GENETIC VARIATIONS AND RELATIONSHIP AMONG DENGUE VIRUS TYPE 3 STRAINS ISOLATED FROM PATIENTS WITH MILD OR SEVERE FORM OF DENGUE DISEASE IN INDONESIA AND THAILAND

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Abstract. Sequence analysis was conducted on structural and non-structural genes of 7 strains of dengue virus type-3 (DENV-3 virus) isolated in Indonesia and Thailand in the year 1973, 1994, and 1998 from patients with different clinical manifestations. In general, sequence similarity among isolates was greater than 93%, indicating that the mutation rate of DENV-3 circulating in this region was not more than 7% in the last 3 decades and suggesting that sequences that may responsible for viral architectures and/or biological function were strictly conserved. Mutations unique to viral strains associated with specific clinical manifestations were not found. Alignment of PrM/M and E nucleic acid sequences followed by parsimony analysis of sequences obtained in this study and published elsewhere allowed generation of phylogenetic trees, demonstrating that DENV-3 strains isolated in Indonesia in 1998 belonged to a separate cluster (subtype 2) from those isolated between 1973-1985 (subtype 1).

INTRODUCTION

Dengue viruses (DENV) (family Flaviviridae, genus *Flavivirus*) are responsible for the most important arthropod-borne virus diseases in humans in terms of morbidity and mortality. Due to global population growth, increased urbanization and spread of both the mosquito vector and the four viral serotypes, dengue is a major emerging problem in tropical and sub-tropical areas worldwide (Halstead, 1997). The dramatic spread of epidemic dengue fever and the emergence of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in Southeast Asia occurred after World War II, where DHF is one of the leading causes of hospitalization and death mainly among children (Gubler and Trent, 1994).

Like many RNA viruses, dengue exhibits substantial genetic diversity, most notably in the existence of four distinct types (DENV1–4), which are no more similar to each other than some different 'species' of flavivirus (Kuno, 1998). Genetic diversity is much more restricted within each type but is still sufficient for clusters of variants – genotypes – to be identified (Rico-Hesse, 1990). A number of factors underpins this biodiversity. It is obvious that dengue virus is highly mutable, as RNA-dependent RNA polymerases are thought to produce approximately one error per round of genome replication (Drake, 1993). However, it is equally apparent that the overall base substitution rate in dengue is less than that observed in other RNA viruses replicated using the similar enzymes (Zanotto *et al*, 1996), which suggests that its rate of replication per year might be somewhat lower than other RMA viruses or that the virus is subject to

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stronger functional constraints.

The burgeoning biodiversity of dengue virus is of little importance if strain variation plays only a minor role in disease. Indeed, there is a great deal of evidence to suggest that host immune response, for example, antibody-dependent enhancement of infection (ADE), is a key factor in DHF and DSS pathogenesis, most notably from epidemiological studies that reveal a higher incidence of severe disease in secondary, compared with primary, viral infections (Burke *et al*, 1988; Thein *et al*, 1997) . Yet, despite this, there are still cases of DHF and DSS that cannot be adequately explained by ADE, particularly in confirmed cases of primary infection (Glaziou *et al*, 1992), where the epidemic situation would be expected to lead to a high number of secondary infections but severe dengue is rare (Watts *et al*, 1999). Such epidemiological observations suggest that strains of dengue virus with a wide variety of pathogenic properties exist in nature, although there is a need for long-term prospective studies of dengue infection within communities.

The most consistent argument for a strain basis to DHF and DSS has been put forward by Rico-Hesse and colleagues (Rico-Hesse, 1990; Rico-Hesse *et al*, 1997). In brief, they propose that a low-virulence strain of DENV-2 has circulated in Latin America since the late 1960s and that epidemics of DHF and DSS did not occur in this region until the arrival of a strain of higher pathogenicity originating from Southeast Asia, where serious dengue disease is more common: this suggestion is supported by a recent epidemiological study of a clinically mild DENV-2 infection in Peru (Watts *et al*, 1999). The complete genome sequences of the putative high (Asian) and low (American) virulence strains have now been compared and candidate mutations – those that differed consistently between the two sets of strains – have been identified (Leitmeyer *et al*, 1999).

