# 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

Muhareva Raekiansyah<sup>1</sup>, Andri Pramesyanti<sup>1</sup>, Budiman Bela<sup>1</sup>, Herman Kosasih<sup>2</sup>, Chairin N Ma'roef<sup>2</sup>, Sherley Yurike Tobing<sup>2</sup>, Panji I Rudiman<sup>3</sup>, Bachti Alisjahbana<sup>3</sup>, Timothy P Endi<sup>4</sup>, Sharone Green<sup>5</sup>, Siripen Kalayanarooj<sup>6</sup>, Alan L Rothman<sup>5</sup> and T Mirawati Sudiro<sup>1</sup>

<sup>1</sup>Department of Microbiology, Medical Faculty, University of Indonesia, Jakarta; <sup>2</sup>NAMRU-2 Jakarta, Indonesia; <sup>3</sup>Internal Medicine Department, University of Padjadjaran, Indonesia; <sup>4</sup>Walter Reed Army Institute of Research, Washington, DC, USA; <sup>5</sup>Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School, Worcester, MA, USA; <sup>6</sup>Queen Sirikit National Institute of Child Health, Bangkok, Thailand

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

Correspondence: Muhareva Raekiansyah, Department of Microbiology, Faculty of Medicine, University of Indonesia, JI Pegangsaan Timust 16, Jakarta 10320, Indonesia. Fax: 62 213100810 E-mail: tjahjanims@cbn.net.id

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 denque is rare (Watts et al, 1999). Such epidemiological observations suggest that strains of denque 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 -80°C. RT-PCR was carried out at 37°C for 60 minutes following incubation at 90°C for 2 minutes in 25  $\mu$ l of reaction mix containing 10x RT buffer (QIAGEN), 5 mM dNTPs, 10 U RNAse inhibitor (Promega), 2.5 U/ $\mu$ l of Sensicript RT (QIAGEN), 2  $\mu$ l of 10 pmol primer, and 15  $\mu$ l 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-

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	AY912450 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 <i>et al</i> , 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

Table 1 Dengue virus type 3 strains analyzed.

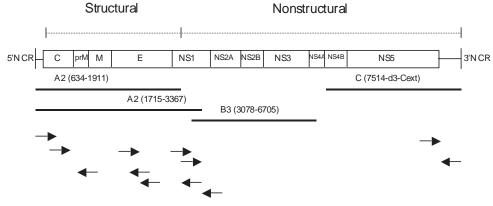


Fig 1–Schematic of the DENV-3 RNA genome and amplification strategy. Arrows pointing to the right indicate regions sequenced 3'→5' on the minus-sense strand; arrows pointing to the left indicate regions sequenced 3'→5' on the plus-sense strand. NCR, non-coding region.

plify 0.5-1.4 kb fragments. The PCR amplification was perform using Pfu Turbo DNA Polymerase (Stratagene). The 50  $\mu$ l reaction mix contained PCR buffer 10x, 10 mM dNTPs (final concentration 0.2 mM each), 1U Platinum Taq DNA Polymerase (Invitrogen), 2.5-4  $\mu$ l 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 esti-

## Table 2 Oligonucleotide primers used for amplification and sequencing.

Primer	Nucleotide sequence $(5 \rightarrow 3)$
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

mate 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.

