1. Imported dengue cases in Taiwan, 2008

A total of 226 laboratory confirmed imported dengue cases were identified in 2008, Taiwan. Among these patients, 102 (45.1%) were detected through the airport fever screening surveillance. Table 1 showed the summary of countries of origin and DENV serotypes of imported cases. The majority of these imported cases were infected in Southeast Asian countries with four exceptions, which were Tonga, India, Bangladesh, and Honduras. Similar to our previous report during the 2002-2007 periods, Indonesia, Vietnam, the Philippines and Thailand are on the top list of importing countries (4). The results reflected the frequency of air travel between Taiwan and these nations, as well as the intensity of massive dengue outbreaks in the country origin during the same period. From the acute phase serum samples of all imported cases, 44 DENV-1, 15 DENV-2, 13 DENV-3, and 7 DENV-4 strains were isolated. The nucleotide sequences of partial NS5 gene fragment and full-length structure gene region of DENV strains isolated were determined and deposited in Taiwan Pathogenic Microorganism Genome Database for molecular epidemiological analysis.

2. Multiple dengue epidemics in southern Taiwan, 2007

For local dengue outbreaks in Taiwan, a total of 488 dengue patients were laboratory confirmed with 3 cases of DHF in 2008. The nucleotide sequences of partial NS5 gene fragment were routinely determined from the acute phase serum samples of confirmed cases. The full-length structure genes of DENV strains isolated from representative indigenous cases were determined. Representative indigenous cases were selected based on the information of epidemiological investigation and preliminary results of partial NS5 gene sequences showing different infection time, infection place, and DENV serotypes and genotypes of these patients. Sequence analyses from more than 111 DENV isolates obtained from acute phase serum samples of indigenous cases showed that 8 different DENV strains (5 DENV-1, 2 DENV-2, and 1 DENV-4) were circulated in Kaohsiung City, Kaohsiung County, Taipei County, Taoyuan County, and Taipei City with limited overlap in the transmission areas.

3. Nucleotide sequencing and phylogenetic analysis

Figure 1 showed the phylogenetic trees derived from full-length E gene sequences of DENV-1 strains isolated from confirmed dengue cases in Taiwan and sequences available from GenBank. Phylogenetic tree was constructed by neighbor-joining and the DENV strains isolated from Taiwan in 2008 were color-marked. Phylogenetic analyses showed that the 5 DENV-1 strains (DENV-1-1 to DENV-1-5) in the genotype I causing local outbreaks were most closely related to D1/Thailand/0707aTW/2007, D1/Myanmar/0807aTW/2008,

D1/Vietnam/0601aTW/2006, D1/Vietnam/0804bTW/2008, and D1/Vietnam/0809dTW/2008 imported strains, respectively.

4. CHIKV identification and characterization

For other arboviruses, we identified 13 imported chikungunya cases from airports fever screening surveillance during 2006-2008. Among these travelers, 7, 3, 1, 1, and 1 case(s) were returned back from Indonesia, Malaysia, Singapore, Bangladesh, and India, respectively. The most common symptoms were fever (n=13), myalgia (n=6), arthralgia (n=5) and rash (n=4). All were detected within 2 days of symptom onset. The CHIKVs were isolated from the acute phase serum samples and the partial nucleotide sequences of envelope protein 1 (E1) gene (1044 bp) were determined. Phylogenetic tree was constructed by the neighbor-joining method using 60 CHIKV strains including 13 imported strains isolated in Taiwan. O'nyong-nyong (ONN) virus sequence was used as the outgroup virus. The results showed that all CHIKV strains from Indonesia belong to Asian genotype, whereas other isolates belong to African genotype (Figure 2).

Discussion:

Studies on returned travelers have provided valuable information regarding the geographic distribution and global movement of DENV strains. The successful application of fever screening surveillance at airports in Taiwan helps to identify a large numbers of imported dengue cases since 2003. A total of 345 imported dengue cases were identified so far by this active surveillance system. The results witness the growing global threat of dengue because of increasing worldwide travel. To strengthen surveillance of arboviruses, screening of Flavivirus and Alphavirus from the acute phase serum samples of fever patients was started in January 2006. We have so far detected 13 CHIK imported cases in Taiwan by airport fever screening surveillance. The results suggest that the cocirculation of dengue and chikungunya would be increased in many Southeast Asian and African countries due to the rise in international travel and the wide distribution of the competent vectors, *Ae. albopictus* and *Ae. aegypti*. The genetic database generated from these isolated virus strains provides useful information for the understanding of global distributions and movements of various DENV and CHIKV serotypes and genotypes. Understanding the genetic changes and the mode of transmission of these viruses is important toward the development of effective control measures.