Particularly for DENV-3 few structural and non structural gene sequences have been published although the complete genome sequence for the highly adapted H87 prototype has been published (Osatomi and Sumiyoshi, 1990). In this study we conducted partial sequencing on various regions of 7 DENV-3 strains to identify genome structural differences that might correlate with pathogenesis. We also studied, using phylogenetic methods, the genetic relationship among the strains, including the reference strain H87.

MATERIALS AND METHODS

Virus strains

The DENV-3 strains used in this study were isolated in Indonesia and Thailand from patients with different clinical manifestation in the year 1973, 1994, and 1998. The clinical diagnosis and the disease severity grading were classified according to the World Health Organization (WHO, 1997). Four virus strains, C0331/94, C0360/94, 98901590, and 98901604, were isolated directly from plasma and the other three strains (KPS-4-0657/207, KPS-04-0461/551, and KPS-04-0657/207) were isolated in C6/36 mosquito cells and identified by indirect fluorescence monoclonal antibody (Table 1).

Primer design

Synthetic oligonucleotide primer pairs were designed to amplify overlapping fragments of the dengue genome based on the published sequence of the H87 prototype strain. Primers were chosen so that their potentially conserved sequence flanked a variable region (Table 2). A diagram of the genome amplification and sequencing strategies is shown in Fig 1.

RNA extraction and RT/PCR

RNA was extracted from plasma or the supernatant of infected cells using QIAamp Viral RNA Mini Kit (QIAGEN) following the manufacturer's protocol. Extracted RNA was stored at -80ºC. RT-PCR was carried out at 37ºC for 60 minutes following incubation at 90ºC for 2 minutes in 25 μl of reaction mix containing 10x RT buffer (QIAGEN), 5 mM dNTPs, 10 U RNAse inhibitor (Promega), 2.5 U/μl of Sensicript RT (QIAGEN), 2 μl of 10 pmol primer, and 15 μl of sample. The reverse primers used were D3-6705c, D3-ext, or D3-2716c.

Two-step PCR was conducted as follows. The first PCR reaction produced a long fragment used as a template for the second PCR to am-

Table 1
Dengue virus type 3 strains analyzed.

Strain	Year	Origin	Sequence source	Passage history	Grade of illness	GenBank accession no
98901590	1998	Indonesia	This study	Plasma	DHF II	AY912453 AY912454
98901604	1998	Indonesia	This study	Plasma	DHF II	AY912455 AY912456
KPS-4-0657/207	1998	Thailand	This study	C6/36 -1	DHF III	AY912457 AY912458
KPS-04-0461/551	1998	Thailand	This study	C6/36 -1	DF	-
CO331/94	1994	Thailand	This study	plasma	DHF III	AY876494
CO360/94	1994	Thailand	This study	plasma	DF	AY923865
CH53489	1973	Thailand	This study	C6/36	DHF I	-
1153	1973	Indonesia	Lee *et al*, 1993	?	DSS	-
H87	1956	Filipina	GenBank	[C6/36;SMB]-35	-	L11423
228761	1973	Indonesia	GenBank	Mosq-1	-	L11425
1280	1978	Indonesia	GenBank	C6/36-2	-	L11426
85-159	1985	Indonesia	GenBank	C6/36-1	-	L11428