## 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

H87	AGTTOTTAGTCTACGTGGACCGACAAGAACAGTTTCGACTCGGAAGCTTGCTT
98901440	
	T
	T
C0380/94	T
CH53469	

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

#### Genetic Variations of DENV Type 3

HET VED03440 00903800 RPE-4-Q687/207 RPE-4-Q687/207 RPE-4-Q687/207 C0353/94 C0353/94 C0352480	TOSAVALENTIALADALATOOSCUTTAGCACCA 0000TTACCETALCTEDCCCETTECCTACCATEGOCACTTCCTTELECCCENELETTOOT
HET DEU0304D DEU0304D DEU03090 DEU-4-DEU7/207 RED-04-D461/881 C036D/06 C036D/06 C04D48P	TATTTT TATACTAL TO CONTRACTOR CONTROL COLLECT A DUAL A TOUR DATA TANDA A CANADA TO TO CONTROLOGY TA CONTROL C. T. C. T.
1011 101003550 101003550 101003550 101004-0462/855 C0133756 C0133756 C0133456 C0133456 C0133456 C013347 C013347 C01347	$\begin{array}{c} u = 0 \\ u = 0 \\ v = 0 \\$
199 Y 00000 2040 0000 2040 0000 2040 000 - 0400 000 - 000 000 - 000 0000 - 000 0000 000 - 000 000 - 000 0000 0000 0000 0000	$ \begin{array}{c} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 $
1017 Ch5053.4411 PHEPD3.4511 R22-4-00577.207 R22-4-00577.207 C0333.794 C0334.794 C0334.794	CTADAANTACOTUTUTAADCATACATACCTOOOACADADUU TOOODAAACOOTUTUTTTTTTTTTUTTUUCAADOOTACACTOOTUACATUCOUAAATTTCAA
1877 90503840 90503880 8752-4-0647/107 875-06-0445/151 00334/94 00340/94 00340/94	THITT TRUE ALTACIADO GRARANTO TOUCAAC ATMAINANCE TO ARATAC ACCOUNT & TOUT A CAST OCAC ACAMINANCE ALC ACCOUNT & TOURA & TO
1017 201903-0410 00003-0500 8792-0-0481/3311 0350-04-0481/3311 0350-354 0310-1350 0300-1350 0300-1350 0300-1350 0300-1350 0300-1350 0300-1350 0300-1350 0300-1350 0300-1350 0300-1350 0300-1350 0300-1350 000-1350 000-1350 000-1000-1000-1000-100000000000000000	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$
1007 100901.640 00901.550 200-4-0407/207 200-4-04-0407/207 200-04-04-04 2005.02/04 2005.02/04 2005.02/04 2005.02/04	LENGT TTO ALT TO ALL THE LATINE TTO THE TO ALL BALE ALL CATOOL TO OT ALL CATOOL TO OT ALL BALE ALL CATOOL TO OT ALL BALE ALL CATOOL TO OT ALL BALE ALL CATOOL TO OT ALL C
1001 00001600	
949901590 1975-4-09577207 1976-04-046673864 00224746 7023902594 6863-988	ФЛАЙСТВИ ВИТАЛАЛИСЯСТВО АЛИ ИТИЛАЛСЯЙЛАЛЬНЫ ТТГТТИТИЛИ И ТТТЛАЛАЛАТИИ В САТИО РАЛАЛАЛИ АЛИЛИ И СТОЦАУТИИ СТОЦАУТ 
10/5-0-01557/207 EPG-04-0461/284 C0321/04 C0303/94	UNADE TABLES ANALASE ACTIVATED TOTALS AND AND ANALASES TOTO TOTO TOTALS ANALASES (ATT TOTAL ANALONG ANALASES (ATT TOTO) (ATTOL) (ATTOL
807-4-0557207 878-04-0517854 (0331794 (0350794) (0851400 807-04-0557207 809-01800 809-04557511 (0314794	$ \begin{array}{c} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 $
889-04-0557/207 879-04-0457/884 C0331/84 C0321/84 C0324/84 C0324/84 099011840 8990-0180 8990-0180 8990-0452/551 C0347/84 C0354/99 00552/99 000-0452/207 8990-0452/207 00552/34 00552/34	UNADO TRE REFERENCE OF THE ALE OF THE THE THE ALE AND ALE AND THE THE THE ALE ALE OF THE ALE ALE OF THE OFFICE ALE ALE OFFICE AL
Bittle-a-UB ST/201 Bittle-a-UB ST/201 CD3 20/94 CD3 20/94 CD3 20/94 CD3 20/94 CD3 20/94 CD3 20/94 DD4 DD4 DD4 DD4 DD4 DD5 201-0450 CD3 20/94 CD3 20/94 C	000000000000000000000000000000000000