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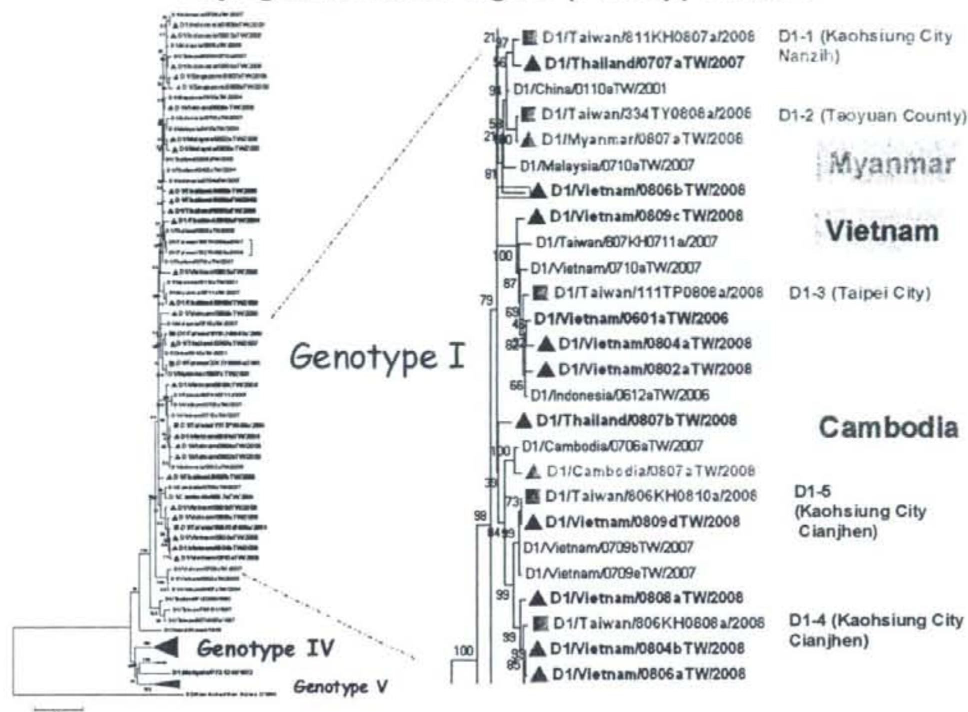
Table 1. Countries of origin and DENV serotypes of imported cases in 2008, Taiwan

Country of origin	Total cases	DENV Serotype				
		DENV-1	DENV-2	DENV-3	DENV-4	unknown
Vietnam	73	30	3	5		35
Indonesia	48	8	11	3	10	16
Thailand	30	11	2	2		15
Philippines	25		3	9		13
Myanmar	15	3		1		11
Cambodia	10	1	1	1		7
Malaysia	8	3	3	1		1
Singapore	7	3	1			3
Tonga	4	1				3
India	3	1				2
Bangladesh	1			1		
Honduras	1	1				
Unknown	1	1				
Total	226	63	24	23	10	106

Table 2. Imported chikungunya cases identified by fever screening at airports during 2006-2008, Taiwan

Date of Detection	Importing Country	CHIKV Genotpye	CHIKV Strain
20061120	Singapore	East/Central/South African	India
20070621	Indonesia	Asian	Indonesia
20071228	Indonesia	Asian	Indonesia
20071230	Indonesia	Asian	Indonesia
20080212	Indonesia	Asian	Indonesia
20080419	Indonesia	Asian	Indonesia
20080705	Indonesia	Asian	Indonesia
20081016	Bangladesh	East/Central/South African	India
20081022	Malaysia	East/Central/South African	India
20081126	Indonesia	Asian	Indonesia
20081208	Malaysia	East/Central/South African	India
20081209	Malaysia	East/Central/South African	India
20081210	India	East/Central/South African	India

1A.



