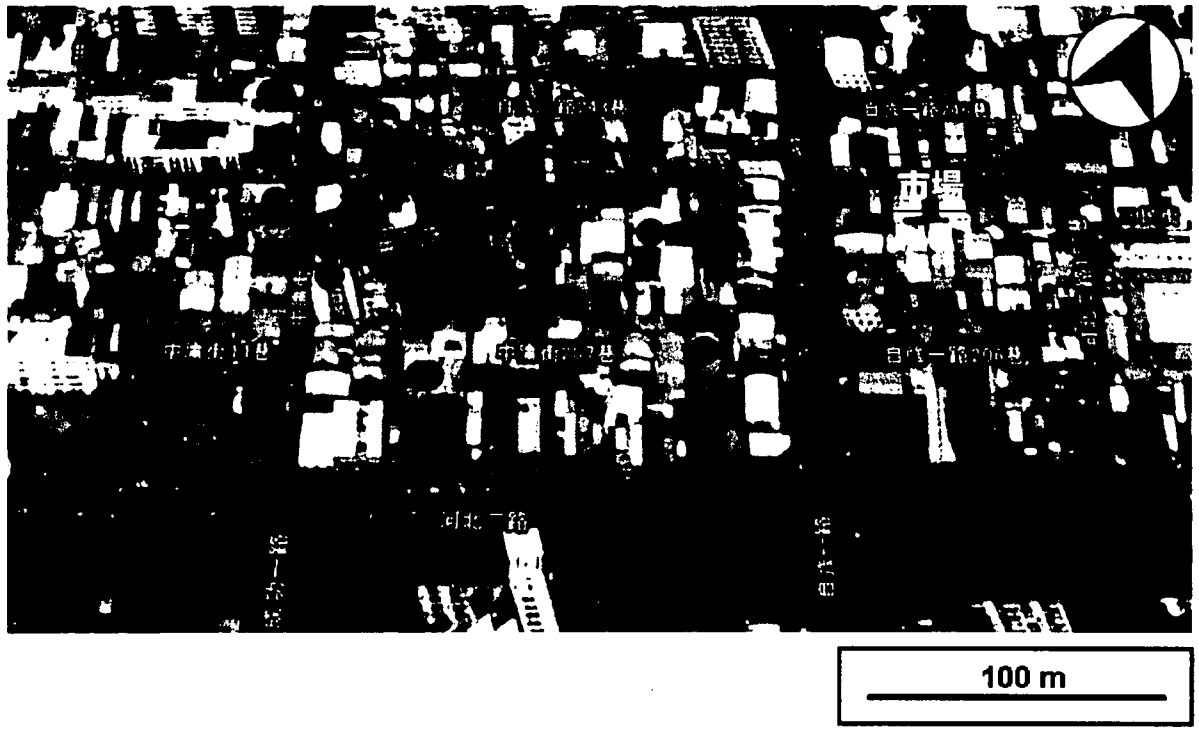

図３．台湾高雄市三民区における２００７年７月から８月までのデング熱流行状況：丸印がデング患者発生地点である．約１００m四方の狭い地区にデング患者が集中している．

Virological and serological surveillance of dengue fever/dengue hemorrhagic fever, Japanese encephalitis and chikungunya in Thailand, March, 2007 –February, 2008.

Surapee Anantapreecha
National Institute of Health, Nonthaburi, Thailand

1) Dengue fever/dengue hemorrhagic fever

From March 2007 to February 2008, National Institute of Health, Department of Medical Sciences received blood specimens from 2,722 suspected dengue case-patients (Table 1). According to the results of IgM and IgG antibody capture ELISA and/or virus isolation and/or RT-PCR, 74.1% of total cases were confirmed to be infected by dengue virus. Confirmed dengue cases were found all year round, but the most prevalent between June to October (Table 2). The majority of confirmed dengue cases were in the age group 10-14 years with 48.4% (Table 3). Of the total, 90.4% of confirmed dengue cases experienced secondary infection (Table 4). All four dengue serotypes were identified of which DEN-1 was the predominant with 54.9% (Table 5). All of the DHF cases caused by DENV-2 and DENV-4 were in secondary infection. 9.9% and 6% of the DHF cases caused by DENV-1 and DENV-3 were in primary infection (Table 6). During the period of study, twenty four isolated dengue viruses were selected to sequence which compose of DENV-1 from human 21 cases, from mosquito 1 case and DENV-4 from human 2 cases. Complete envelope protein of those viruses was characterized. The DENV-1 virus sequences were compared with DENV-1, which was isolated in 1964 in Thailand. We found that the genetic diversity of envelope protein of new and old DENV-1 virus is not so much about 8%. In addition to, 22 dengue viruses from Cambodian children plasma were sequenced. Ten cases were complete sequence of envelop protein and data were not yet analyzed.