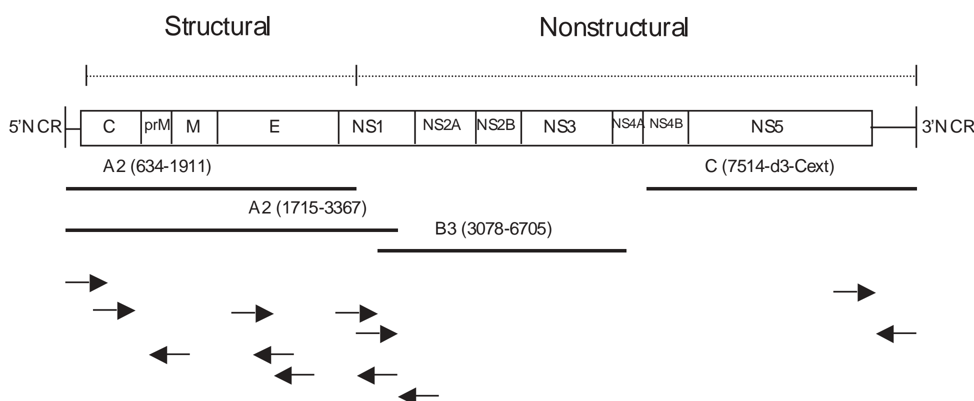

Fig 1–Schematic of the DENV-3 RNA genome and amplification strategy. Arrows pointing to the right indicate regions sequenced 3´→5´ on the minus-sense strand; arrows pointing to the left indicate regions sequenced 3´→5´ on the plus-sense strand. NCR, non-coding region.

plify 0.5-1.4 kb fragments. The PCR amplification was perform using Pfu Turbo DNA Polymerase (Stratagene). The 50 μl reaction mix contained PCR buffer 10x, 10 mM dNTPs (final concentration 0.2 mM each), 1U Platinum Taq DNA Polymerase (Invitrogen), 2.5-4 μl of template DNA and distilled water. PCR conditions were 95°C for 45 seconds, followed by 40 amplification cycles of 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 5 minutes, with a final extension at 72°C for 7 minutes. When products of PCR reaction were not specific, targets bands were excised and purified by using the Qiagen QIAquick Gel Extraction kit (QIAGEN) following the manufacturer's instructions. All remaining PCR reaction products were purified by using the Qiagen PCR Purification kit following the manufactures's protocol.

DNA sequencing

Purified PCR products were sequenced at the Eijkman Institute for Molecular Biology, Jakarta, Indonesia, by the cycle sequencing dye terminator method (Perkin-Elmer/Applied

Biosystem, USA).

Sequence and phylogenetic analysis

The sequence analysis was performed by using GENETYX-WIN Ver.2.0 (Software Development, Tokyo, Japan) and Bioedit 2.0 software (http://www.mbio.ncsu.edu/Bioedit.bioedit.html). Phylogenetic trees were constructed using Mega 2 software (http://www.megasoftware.net) using maximum parsimony algorithm. The bootstrap method, with 100 replications, was used to estimate the reliability of the predicted tree. Representative published sequences from DENV-2 and four subtypes of DENV-3 which have been submitted to GenBank were used for comparison.

Table 2
Oligonucleotide primers used for amplification and sequencing.

Primer	Nucleotide sequence (5´→3´)
D3–SP6-1s	GACCCGCGGATTTAGGTGACACTA TAGAGTTGTTAGTCTACGTGGAC
D3–634s	TTGGTGCAACCTTACATCGA
D3–1021s	GTGTGTGACCACCATGGCT
D3–1715s	GCACTGACAGGAGCTACAGA
D3–1996s	AGTGGTGACCAAGAAGGA
D3–2580s	GAATCAGGTCAACAACCAGA
D3–3078s	CTGCACATGGCCAAAATCAC
D3–3581s	TTCACGTTTGTGCTCCTCCT
D3–7514s	GGAGCTGGGCTTGCTCTTTC
D3–358c	ATCTCCTTCTTGAAGCCTTT
D3–800c	TCTCGACTTGTCTCCAAGCT
D3–1911c	CCTTTGTACTCAACCTTAATGA
D3–2716c	TTTCCCTTGCTCTAAGACCC
D3–3367c	AAGTGTGCACGAGCGGCAAC
D3–3778c	TCAGTTTAAGAGCCATGAGC
D3–4308c	CCATGTTACATCTGCTGCTT
D3–10206c	ACTACATGCCTTCGATGAAG
D3–cext	TCGCGATCGCAGAACCTGTTGATT

S: sense; C: antisense

RESULTS

Nucleic acid and deduced amino acid variation

In this study, we analyzed nucleotide sequence of C, prM/M, E, and NS1 genes and both non-coding regions of seven DENV-3 strains isolated from patients with different clinical manifestations in Indonesia and Thailand. These genes were suggested to be important for molecular markers of dengue pathogenicity (Leitmeyer *et al*, 1999). We also compared prM/M and E sequences obtained in this study with data published in GenBank; sequence data for C, NS1, and non-coding regions of DENV-3 are limited.