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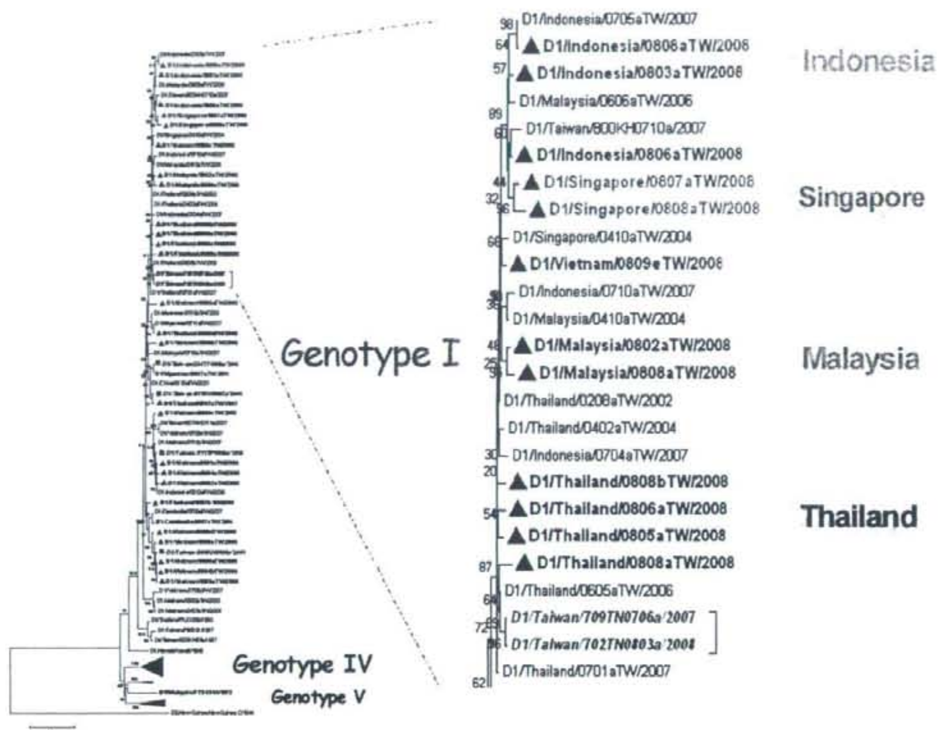

1C.

Phylogenetic tree of E gene (1485 bp) in DEN-1

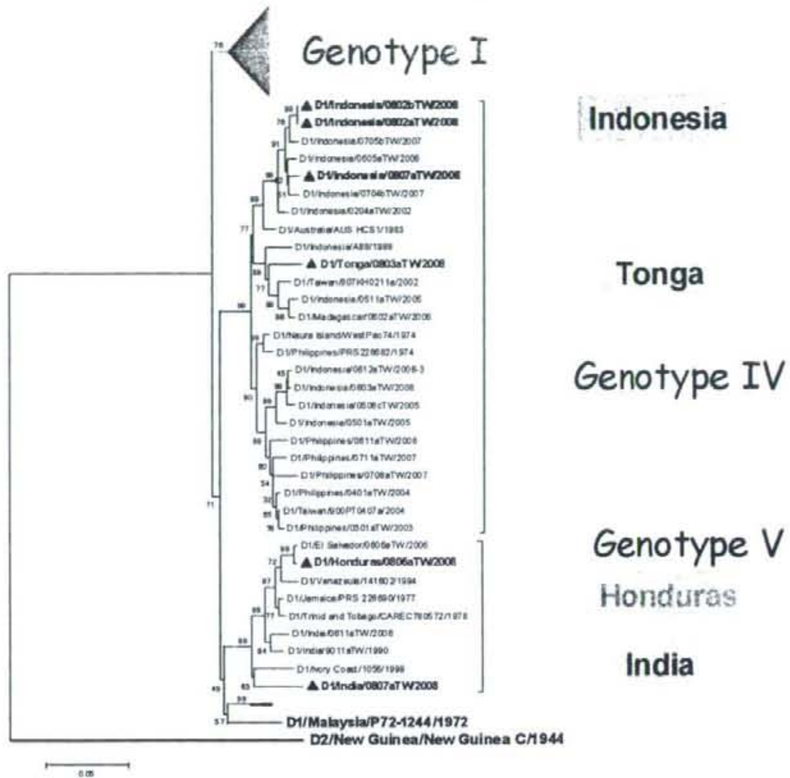

Figure 1. Phylogenetic trees derived from full-length E gene sequences of DENV-1 strains isolated from confirmed dengue cases in 2008, Taiwan. The strains isolated from imported and indigenous cases are designated in full triangles and full squares, respectively. The trees were constructed by the neighbor-joining method. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation. GenBank accession numbers are shown in the parentheses.