2) Japanese encephalitis

From March 2007 to February 2008, a total of 689 cases of clinical diagnosed viral encephalitis were submitted from hospitals all over the country for laboratory examination.

According to the results of IgM-Capture ELISA, 106 cases (15.4%) were determined to be JE. Confirmed JE cases occurred in all the 4 regions in Thailand (Table 7). Confirmed JE cases occurred most in May (Table 8). The most affected age group was 10-14 years (Table 9)

3) Chikungunya

Form March 2007 to February 2008, National Institute of Health received sera from 27 suspected Chikungunya case-patients. According to the results of HI test for Chikungunya all cases were negative. We also did HI test for Chikungunya in 15 PUO case-patients and in 60 suspected dengue case-patients which serological and/or virological test for dengue were negative. There were all negative in PUO case-patients.There were 2 presumptive Chikungunya virus infection cases and 58 negative cases in suspected dengue virus case-patients.

Table 1 IgM and IgG antibody capture ELISA and/or virus isolation and/or RT-PCR results of suspected dengue case-patients, March, 2007 –February, 2008

Suspected dengue cases	Confirmed dengue cases	Negative	Uninterpretable
No.	No. (%)	No. (%)	No. (%)
2,722	2,016 (74.1)	222 (8.2)	484 (17.8)

Table2 Month distribution of confirmed dengue cases, March, 2007 –February, 2008

YEAR	Month	No.	%
2007	March	94	4.7
	April	87	4.3
	May	113	5.6
	June	260	12.9
	July	212	10.5
	August	271	13.4
	September	238	11.8
	October	268	13.3
	November	199	9.9
	December	183	9.1
2008	January	89	4.4
	February	2	0.1
Total		**2,016**	

Table 3 Age distribution of confirmed dengue cases, March, 2007 –February, 2008

Age group	No.	%
< 1	24	1.2
1-4	150	7.5
5-9	528	26.5
10-14	964	48.4
15-24	230	11.5
25-34	52	2.6
35-44	27	1.4
45-54	9	0.5
55-64	7	0.4
65 up	2	0.1
Total	**1993**	

Table 4 Laboratory feature of confirmed dengue case-patients with primary and secondary infection, March 2007-February 2008

Age	primary		secondary	
group	No.	(%)	No.	(%)
< 1	16	84.2	3	15.8
1-4	19	18.4	84	81.6
5-9	36	9.2	354	90.8
10-14	65	8.6	690	91.4
15-24	5	3.1	154	96.9
25-34	0	0.0	35	100.0
35-44	2	9.5	19	90.5
45-54	0	0.0	6	100.0
55-64	0	0.0	5	100.0
65 up	0	0.0	2	100.0
Total	**143**	**9.6**	**1352**	**90.4**

Table5 Proportion of each of the four serotypes, March 2007-February 2008

Suspected dengue cases	DENV-1		DENV-2		DENV-3		DENV-4	
	No.	%	No.	%	No.	%	No.	%
1069	587	(54.9)	140	(13.1)	233	(21.8)	109	(10.2)

Table6 Ratio between primary and secondary infection among DF and DHF cases caused by each of four dengue virus serotypes from Mar 2007-Feb 2008