#### SOUTHEAST ASIAN J TROP MED PUBLIC HEALTH

1877 00901440 99501390 89501390 895-04-0401/207 203-04-0401/201 00260/94 00260/94	004T0441Y004070000000000000000400414447007000400000000
9077 909901.590 909901.590 9092-4-0659/107 895-04-0461/551 00940/94 00940/94 00940/94 00940/94	A A A T T UGA AT A OUT T O C T C T T LA C C T UGA T A OUT T O A AT T O A A A C T C T A T UT C T T C T UC AT T UC AT A C O A T A OUT T C T A T C T C T A T C T C T A T C T C
107 00901.000 00901.000 009-1-0.057/207 073-04-0402/351 01321./94 01340/94 01340/94	00000000000000000000000000000000000000
1077 905901.540 905901.550 805-4-0459/207 805-04-0465/351 00350/94 00550/94 00550/94	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
1801 00001.640 00001.640 KP3-4-18.87/287 KP8-04-0441/851 C0360.784 C0360.784 C0360.784	CC AGG & TOCIDATO TOTO TOTO TOTO A BUT DA ATTACCO DA TOTALE TOT
1807 200501.000 2005-0.0557/207 205-0.4-0461/551 20551/24 20560/98 20560/98 20560/98	TO OCOTE TEREBOLIAR ON DERABBILAR TALCE LEAGE LANGE LATING AND TARABETARTE TO UNROPORT TO OC BARBATROTORIC ADD. TO AR ARA I O ARA 10 10 10 10 10 10 10 10 10 10
807 00001440 80001440 8000440427/207 8020440442581 202040/94 202040/94 202040/94 202040/94 80001488 80004442581 8000444581 80004442581 8000444581 800044581 80004581 800	LC AO & & A ATTOC TOTTE A TRANSPORTANTO CONTACTO & CONTACTO CONTAC
887 99301440 99314500 893-4-0457/201 893-04-0457/201 893-04-0457/201 893-04-0457/201 893-04-0457/201 893-04-0457 893-040 803-040	AC AN OCCULAR A TROOP TA Y TOTAL TALLA ANTITAL A ANTITAL A ANTITAL A ALCO A TOPO TILA TALLAND TOLLA A TOPO TA A 0
10074 009501.6403 8096-0.5500 8096-0-04.57/207 905-04-04057/207 905-04-04057/207 00300/54 00300/54 0153468	ТС АЛАЛАГЫ ТТ ТТЫНАЮ АА ТООТОТОС 7 АЛАЛАОТОКТАТАТИА ГТАТО СТАААЛАЛЫТ. ТАНК ТООТОСО АТ ТТООС ААЛАЛАЛАЛАЛАЛАЛ АЛИАЛАЛ АЛИАЛ ТТАТО СТАААЛАЛЫТ. ТАНК ТООТОСО АЛ ТТООС ААЛАЛАЛАЛАЛ АЛИАЛАЛ АЛИАЛ АЛИАЛАЛ АЛИАЛ АЛИАЛАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛАЛ АЛИАЛАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛ АЛИАЛИАЛ АЛИАЛИАЛИАЛ АЛИАЛИАЛИАЛ АЛИАЛИАЛ АЛИАЛИАЛИАЛИАЛ А
807 949401,8440 949901,8940 8695-8-04-04-03/2897 8695-94-04-03/2897 8695-94-04-03/2897 8695-94-04-03/2897 802402/54 63452.980	AF ACC CEALACTOC ACC TO CONCENT TO COLA AF ACT TO COLOR TO CALC TO CALC TO CALC ACC ACC ACC ACC ACC ACC ACC ACC AC
8997 099201.400 099204.600 8793-4-04877.507 8293-4-04637.504 00331/394 00330/094 0350-999	$ \begin{array}{c} an initial is a first a france of the Constraint is in the arm of a first a france of the optimal is a first of the optimal is a france of the optimal is a first of$
1827 00901.640 989901.590 8290-4-0487/207 825-04-0482/051 0331/94 00380/98 0388/98 0388/98	000 THE TOO TATUOE ATHONA ATTO & DAOCO CATTAR TURH A ROA ROA ROA AC A THE TAR ROTO TO TO BOO CTURIO A ROA ROA ROA ROA ROA ROA ROA ROA ROA