Phylogenetic tree of partial E1 gene (1044 nt) of CHIKV

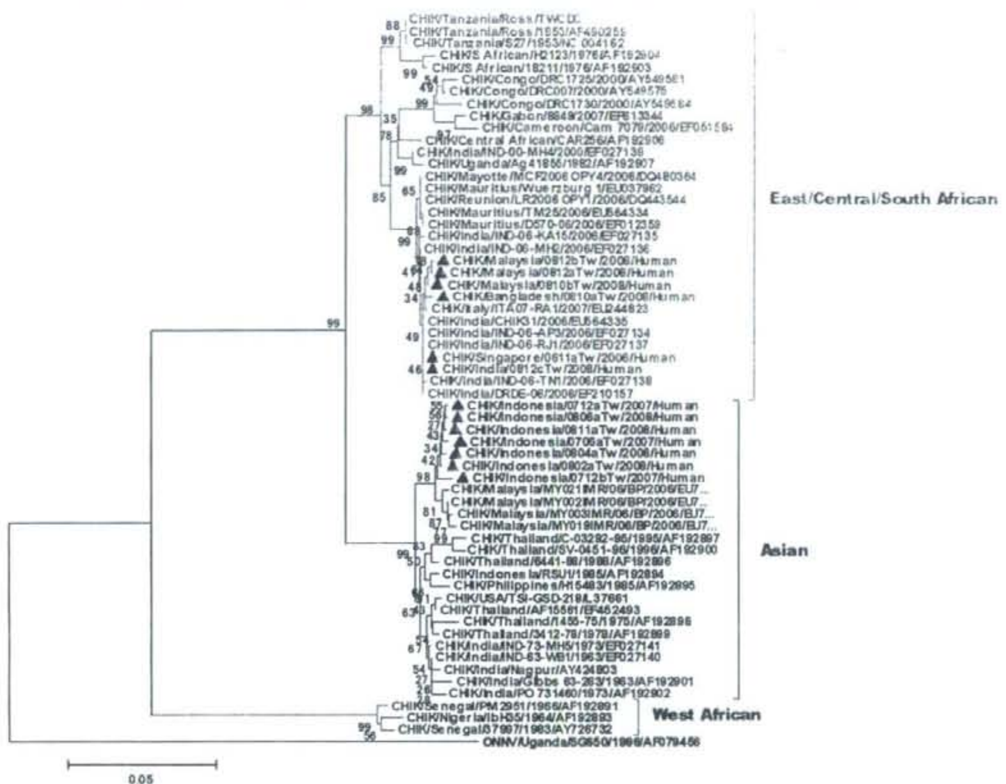

Figure 2. Phylogenetic relationships of chikungunya virus (CHIKV) isolates from 13 imported cases in Taiwan. The tree was constructed by the neighbor-joining method using partial nucleotide sequences of envelope protein 1 (E1) gene (1044 bp) of 60 CHIKV strains. O'nyong-nyong (ONN) virus sequence was used as the outgroup virus. The 13 imported CHIKV strains in Taiwan are designated by triangles. Viruses were identified using the nomenclature of virus/country/strain/year of isolation/GenBank accession number. The scale bar on the left indicates substitutions per site.

ANALYSIS OF GENE AND POLYPEPTIDE SEQUENCES OF DENGUE VIRUSES ENVELOPE AND NS1

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INTRODUCTION

Since first reported in 1968 (Sumarmo, 1987) dengue remain a major problem in Indonesia. During Jan-Nov 07, 127,687 cases with 1296 death were reported, which is 20% increase in morbidity compared to the same period in 2006 (WHO/SEARO). In Indonesia all serotypes are endemic. DHF have been reported in all provinces. (Setiati, 2007).

Dengue infections can manifest as mild dengue fever (DF) to severe dengue hemorrhagic fever/ dengue shock syndrome (DHF/DSS) or even death (WHO, 1997). Dengue infection is still a major problem in Indonesia. Morbidity rate of dengue infection is Indonesia is relatively higher compare to other countries. This is mainly due to the late treatment of severe cases. On the other hand, lack of early diagnostic tool, has lead to unnecessary hospitalization of unproven cases which cause inefficient use of limited facilities. Therefore availability of a simple laboratory test that can detect early cases of dengue infection is very important. Also, until now a test that can be prognostic in differentiating cases that may progress into DHF/DSS is not available. Availability of the tests mentioned above will be very useful in the management of dengue infection, and it will also improve efficiency of limited hospital facility usage and finally will reduce mortality caused by DHF.

At present, various approaches and methods for dengue diagnosis have been developed and widely used (Shu, 2004). However, there are still some weakness that must be overcome, such as : Hemaglutination inhibition assay needs pair samples (WHO, 1997), and antibody capture-based methods are usually positive on samples taken later in infection (3-7 days after infection); RT-PCR although sensitive and specific (Lanciotti, 1992), needs special care of specimen and can be done only in well-equipped laboratories; antigen-capture assays are more sensitive but until now the sensitivity to detect infection of all serotypes in both primary and secondary infection and specificity to determine serotype in secondary infection still must be improved. Recently NS-1-based diagnostic kits are becoming widely available (Alcon, 2003). However, the present ELISA format, despite quite easy to use is still expensive for low-income countries such as Indonesia.

In this study we will be more focus to continuing molecular epidemiology study based on env genes, as well as proteins which is the target of diagnostic test (NS-1), and the potential target of antiviral drugs and

vaccine, i.e. NS-3 protein. By studying those genes, we hope we can be able to evaluate use of NS-1 based giagnostic kits, and in the future develop diagnostic kits based on recent, indigenous, Indonesian strains.