In general, the features of the nucleic acid and amino acid sequences can be summarized as follows. There were no areas of high sequence variability identified. Nucleotide sequence variations were present as single-base substitutions scattered throughout the entire length of the genome, except for the 5´ NCR which varied only in the downstream region (Fig 2). Compared to the DENV-3 prototype strain H-87, nucleotide changes occurred at a maximum of 6.9% of the gene fragment (NS1). The majority of nucleotide changes within the genome (70-80%) were transitions. This indicates that divergence of the studied region was no more than 7% and corresponded to the distance between early (1973) and recent (1998) Indonesian and Thailand DENV-3 strains. No nucleotide deletions or insertions were observed within the coding region

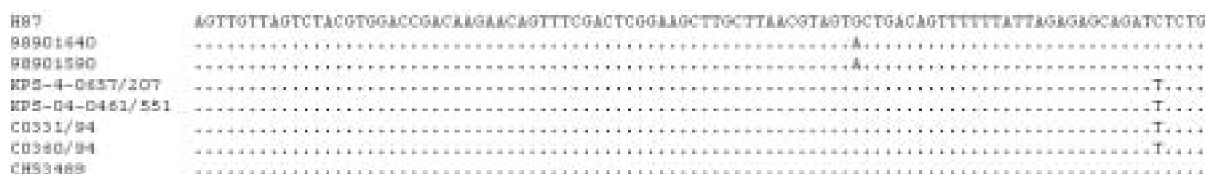

Fig 2–Nucleotide sequences of the 5´ non-coding region of DENV-3 strains compared with prototype H-87.