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

#### GENETIC VARIATIONS OF DENV TYPE 3

887 94901490 94901590 8793-4-0657/207 8793-04-0462/861 00313/94 00350/94 0035094	а —
1887 98901.640 98901.590 KP3-4-0637/207 KP3-04-0461/881 (0336/94 C0360/94 C0352.409	ПАЛИТСАЮВСИСАДАЛОС ДООТ ТИПАВСАДАСНИК ПИСТИТАЮС ТОСОТСИТОВИНАСТАДАРСС ТОВОДНОСТОСАДАРСИ ИНИОДАЛСТИТАС 
1887 99901.690 99901.590 KP3-4-0639/207 KP3-04-0481/881 (0331/94 C0360/94 C2852409	0 C 2000 TO TARC AGACTAO TO TTADA ORBINACCOL TO CLATIBLE AL OCLARC AND DO DO CO DARE A CLOADO BLA NET TO TARC TELETTRE AL ADO A. C. C. C. C. C. C. C. C. C. C
1887 90901.600 90901.500 KPB-04-0639/207 KPB-04-0481/881 003501/94 00360/94 CB53409	СТ ТЕЛЕОВИТ ТАТ БОЛАФЫСС СС ОСОССАБЫЕ ВЫЛЬВАСАОСАТАТ ТОАС ОСТИОВАЛЛАССЬ ОВАЛАТСС ТОСТОТС ТОСТОВСАТ САТТО С БЛОСАСАДАА 
987 98901590 8791-4-0859/207 879-94-0461/881 0031/94 00360/94 0380/94	COCCAGRARATOURA TGOTOC THT TGARTCARCAGOT D.T.

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

(Fig 3), although the 3<sup>°</sup> 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