Here we report the overall results from the study started in 2005.

METHODS:

1.. Source of the viruses, serology and RT-PCR.

Specimens were collected from hospitalized patients with suspected dengue infection in Jakarta within the period of February 2006 to march 2007. Serum samples collected in 2006 were serologically examined by Hemaglutination inhibition test. Rapid immunochromatographic assay (PanBio Inc., Brisbane, Australia) were done to year 2006 and 2007 specimens. The sera were also tested by RT-PCR (Lanciotti, 1992) to see the presence and type of virus.

2. Determination of viral nucleotide sequences

2.1. *RT-PCR and DNA sequencing*

To genotypically characterize the isolated viruses, nucleotide sequencing will be done. RNA were extracted from 140 ul of plasma using Viral RNA Isolation Kit (Qiagen, GmbH, Germany or Roche) according to the manufacturer's instruction. Complementary DNA (cDNA) strands were reverse-transcribed using Super Script III First Strand Synthesis System with Random hexanucleotide primer according to the manufacturer's instructions (Invitrogen). Amplification of DNA fragments were performed according to the annealing temperature corresponding primers used (Table 1) and length of the expected product. After amplification and purification, the DNA were sent to the DNA sequencing facility at the Eijkman Institute, Jakarta or Microbiology Laboratory of the Faculty of Siences, University of Indonesia. The sequences were determined using Taq Big Dye Deoxy Terminator Cycle sequencing kits.

RESULTS AND DISCUSSION:

Specimens

During year 2008 we continued analyzing 215 samples collected in 2006 and 2007. Viral isolation was attempted, but we failed to get virus isolated. The sequences studie were from RNA isolated from plasma specimens stored in –70°C. In total, cDNA had been prepared from 40 samples using random primers. Sequencing had been done to total . 10 samples (table 1). We sequenced mainly env and NS1 regions. Sequencing of NS3 is now in progress

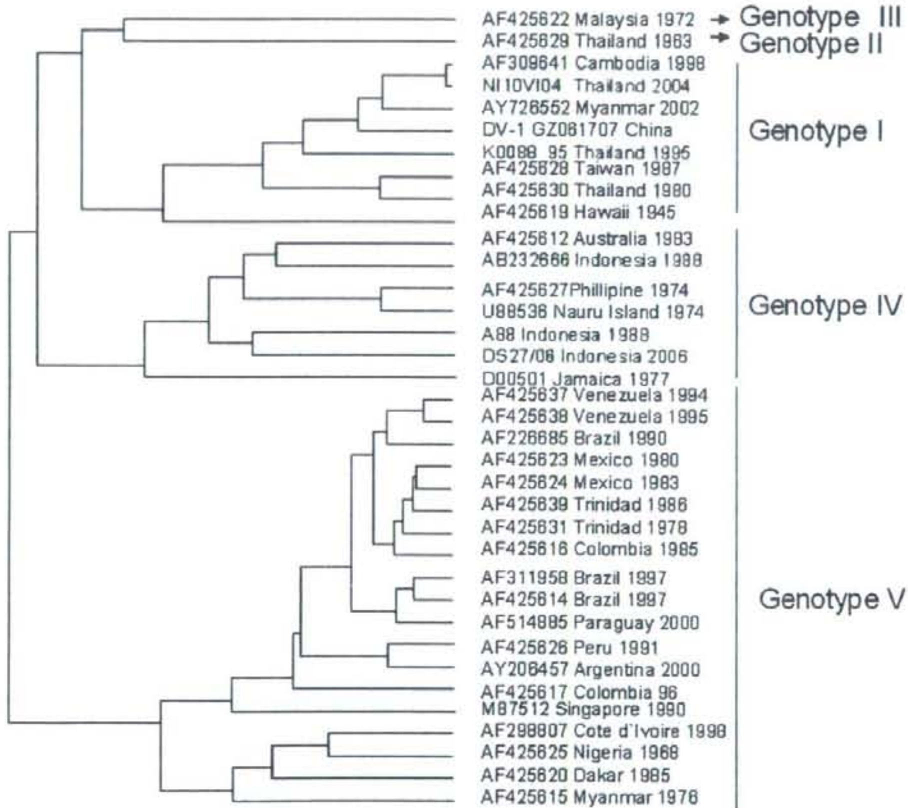
Table 1 : Dengue virus strains sequenced in this study