	DENV-1		DENV-2		DENV-3		DENV-4		Total	
Diagnosis	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**
DF										
Primary	26	17.7	0	0.0	11	15.7	1	2.9	38	12.7
Secondary	121	82.3	47	100.0	59	84.3	34	97.1	261	87.3
total	147		47		70		35		299	
DHF 1, 2										
Primary	21	9.7	0	0.0	6	6.9	0	0.0	27	7.3
Secondary	195	90.3	32	100.0	81	93.1	37	100.0	345	92.7
total	216		32		87		37		372	
DSS										
Primary	3	11.5	0	0.0	0	0.0	0	0.0	3	5.9
Secondary	23	88.5	8	100.0	13	100.0	4	100.0	48	94.1
total	26		8		13		4		51	
DHF 1, 2+DSS										
Primary	24	9.9	0	0.0	6	6.0	0	0.0	30	7.1
Secondary	218	90.1	40	100.0	94	94.0	41	100.0	393	92.9
total	242		40		100		41		423	
DF+DHF 1, 2 +DSS										
Primary	50	12.9	0	0.0	17	10.0	1	1.3	68	9.4
Secondary	339	87.1	87	100.0	153	90.0	75	98.7	654	90.6
total	389		87		170		76		722	

Table 7. Region distribution of confirmed JE cases.

Area	No.	%
Central	39	36.8
North	46	43.4
Northeast	11	10.4
South	10	9.4
Total	**106**	**100.00**

Table 8. Month distribution of confirmed JE cases

YEAR	Month	No.	%
2007	March	10	9.4
	April	11	10.4
	May	21	19.8
	June	16	15.1
	July	18	17.0
	August	14	13.2
	September	4	3.8
	October	6	5.7
	November	3	2.8
	December	3	2.8
2008	January	0	0
	February	0	0
Total		**106**	**100.00**

Table 9. Age distribution of confirmed JE cases

Age group	No.	%
< 1	1	0.9
1-4	15	14.1
5-9	28	26.4
10-14	44	41.6
15-24	8	7.6
25-34	3	2.8
35-44	2	1.9
45-54	2	1.9
55-64	1	0.9
65 up	2	1.9
Total	**106**	**100.00**

Characterization of dengue virus prevalence in Taiwan for establishment of the laboratory network for molecular epidemiology of dengue and other mosquito-borne viruses prevalent in Asia

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Summary:

Recent molecular epidemiological study had demonstrated that dengue is not endemic in Taiwan and constant importations of multiple dengue viruses (DENVs) from the neighboring Southeast Asian countries were responsible for each year's local outbreaks. To reduce the imported DENVs and their local spread, we establish laboratory-based dengue surveillance system for early identification of imported cases. In addition, we conducted molecular epidemiological study to monitor the local transmissions of multiple DENV strains co-circulated in southern Taiwan. Among the 170 confirmed imported dengue patients in 2007, 71 (41.8%) were detected through airport fever screening surveillance system. From the acute phase serum samples of all imported dengue cases, 45 DENV-1, 27 DENV-2, 20 DENV-3, and 5 DENV-4 strains were isolated. For local dengue outbreaks in Taiwan, a total of 1978 dengue patients were laboratory confirmed with 11 cases of dengue hemorrhagic fever (DHF) and 1 death. Sequence analyses from more than 171 DENV isolates obtained from acute phase serum samples of indigenous cases showed that 4 different DENV strains (3 DENV-1, and 1 DENV-2) were co-circulated in Tainan City, Tainan County, Kaohsiung City and Kaohsiung County with limited overlap in the transmission areas. Phylogenetic analysis showed that these DENVs were imported from Thailand, Malaysia, Vietnam, and Vietnam, respectively

For other arboviruses, we identified 3 imported Chikungunya (CHIK) case from airport fever screening surveillance in 2007. All 3 travelers were returned back from Indonesia. Phylogenetic analyses of isolated CHIKVs showed that these CHIKV strains belong to Asia genotype.

Purpose:

Emergence of pathogenic microorganisms is an increasing concern. Infection by mosquito-borne viruses is a foremost problem in Asia. Understanding the epidemiological situations of the diseases and the phenotypic and genotypic characteristics of viruses contributes to

the development of new strategies for control and prevention. In order to promote communication and exchange of the information of dengue and other mosquito-borne viruses, laboratory network between Asia and Pacific Rim should be developed and strengthened.