#### SOUTHEAST ASIAN J TROP MED PUBLIC HEALTH

HE7 D0001440 H001890 KF2-4-0487/207 KF2-04-0481/201 00311/34 (0350/94 (0550/94 (0550/94	REAL PARTIES OF THE LEPATHON TO BUC AND THE CLASS OF THE URAF LATER LATER LATER THE RECALDED ADDRESS TO THE PART OF THE PART O
HE7 HE91240 90901590 879-04-0687/207 879-04-0481/301 00960/94 00960/94 cm3460	TELLERBLATLATES TO BE THE THE DEPENDENT OF THE ASSAULT FOR THE SECOND STUTTED FOR THE
887 96901640 96901640 9690-6697/207 979-64-0402/501 00901/94 00901/94 00900/94	RECERTENENT OF TOTAL AS A STATE OF THE ASSAULT AND OF TELSAR AND THE TOTAL TOTAL TOTAL TOTAL AND THE T
HE7 HE901840 PE001840 H95-4-0057/207 H95-04-0465/201 0051/94 C0180/94 C0280/94	VUVVLEROOCVITTRARNEPTLDIELORTEATOLATLERILCIEOXITHITTERECTOGRAFILPEEODORTVCENTYVERONOCOLPOXORLVTCAREO
887 99901440 89901440 899-4-0657/207 895-4-0451/553 0954/04 20340/04 20340/04 20340/04	CLEBIESSRVUEBEER FYVIITUNTOLOKAVUNETGOVTAETTELAITAEAILFEVOTLOLECOPKTELOFERILLTHENPANNVRHANFFLAFFLFTE P
HE7 90901340 90901590 899-8-06597200 899-8-06592200 00311288 00311288 00311288	TATTETETETERELLUTTERIAHAMKGEVVVLOTULENARIETAL TOATEIGTIJOTIIFANNLEUPLENENELLENKEVARUNTVLENEVNETGHTILI 
1897 949901.640 949901.590 8796-9-0557/207 8761.04.04657/207 8761.04.04657/207 8761.04.04657/207 8761.046 870.4657 870.4557 870.4	EVETROLED APCKLEFELTER OGORANDORL TAMP/VTREEEPVELTAEP FFIREINTYLOLIND KALELINT TAKON TAKONAN MAALAD TAKO F T T T T T T T T T T T T T
807 90901840 80901890 879-4-0687/207 879-404-0485/207 879-04-0485/207 0050/294 0050/294 0050/294 0050/294	GEVGOVLED.GETVELFOGETTELFOUNDEIHEEGUVLETWEGLENDEFECTEIGTEGTUNGVOGEDBOCVIBMEGRELECODGEFVTERVET V V V V V V V V V V V V V V V V V V
007 0001240 0001500 EP5-4-0657/207 KF5-4-0657/207 C0211/24 C0212/24 C0212/24 C0210/24 C0210/24	NTEGRERÇAS EFERVATAT ADA MEMOUCUTHET TRHEHLL MELTATELMENT HILTVVVOG TITVLEGNERTLTPGF BELEVINET WILLAR IV TAE 
937 94901840 94901840 849-4-0487/207 899-04-0481/853 0331/94 0346/94 0346/94 034699	TONODE ILLOGES THE CENTRE OF NUMERAL SECTOR OF THE LEVEL OF THE CENTRE CENTRE CENTRE OF A SECTOR OF THE CENTRE OF A SECTOR OF
HE7 98901640 98901590 HF5-4-0657/207 KF2-04-04612581 C0380/94 CKE1460	11 HTL NUMBER LEP KOLL OF ENGINEEP-FTHTUTAGE MELDELELOPHYC ROT TVV LIEHC OTHINFILLET TVGGRL ER BCCRBC TLPFLETHORD Y Y T T T T T T T
807 90901840 90801840 879-4-0687/207 879-64-0687/207 879-64-0687/207 879-64-0687/207 80381/94 00340/94 00340/94	GOWFINE DP INE REINWITH, ANA 

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).

#### 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

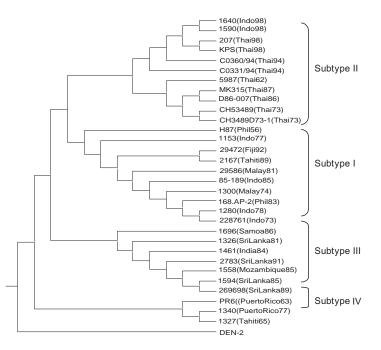


Fig 6–Phylogram generated by parsimony analysis of amino acid sequences for prM/M and E genes of DENV-3 strains.

*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 hypothesis 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

# ACKNOWLEDGEMENTS

This study was funded in part by NIH Grant P01 A134533. We thank Dr Masanori Terajima for suggestions and critical reading of the manuscript, and Dr Ananda Nisalak, Dr Francis A Enis, and Dr Agus Sjahrurachman for their support in completion of this study.

# REFERENCES

- Burke DS, Nisalak A, Johnson DE, Scott, RM. A prospective study of dengue infections in Bangkok. *Am J Trop Med Hy*g 1988; 38: 172-80.
- Bray M, Men R, Tokimatsu I, Lai CJ. Genetic determinants responsible for acquisition of dengue type 2 virus mouse neurovirulence. *J Virol* 1998; 72: 1647-51.
- Chungue E, Deubel V, Cassar O, Laille M, Martin PMV.

Molecular epidemiology of dengue 3 viruses and genetic relatedness among dengue 3 strains isolated from patients with mild or severe form of dengue fever in French Polynesia. *J Gen Virol* 1993; 74: 2765-70.