No	Strain	Year isolated	Clinical manifestation	Serotype	Genes sequenced
1.	DS27/06	2006	DHF-I	DENV-1	Env, NS1
2	DS31/06	2006	DHF-I	DENV-2	Env, NS1
3	DS24/06	2006	DF	DENV-2	Env
4	DS20/06	2006	DHF-II	DENV-2	Env
5	DSA06/07	2007	DHF-III	DENV-2	Env
6	DS 002/06	2006	DF	DENV-3	Env, NS1
7	DS 029/06	2006	DHF-II	DENV-3	Env, NS1
8.	DSA 02/06	2006	DSS	DENV-3	Env, NS1
9.	17/04	2004	DHF-II	DENV-3	Env, NS1
10.	DS22/07	2007	DHF-1	DENV-3	Env

Envelope region of DENV-1, DENV-2 and DENV-3

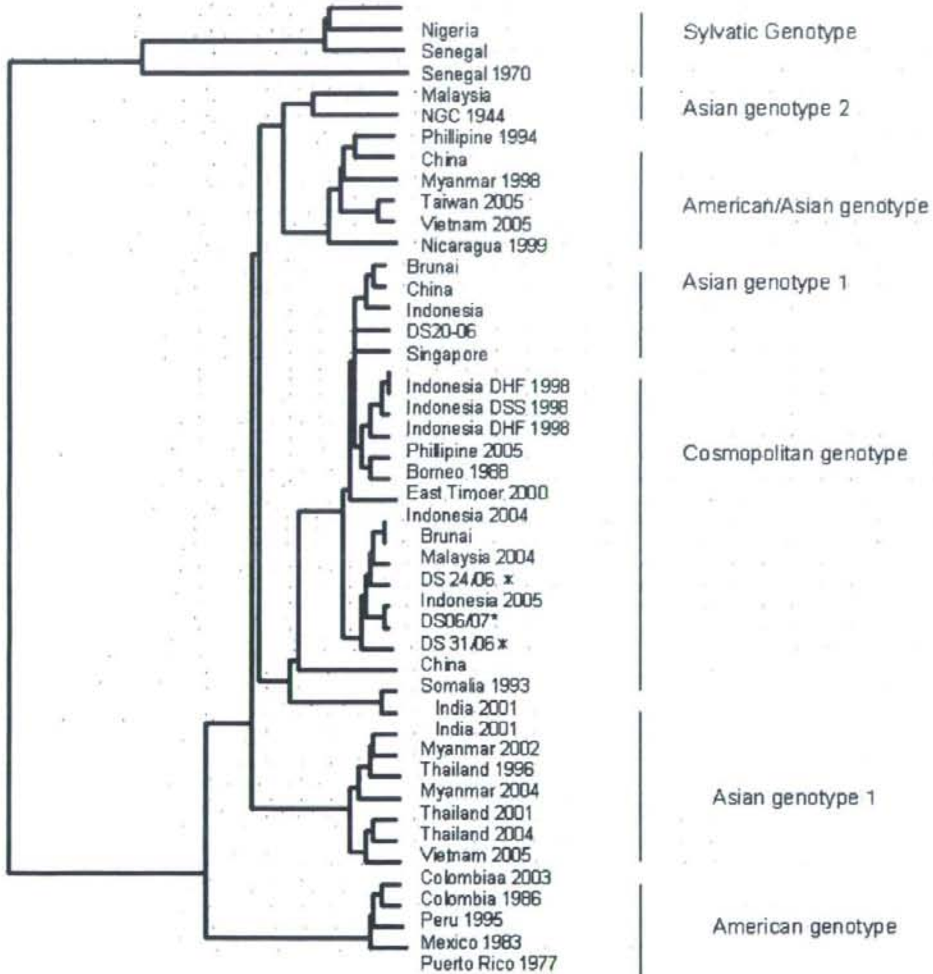
In this study, envelope genes of one DENV-1. four DENV-2 and five DENV-3 strains were sequenced (table 1). Phylogenetic analysis of DENV-1 was done according to the genotype classification by Goncalves et.al. (Goncalves, 2002). Thirty six strains including 1 strain from Indonesia and 33 other strains data obtained from Genbank were indluded in the analysis. The DENV-1 sequenced in this study was identified as genotype IV, together with the strains of 1988 (Fig 1).

Figure 1. A Phylogenetic tree of DENV-1. Maximum likelihood trees showing the phylogenetic relationships among 36 strains of DENV-1. The analysis was based on nucleotide sequences of the E gene. The genotype clusters are labeled according to the scheme of Goncalves, *et al.*, 2002. The name of isolates refer to country of origin, year of isolation and also diseases severity if available in GeneBank.