Methods:

1 Clinical samples and laboratory diagnosis

Human serum samples from clinically suspected DENV and other arbovirus infections were submitted to the Vector-Borne Viral and Rickettsial Diseases Laboratory, Research and Diagnostic Center, Taiwan Centers for Disease Control (Taiwan CDC), Department of Health, for laboratory diagnosis. A confirmed dengue or CHIK case was defined as febrile illness associated with a positive real-time RT-PCR test (1-3), the detection of DENV- or CHIKV-specific IgM and IgG antibodies (4-5), or isolation of DENV or CHIKV (3). A multiplex one-step real-time RT-PCR was developed to simultaneously detect and differentiate various flaviviruses and alphaviruses in the acute-phase serum samples using group-specific and virus-specific primers. In addition, a specific flavivirus/alphavirus-specific capture ELISA was developed to detect and differentiate various flavivirus/alphavirus infections.

2 Virus isolation and identification

DENVs and CHIKVs were isolated from the acute phase serum samples of confirmed cases. The virus isolation was performed using mosquito cell line (clone C6/36 of *Aedes albopictus* cells). For each acute phase serum, 4 μl of serum sample was diluted in 200 μl cultured medium (RPMI, Gibco/BRL, Life Technologies, containing 1% FCS) and added to a 96-well microtiter plate, 50 μl/well in quadruplicate. Then, 10^5 cells/100 μl/well of C6/36 cell line were added into the microtiter plate and incubated at 37℃ for 2-7 days. Cells were harvested and virus isolates were identified by the indirect fluorescent antibody test with virus group-specific and serotype-specific monoclonal antibodies.

3. Primers used for RT-PCR and nucleotide sequencing of DENV

The diagnostic tests for flavivirus infection from fevered patients on the basis of the results of one-step SYBR Green I-based real-time reverse transcription (RT)-PCR and envelope/membrane-specific capture IgM and IgG ELISA had been described previously (1). To screen viremic fever patients with alphavirus infection, a multiplex one-step SYBR Green I-based real-time RT-PCR was

developed. A cocktail consisted with three sets of primers were mixed and used for RT-PCR screening. The alphavirus-specific primer set (AL-2: 5'-AAG CTY CGC GTC CTT TAC CAA AG-3' and AL-3: 5'-GTG GTG TCA AAC CCT ATC CA-3') targeted a consensus region of the nonstructural protein 1 (nsp1) genes to detect all alphaviruses. The CHIKV-specific primer set (F-CHIK: 5'-AAG CTY CGC GTC CTT TAC CAA AG-3' and R-CHIK: 5'-CCA AAT TGT CCY GGT CTT CCT-3') targeted a region of the envelope protein 1 (E1) gene of CHIKVs (6). The Ross River virus-specific primer set (RRV-1: 5'-GGG TAG AGA GAA GTT YGT GGT YAG-3' and RRV-2: 5'-CGG TAT ATC TGG YGG TGT RTG C-3') targeted a region of the envelope protein 2 (E2) gene of Ross River virus. Positive results were then confirmed by gene sequence analysis, virus isolation, and serological test.

4. **Preparation of viral RNA, RT-PCR amplification and nucleotide sequencing**

Viral RNAs were extracted from either acute phase serum samples or culture supernatant of C6/36 cell line infected with each of the isolated DENV or CHIKV strains using the QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nucleotide sequences of partial NS5 gene fragment of DENV were determined directly from acute phase serum samples using RT-PCR product of one-step SYBR Green I quantitative RT-PCR (1). Partial NS5 gene sequencing was routinely performed to detect and differentiate serotype and genotype of the newly identified DENVs. For full-length structure gene sequencing, extracted viral RNA from culture supernatant of C6/36 cell line infected with each of the isolated DENV strains was used as a template for cDNA synthesis, which subsequently was used for PCR amplification. Two overlapping PCR products spanning the full-length structure gene were purified from agarose gels and directly sequenced in both directions using ABI Prism automated DNA sequencing kit and ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer's protocol. Overlapping nucleotide sequences were combined for analysis and edited with the Laser software package (DNASTAR Inc, Madison, WI).

