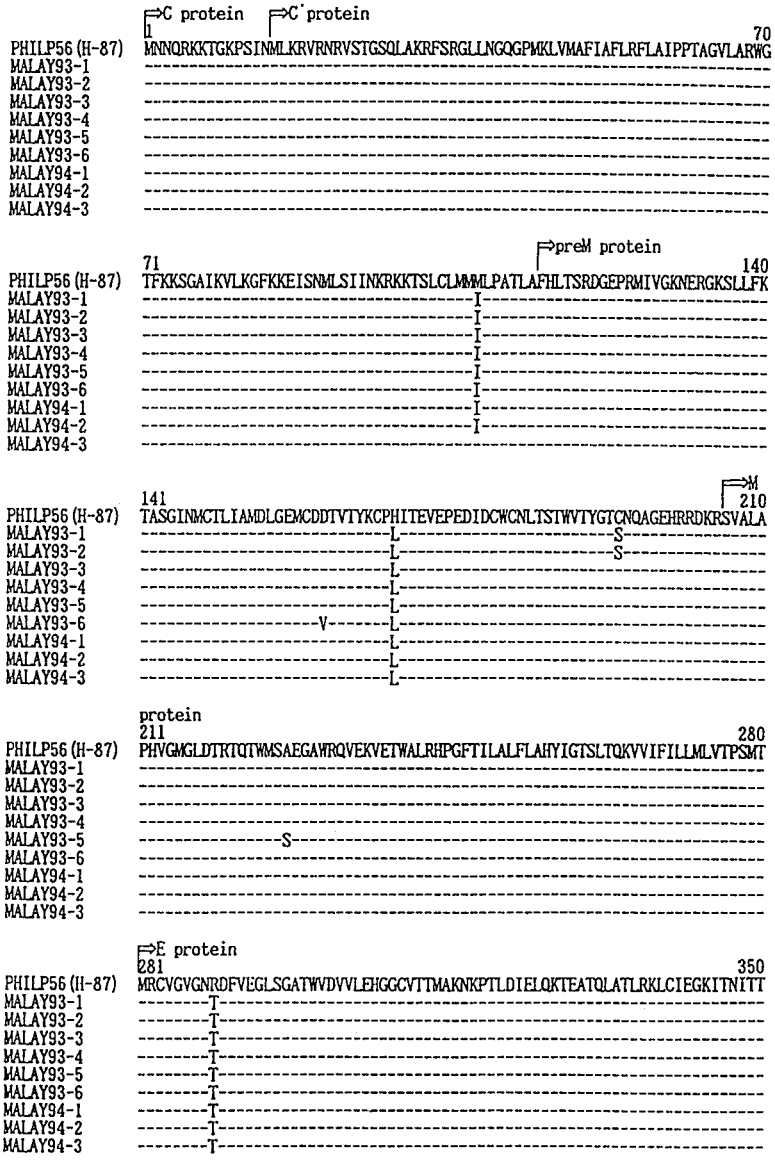

FIGURE 2. Comparison of the deduced amino acid sequences of C, C′, preM, and M proteins and partial E protein from 10 dengue type 3 virus strains. Amino acid residues are numbered from the C protein region of the genomic RNA. For definitions of abbreviations, see Figure 1.

TABLE 1

Description of the Malaysian dengue type 3 viruses isolated in this study

Strain	Code	Year	Symptom*	Passage history†	Accession number‡
JM006	MALAY93-1	1993	DF	6	AB010982
JM068	MALAY93-2	1993	DF	7	AB010983
JM086	MALAY93-3	1993	DHF	7	AB010984
JM091	MALAY93-4	1993	DHF	7	AB010985
JM098	MALAY93-5	1993	DF	7	AB010986
JM119	MALAY93-6	1993	DF	7	AB010987
JM180	MALAY94-1	1994	DHF	7	AB001988
JM181	MALAY94-2	1994	DHF	7	AB010989
Z026	MALAY94-3	1994	DF	7	AB010990

* DF = dengue fever; DHF = dengue hemorrhagic fever.
† Passage numbers in the *Aedes albopictus* cell line C6/36 are shown.
‡ GenBank accession numbers are shown.

1, and the amino acid sequences deduced from them are shown in Figure 2. (H-87 was previously classified as the subtype I of DEN-3[4].)

The features of the nucleic acid and amino acid sequences of the nine isolates can be summarized as follows. 1) The nucleic acid sequences of the nine isolates differed from each other by 0–1.2%, and from that of H-87 by 3.7–4.0%, indicating that the nine isolates are closely related to each other, but distantly related to H-87. 2) The variations in the amino acid sequences among the nine isolates were not significant compared with the nucleic acid differences noted above, with 0–2 amino acid changes (0–0.6%) throughout the compared region. Similarly, the differences in the amino acid sequences between the nine isolates and H-87 were not marked, with 2–4 amino acid changes (0.6–1.1%). These results indicate that most of the mutations detected in the protein coding region

TABLE 2
Oligonucleotide primers used for amplification and sequencing

Primer	Sequence	Position*	Orientation†
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