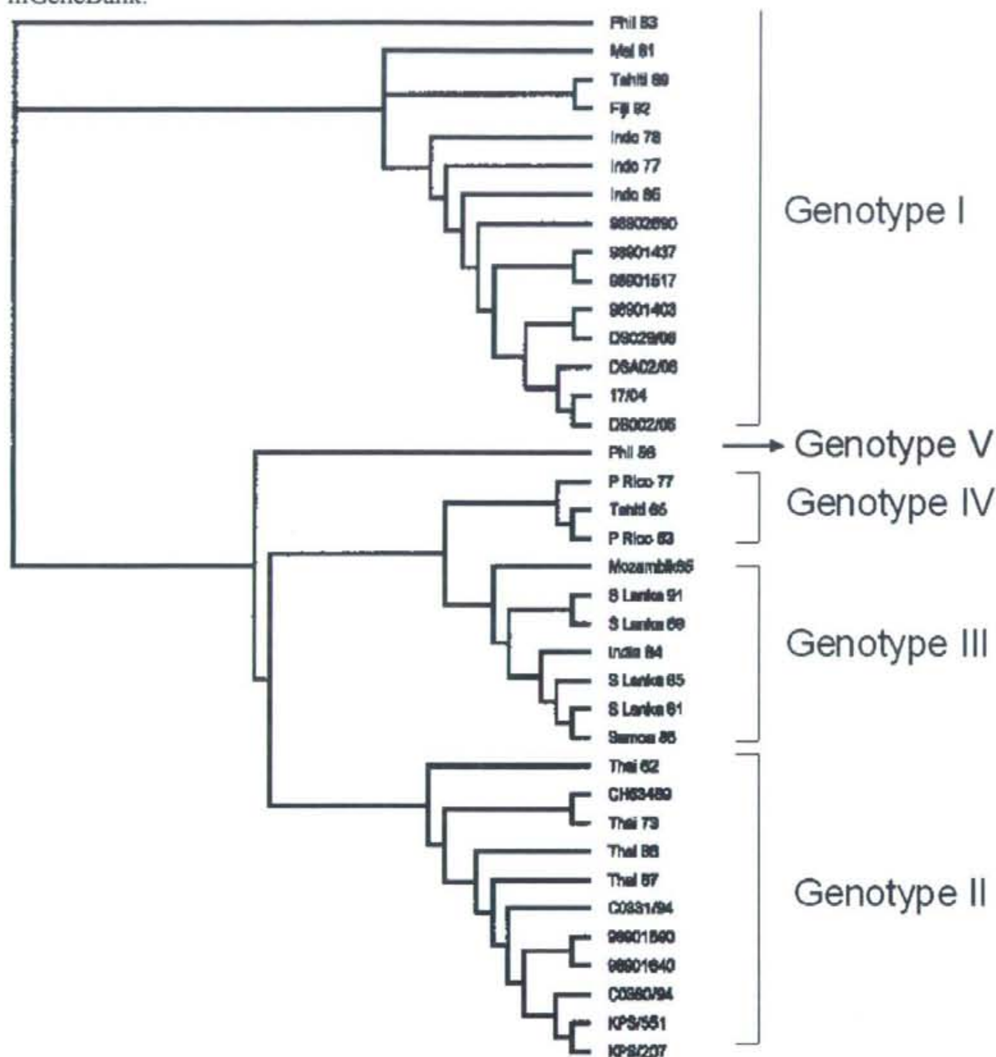
Phylogenetic analysis of DENV-2 was done according to the genotype classification by Huang et.al. (Huang, 2007). Forty-three strains data were included in the analysis. Four strains sequenced in this study, as well as 5 other strains from Indonesia and 33 from other countries data obtained from Genbank were included. The DENV-2 sequenced in this study were identified as cosmopolitan genotype and Asian genotype I. (Fig 2).

Figure 2. A Phylogenetic tree of DENV-2. The analysis was based on nucleotide sequences of the E gene. The genotype clusters are labeled according to the scheme of Huang, et al.(Huang,2007). Roman numerals denote the different genotypes of DV-2. An asterisk (*) indicates the strains sequenced in the present study. The name of isolates refer to country of origin, year of isolation and also diseases severity if available in GeneBank.



Phylogenetic analysis of DENV-3 was done according to the genotype classification by Zhang et.al. (Zhang, 2005). Thirty-seven strains data were included in the analysis. Five strains sequenced in this study, as well as 9 strains from Indonesia and 20 other strains data obtained from Genbank were included. The DENV-3 sequenced in this study were identified as genotype I. (Fig.3)

Figure 3. A Phylogenetic tree of DENV-3. The analysis was based on nucleotide sequences of the E gene. The genotype clusters are labeled according to the scheme of Zhang, et al., 2005. The name of isolates refer to country of origin, year of isolation and also diseases severity if available inGeneBank.



NS-1 region of DENV-1, DENV-2, and DENV-3.