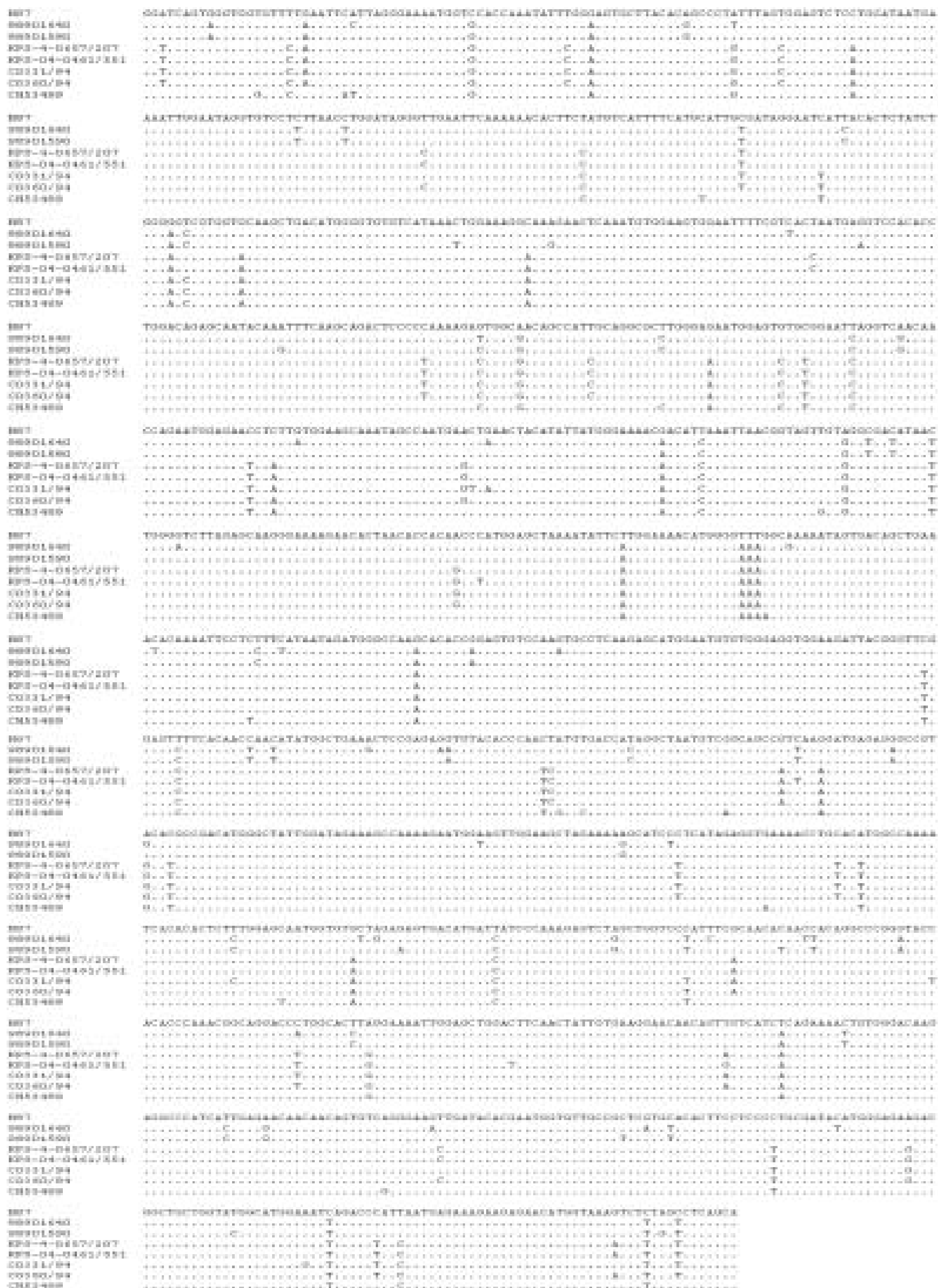

Fig 3–Nucleotide sequences of the C, prM/M, E, and NS1 genes of DENV-3 strains compared with prototype H-87.