* Positions in the sequence of H-87 (a standard dengue type 3 virus strain).[17]
† S = sense orientation; C = antisense orientation.

were silent, and are in agreement with the previous observation by Lanciotti and others.[4] 3) Five of the nine isolates were derived from patients with DF, and four were from patients with DHF (Table 1). However, no change common to either disease was found in either the nucleic acid sequences or the deduced amino acid sequences (Figures 1 and 2). This is consistent with the previous finding that 27 DEN-3 virus isolates from patients with different disease severities had the same amino acid sequence of E protein.[3]

Evolutionary relationship among the nine Malaysian and previously established DEN-3 virus isolates. The nucleic acid sequences of the C and C′ protein genes have not been reported for most DEN-3 isolates, as they have for H-87.[17] However, the sequences of the preM, M, and E genes from 23 distinct DEN-3 isolates that have been obtained from various geographic regions and at various times were available.[4] Thus, we aligned the 720-nucleotide sequences encompassing the preM and M protein genes and part of the E protein gene from the nine Malaysian isolates and 23 previously established isolates (Table 3). Using the aligned 720-nucleotide sequences, we constructed a neighbor-joining phylogenetic tree[12] (Figure 3).

TABLE 3
Nucleic acid sequences of dengue type 3 viruses used as references for phylogenetic analysis

Strain	Code	Geographic origin	Year	Access no.
29472	FIJI92	Fiji	1992	L11422
1416	INDIA84	India	1984	L11424
228761	INDON73	Indonesia	1973	L11425
1280	INDON78	Indonesia	1978	L11426
85-159	INDON85	Indonesia	1985	L11428
1300	MALAY74	Malaysia	1974	L11429
29586	MALAY81	Malaysia	1981	L11427
1558	MOZAM85	Mozambique	1385	L11430
H-87	PHILP56	Philippines	1956	L11423
168.AP-2	PHILP83	Philippines	1983	L11432
PR6	PUERT63	Puerto Rico	1953	L11433
1340	PUERT77	Puerto Rico	1977	L11434
1969	SAMOA86	Samoa	1986	L11435
1326	SRILA81	Sri Lanka	1981	L11431
1594	SRILA85	Sri Lanka	1985	L11436
260698	SRILA89	Sri Lanka	1989	L11437
2783	SRILA91	Sri Lanka	1991	L11438
1327	TAHIT65	Tahiti	1965	L11439
2167	TAHIT89	Tahiti	1989	L11619
5987	THAIL62	Thailand	1962	L11440
CH3489D73-1	THAIL73	Thailand	1973	L11620
D86-007	THAIL86	Thailand	1986	L11441
MK315	THAIL87	Thailand	1987	L11442