As we have reported in 2008, we analyzed the NS-1 region of DENV-3 strains 17/04, DS 002/06, DS 029/06 and DSA 02/06 and compared them with 14 other strains data from the GenBank. The results showed that isolates from Thailand belong to one cluster, and strains from Indonesia belong to one other cluster. The highest nucleotide homology was between strain 98901517 (DHF) and strain 98901437 (DSS), and the lowest was between strain KPS/551 (DF) and strain DS 029/06 (DHF). We also analysed four B-cell epitopes reported by Roehrig et al.(1997) and Falconar (1997, 2008), For DENV-3 the. Epitopes analysed were LD2 (aa 25-33 : VHTWTEQYK), epitope 24A (aa 61-69: TRMENLLWKQ), epitope LX1 (aa113-119: YSWKTWGK) and epitope 24C (301-309: TTVSGKLIH). We found that those four epitopes were conserved in all 14 strains analysed.

In this year report we extend our study on NS-1 region of DENV-2 and DENV-1. we found that the strains we analyzed have the four B-cell epitopes stated above. Figure 6 shows the multialignment of NS1 peptide sequences of the three serotypes.

Figure 4. Phylogenetic tree of NS-1 region of DENV-3

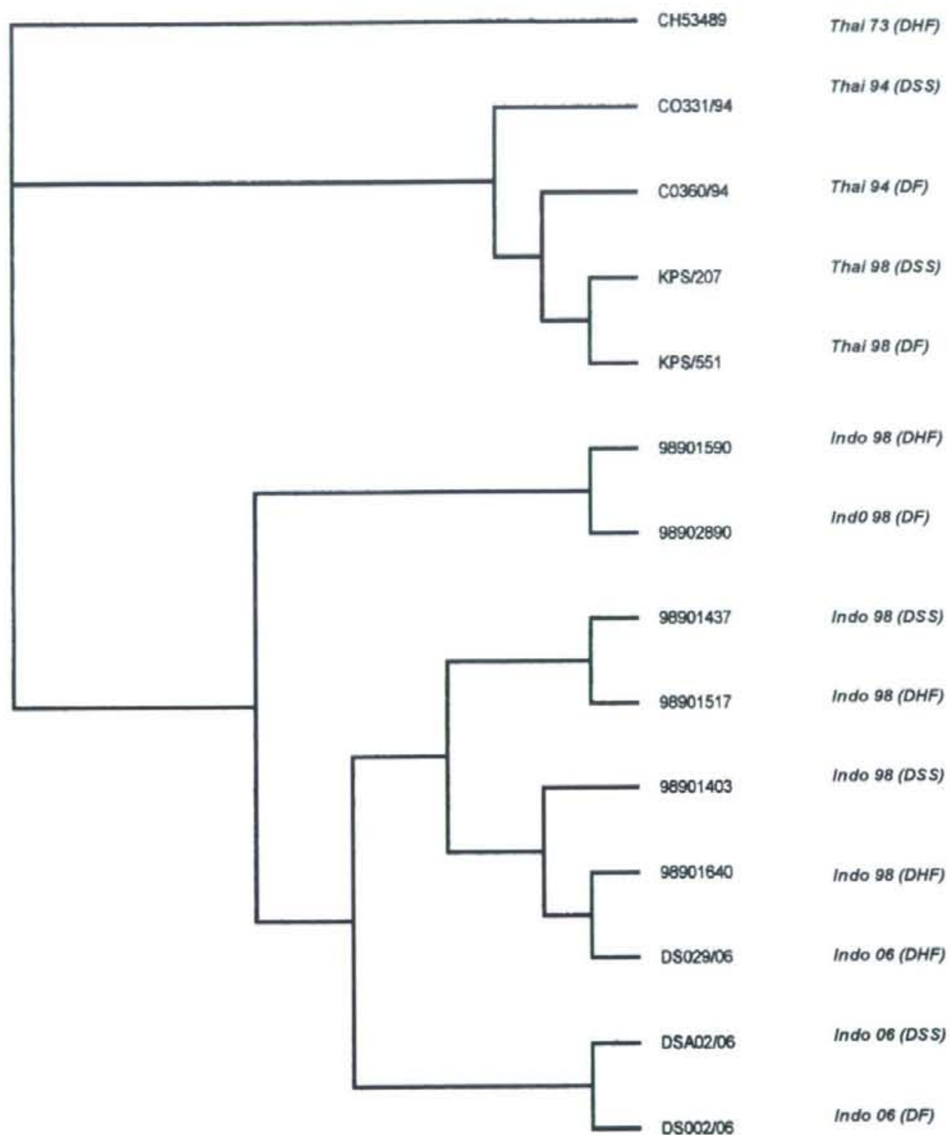


Fig. 5. Philogenetic analysis of the NS1 region of DENV-1