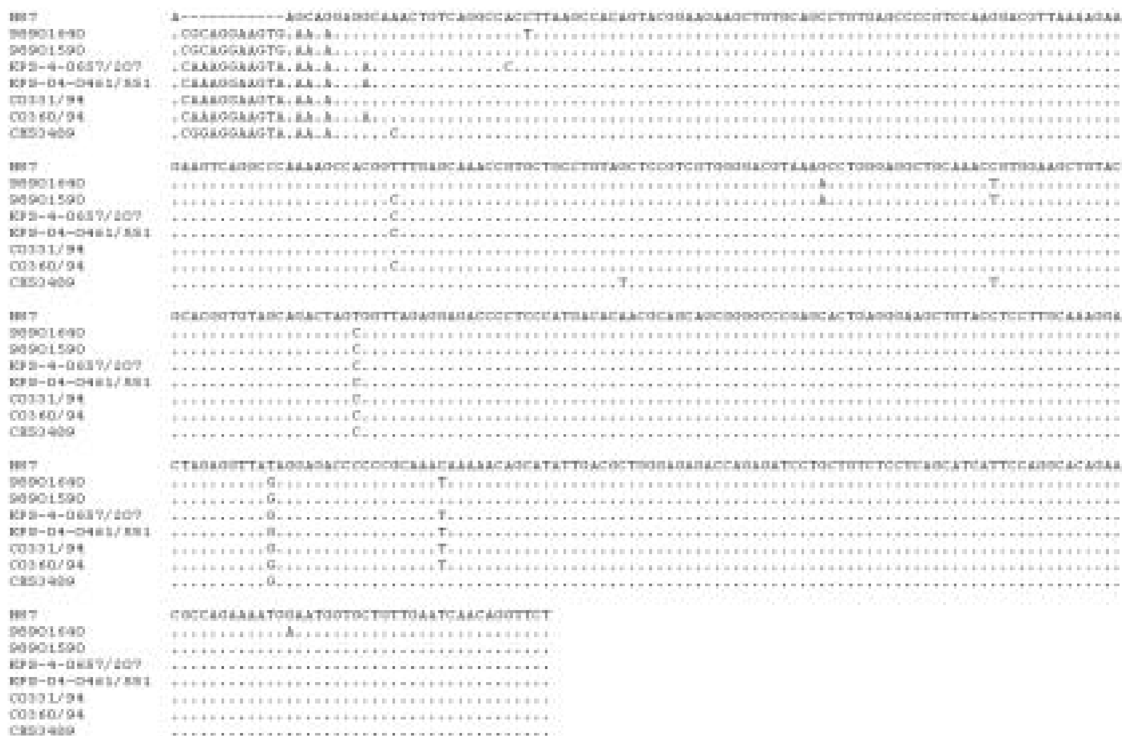

Fig 4–Nucleotide sequences of the 3´ non-coding region of DENV-3 strains compared with prototype H-87.

(Fig 3), although the 3´ NCR of all DENV-3 strains analyzed showed an eleven nucleotide insertion, compared with H-87 (Fig 4).

The homology in the amino acid sequences was 99.6-100% and 95.6-100% among the Indonesian and Thai isolates, respectively. Most of the nucleotide mutations detected in the protein-coding region were located at the third position of the codon and were silent. These results indicate that the protein sequences of all DENV-3 strains analyzed were highly conserved. For the E and NS1 proteins, the majority of the amino acid changes occurred in clusters common to all strains (Fig 5). All DENV-3 strains possessed the coding sequences for amino acids postulated to play a major role in processing and folding of the E protein (Henchal *et al*, 1990). The 12 cysteine residues, predicted N-linked glycosylation sites, proteolytic cleavage sites, and membrane anchor present in flavivirus prM/M and E proteins were identified in all DENV-3 strains analyzed. The 14 amino acid region from position 97-111 in the E protein which is predicted to form fusion domain (Roehrig *et al*, 1998) was conserved. However, within the core protein, we observed three amino acid changes (Val 65 Ile, Lys 82 Arg, and Lys 96 Arg) that were only identified in Indonesian isolates.

In this study, we did not find unique mutations of the genes analyzed in DENV strains associated with specific disease profiles. Particularly for the E protein, this is consistent with the previous finding by Chungue *et al* (1993), in which the relatedness between nucleotide sequence of the E protein and the severity of disease could not be in 27 DENV-3 strains isolated from patients with different disease severity.

Evolutionary relationships among the Indonesian and Thai strains and previously published DENV-3 isolates

To determine the genetic relationships among DENV-3 strains, a phylogenetic analysis was done using the prM/M and E genes of the seven strains studied, the prototype H-87 strain, and 23 additional viruses. This analysis clearly

Fig 5–Amino acid sequences of the C, prM/M, E, and NS1 proteins of DENV-3 strains compared with prototype H-87. Amino acid sequences were deduced from the nucleotide sequences shown in Fig 4.

confirmed the existence of the four DEN-3 subtypes from various geographic regions and time periods (Table 1). The phylogram generated from prM/M and E genes showed that all Thailand DENV-3 isolates were grouped into subtype II, including the five isolates studied. A different result was observed with regards to Indonesian isolates. The two DENV-3 viruses isolated in 1998 were in a separate subtype from isolates from the period 1973-1985, namely subtype I (Fig 6).