- Drake JW. Rates of spontaneous mutation among RNA viruses. *Proc Natl Acad Sci USA* 1993; 90: 4171-5.
- Gualano RC, Pryor MJ, Cauchi, MR, Wright PJ, Davidson, AD. Identification of a major determinant of mouse neurovirulence of dengue virus type 2 using stably cloned genomic-length cDNA. *J Gen Virol* 1998; 9: 437-46.
- Glaziou P, Chungue E, Goetas P, *et al.* Dengue fever and dengue shock syndrome in French Polynesia. *Southeast Asian J Trop Med Pub Health* 1992; 3: 531-2.
- Gubler DJ, Trent DW. Emergence of epidemic dengue/ dengue hemorrhagic fever as a public health problem in the Americas. *Infect Agents Dis* 1994; 2: 383-93.
- Halstead SB. Epidemiology of dengue and dengue hemorrhagic fever. In: Gubler DJ, Kuno G. eds. Dengue and dengue hemorrhagic fever. Colorado: CAB International, 1997: 23-44.
- Henchal EA, Putnak JR. The dengue viruses. *Clin Micrbiol Rev* 1990; 3: 376-96.
- Kinney RM Butrapet S, Chang G, *et al.* Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. *Virology* 1997; 230: 300-8.
- Kuno G. Phylogeny of the genus *Flavivirus*. *J Virol* 1998; 72: 73-83.
- Lanciotti RS, Lewis JG, Gubler DJ, Trent DW. Molecular evolution and epidemiology of dengue-3 viruses. *J Gen Virol* 1994; 75: 65-75.
- Lanciotti RS. Molecular evolution and phylogeny of dengue-4 viruses. *J Gen Virol* 1997; 78: 2279-86.
- Lee E, Gubbler DJ, Weir RC, Dalgarno L. Genetic and biological differentiation of dengue 3 isolates obtained from clinical cases in Java, Indonesia, 1976-1978. *Arc Virol* 1993; 133: 113-25.
- Leitmeyer KC, Vaughn DW, Watt DM, *et al.* Dengue virus structural differences that correlate with pathogenesis. *J Virol* 1999; 73: 4738-47.
- Messer WB, Gubler DJ, Haris E, Sivanathan K, de Silva AM. Emergence and global spread of a dengue serotype 3, subtype III virus. *Emerg Infect Dis* 2003; 9: 800-9.
- Osatomi K, Sumiyoshi H. Complete nucleotide sequence of dengue type 3 virus genome RNA.

Virology 1990; 176: 643-7.

- Rico-Hesse R. Molecular evolution and distribution of dengue viruses type-1 and type-2 in nature. *Virology* 1990; 174: 479-93.
- Rico-Hesse R, Harison L, Salas R, *et al.* de A. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology* 1997; 230: 244-51.
- Roehrig JT, Bolin RA, Kelly RG. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. *Virology* 1998; 246: 317-28.
- Sanchez IJ, Ruiz BH. A single nucleotide change in the E protein gene of dengue virus 2 Mexican strain affects neurovirulence in mice. *J Gen Virol* 77; 2541-5.

- Sumarmo H. Dengue Haemorrhagic Fever in Indonesia. *The Southeast Asian. J Trop Med Public Health* 1987; 18: 269-74.
- Thein S, Aung MM, Shwe TN, *et al.* Risk factors in dengue shock syndrome. *Am J Trop Med Hy*g 1997; 56: 566-72
- Watts DM Porter KM, Putvatana P, *et al.* Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. *Lancet* 1999; 354: 1431-4.
- WHO. Dengue haemorrhagic fever: Diagonosis, treatment, prevention and control. 2<sup>ed</sup>. Geneva, 1997.
- Zanotto PM, Gould EA, Gao GF, Harvey PH, Holmes EC. Population dynamics of flaviviruses revealed by molecular phylogenies. *Proc Natl Acad Sci USA* 1996; 93: 548-53.