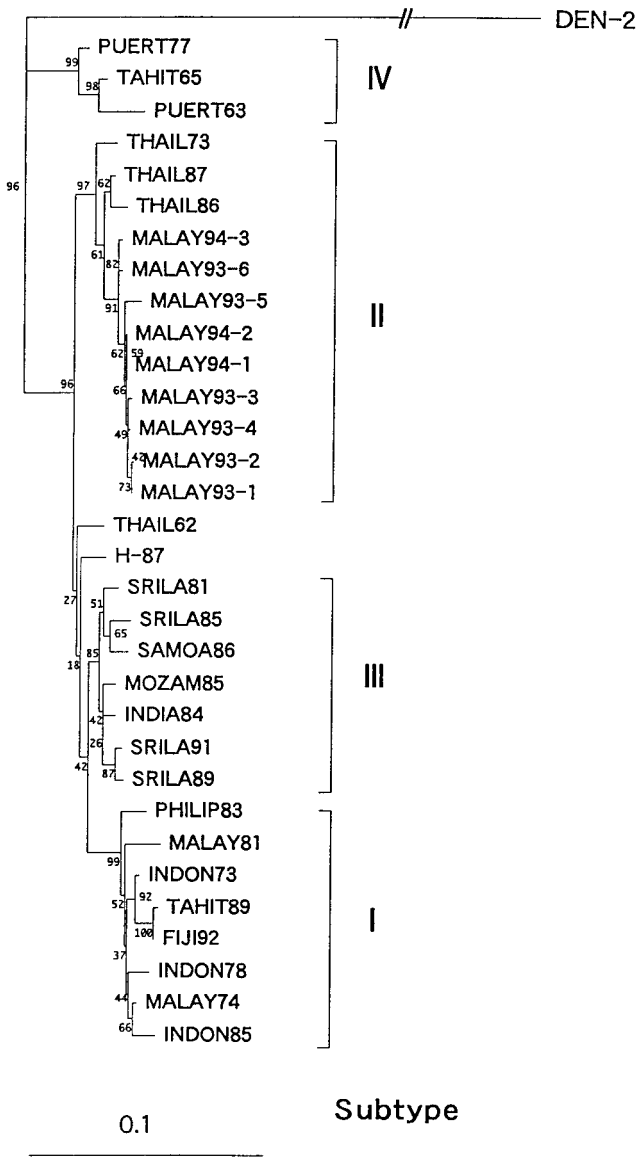

FIGURE 3. Dendrogram showing genetic relationships among nine dengue type 3 (DEN-3) virus strains isolated in Malaysia during 1993 and 1994 and 23 DEN-3 virus strains from different geographic areas of Asia. Alignment of preM, M, and part of the E protein sequences determined in this and other studies (Table 1) generated 720 sequences. A neighbor-joining phylogenetic tree[12] was constructed from the 720 sequences using the CLUSTAL W program.[13] The tree was rooted using a dengue-2 virus strain (Jamaica[19]) as the outgroup. The phylogenetic tree was visualized by the TREEVIEW 1.4 program.[15] The code for each sequence is shown in Table 1. The numbers at the node indicate bootstrap confidence levels obtained by 100 replicates. Subtypes are indicated to the right of the tree. For definitions of abbreviations, see Tables 1 and 3.

Lanciotti and others[4] used a parsimony method to classify the 23 DEN-3 virus isolates into four subtypes: I, II, III, and IV. According to the neighbor-joining tree, most of the DEN-3 isolates, with the exception of H-87 and THAIL62, diverged into four clusters that corresponded to subtypes I, II, III, and IV.[4] Without exception, all nine 1993 and 1994 Malaysian DEN-3 isolates were grouped into subtype II, which

includes three Thailand DEN-3 isolates from the period 1962–1987.

DISCUSSION

Lanciotti and others[4] showed by phylogenetic analysis that seven DEN-3 virus isolates in Malaysia obtained from 1974 to 1981 were classified into subtype I, which contained the 1956 Philippine isolate H-87 and two other Philippine isolates from 1983 and 1984. Thus, the genetic data indicate that in The Philippines, type I viruses had been maintained for nearly 30 years. During this period, the Philippine viruses may have been introduced into Malaysia, followed by their maintenance in a silent transmission cycle without causing a significant dengue epidemic. We speculate that these endemic viruses contributed to the recent dengue epidemic in Malaysia.

In contrast to our speculation, however, all nine DEN-3 isolates obtained during 1993 and 1994 were grouped into subtype II, which includes three Thailand DEN-3 isolates from 1973 to 1987 (Figure 3). Thus, the genetic data indicate that the epidemic DEN-3 viruses in Malaysia during 1993 and 1994 were derived, with some genetic drift, from the endemic DEN-3 viruses in Thailand.

Malaysia and Thailand border each other in the center of the Malay peninsula. This geographic situation may have allowed the gradual movement of epidemic zones in which dengue viruses were circulating between *Aedes* mosquitoes and humans.[1]

Our data together with those of Lanciotti and others[4] suggest that the introduction of DEN-3 viruses from Thailand was directly associated with the recent dengue epidemic in Malaysia in 1993–1994. Alternatively, the Thailand viruses may have been introduced into Malaysia before the recent dengue epidemic and may have been maintained in a silent transmission cycle; these endemic viruses may have contributed to the recent dengue epidemic in Malaysia. If the latter possibility were the case, not only the nine current DEN-3 viruses (subtype II), but also the previously detected DEN-3 viruses (subtype I) would have been involved in the recent dengue epidemic (subtypes I and II are antigenically indistinguishable). However, we detected no type I virus from Malaysia in 1993–1994.

It should be noted that the transmission of dengue viruses between different areas also occurs in the southern Pacific. Chungue and others compared nucleic acid sequences between dengue isolates from the South Pacific during early and recent periods and found that the early and recent dengue viruses classified into different genotypes, suggesting that the recent dengue epidemics in the South Pacific were due to the introduction of a new virus rather than to the re-emergence of the earlier strain.[3,5]

Our study and those of Chungue and others[3] emphasize that the transmission of dengue viruses between different areas is still in progress in various tropical regions, although all four serotypes of dengue viruses have become endemic in most tropical regions.[18] This aspect of dengue epidemiology should be taken into account when a control program for the disease is developed. Detailed ecologic study of *Aedes aegypti*, the carrier of dengue viruses, might be required to prevent the introduction of dengue viruses from neighboring countries.

Acknowledgments: We thank Dr. Y. Yogo (Institute of Medical Science, University of Tokyo) for critical review of the manuscript and Dr. M. T. Arif (Director of the Institute for Medical Research [IMR], Kuala Lumpur, Malaysia) for permission to publish this paper.

Financial support: This study was supported by the IMR-Japan International Cooperation Agency Research Project on Tropical Disease in Malaysia, technically managed by Dr. H. Tanaka, Chief Advisor.

Authors' addresses: Nobuyoshi Kobayashi and Chie Sugimoto, Department of Viral Infection, Institute of Medical Science, University of Tokyo, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108-8639, Japan. Ravindran Thayan, Zainah Saat, Balasubramaniam Vijayamalar, and Mangalam Sinniah, Division of Virology, Institute for Medical Research, Kuala Lumpur, Malaysia. Kazumasa Oda, Japan International Cooperation Agency, Tokyo, Japan. Akira Igarashi, Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

Reprint requests: Nobuyoshi Kobayashi, Department of Viral Infection, Institute of Medical Science, University of Tokyo, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108-8639, Japan.

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