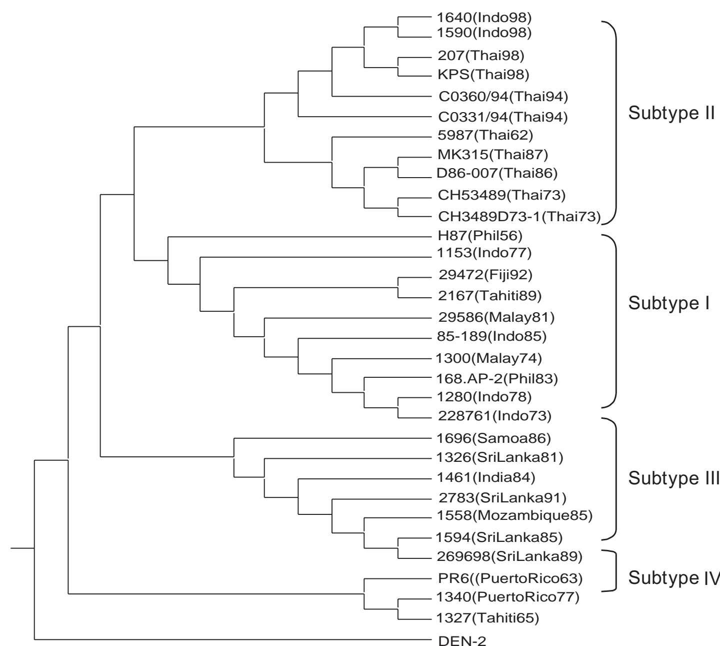

Fig 6–Phylogram generated by parsimony analysis of amino acid sequences for prM/M and E genes of DENV-3 strains.

DISCUSSION

Indonesia and Thailand have become a predominant region in terms of DHF/DSS cases. In Indonesia, the incidence of DHF/DSS has increased steadily since the first reported cases in Jakarta and Surabaya in 1968 (Sumarmo, 1987). By 1990, all 27 provinces of Indonesia had reported cases of DHF (Report of Dir Jen P2M-PLP, Ministry of Health, Republic of Indonesia, unpublished data). An early 2004 DHF outbreak caused nearly 500 deaths (Report of Dir Jen P2M-PLP, Ministry of Health, Republic of Indonesia, unpublished data). In Thailand, a retrospective review of patient records revealed DHF/DSS cases as early as 1950 (Halstead, 1997) and the disease has become a severe and intractable public health problem in the intervening years.

It has been suggested that some DENV strains from the same serotype could cause more severe disease than others because they have a greater ability to be enhanced by heterotypic antibodies. More recent analyses have identified mutations in DENV that are associated with changes in virulence, either by comparing original and attenuated strains (Sanchez and Ruiz, 1996; Kinney *et al*, 1997) or by using chimeric viruses or cDNA infectious clones (Kinney *et al*, 1997; Bray *et al*, 1998; Gualano *et al*, 1998). Some studies have even described the apparent segregation of viruses causing mild and severe disease in nature (Lanciotti, 1997; Messer *et al* 2003). However, as these have generally utilized only small fragment of the viral genome, in reality they tell us little about the contribution of strain variation to dengue pathogenesis.

In this study, we performed a comparison of several DENV strains of serotype 3 isolated from DF, DHF, and DSS cases in a particular outbreak area to clarify the role of genetic variation in the occurrence of severe disease. However, our results showed that none of nucleotide and/or amino acid changes within the structural and non-structural regions analyzed were consistent among DF or DHF samples and could not be correlated with disease outcome. Also, we did not find that specific changes occurred in regions known to affect pathogenicity *in vitro*, such as the 3′ NCR, which is fundamental to viral replication, translation and assembly, or the envelope protein, which stimulates neutralizing antibodies and induces cell-mediated immune responses, as well as being the site of attachment to cellular receptor(s). A similar result was also observed among Thai DENV-2 virus isolates (Leitmeyer *et al*, 1999). This supports the hy-

pothesis that all dengue viruses belonging to the Southeast Asia genotype have the potential to cause severe disease (Rico-Hesse *et al*, 1997). However, the number of strains analyzed in this study is relatively small. Further study involving a greater number of DENV-3 viruses is needed to compare the Southeast Asia genotype and other genotypes. Comparison of viruses from genetic groups with distinct clinical and epidemiologic associations may better identify structural differences that correlate with pathogenesis (Leitmeyer *et al*, 1999).

Many investigators have used viral nucleotide sequence data and phylogenetic methods to understand genetic relationships between viruses as well as the epidemiology of viral disease. Lanciotti *et al* (1994) showed by phylogenetic analysis that seven DENV-3 viruses isolated in Thailand from 1962-1986 and Indonesian isolates from 1973-1985 were classified into subtypes II and I, respectively. Our study shows that, within Thai isolates, subtype II viruses have been maintained for 36 years. This did not occur within Indonesian isolates, which may indicate that DENV-3 viruses circulating in Indonesia have undergone some genetic drift or come from a different progenitor. It is not known whether this change or a different group of DENV-3 isolates in Indonesia has altered the virulence of the viruses.

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