5. **Phylogenetic analysis**

Phylogenetic analyses were conducted using PHYLIP (version 3.6) or MEGA version 3.1. Genetic distances were calculated by using Kimura 2-parameter distance algorithm with 1,000 bootstrap replicates. Neighbor-Joining method was

used to generate the phylogenetic trees.

Results:

1. Imported dengue cases in Taiwan, 2007

A total of 170 laboratory confirmed imported dengue cases were identified in Taiwan in 2007. Among these patients, 71 (41.8%) 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 three exceptions, which were Solomon Islands, India and Bangladesh. Similar to our previous report during the 2002-2006 periods, Indonesia, Vietnam, the Philippines and Thailand are on the top list of importing countries (2). 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, 45 DENV-1, 27 DENV-2, 20 DENV-3, and 5 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 1978 dengue patients were laboratory confirmed with 11 cases of DHF and 1 death in 2007. 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. From more than 171 DENV isolates obtained from acute phase serum samples of corresponding indigenous cases, 159 DENV-1 and 12 DENV-2 were selected for full-length structure genes sequencing. The results showed that 4 different DENV strains (DENV-1-1, DENV-1-2, DENV-1-3 and DENV-2) were co-circulated in Tainan City, Tainan County, Kaohsiung City and Kaohsiung County with limited overlap in the transmission areas.

3. Nucleotide sequencing and phylogenetic analysis

Figures 1 and 2 showed the evolutionary relationship of DENV-1 and DENV-2 strains using E genes sequences available from Taiwan CDC dengue genomic database and GenBank. Phylogenetic tree was constructed by neighbor-joining and the DENV strains responsible for the

local dengue epidemics in 2007 were highlighted. Phylogenetic analyses showed that the 4 DENV strains; DENV-1-1, DENV-1-2, DENV-1-3 and DENV-2 causing local outbreaks were most closely related to D1/Thailand/0605aTW/2006, D1/Malaysia/0606aTW/2006, D1/Vietnam/0709aTW/2007 and D2/Vietnam/0510cTW/2005 imported strains and were imported from Thailand, Malaysia, Vietnam, and Vietnam, respectively.

4. CHIKV identification and characterization

Since the first detection of imported CHIK case at the Taoyuan international airport in Taiwan through fever screening surveillance system on 20 November 2006, 3 more imported cases all returned from Indonesia were detected in 2007. Real-time RT-PCR screening showed high viremia of alphavirus, but not flavivirus on day 2-3 acute phase serum samples of these patients. Serodiagnosis showed positive seroconversions on all cases using immunofluorescent antibody assay (IgM+G+A titers $\geq$ 640) and ELISA. The CHIKVs were isolated from the acute phase serum samples and the full genome sequences were determined. Phylogenetic analysis showed that these CHIKV strains belong to Asian genotype (data not shown). Figure 3A showed the CHIKV-specific IgM and IgG ELISA. The convalescent-phase serum samples from the first two patients with CHIKV infections (CK95 and CK96) showed positive IgM and IgG responses to CHIKV but not to DENV and Ross River virus.

Discussion:

In a world of global village, it is a difficult challenge to effectively control imported infectious diseases. The successful application of fever screening surveillance at airport in the identification of imported dengue cases had led us to expand this program. We started to screen both Flavivirus and Alphavirus from the acute phase serum samples of fever patients in January 2006. We have so far detected more than 250 imported dengue cases and 4 CHIK cases during 2003-2007 in Taiwan by airport fever screening surveillance. Our results witness the growing global threat of dengue in a new era with unparalleled human movement. The study demonstrated the growing spreads and co-circulations of DENVs and CHIKVs in different geographic areas of Southeast Asian countries in recent years. The genetic database generated from these isolated imported 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 Taiwan, 2007

Country of origin	Total cases	DENV Serotype				
		DENV-1	DENV-2	DENV-3	DENV-4	unknown
Vietnam	55	20	10	2	0	23
Indonesia	48	8	10	8	2	20
Philippines	22	3	6	8	0	5
Cambodia	12	2	1	1	0	8
Thailand	10	4	0	0	2	4
Malaysia	7	3	2	1	0	1
Myanmar	7	1	0	2	0	4
Singapore	4	0	4	0	0	0
Solomon Islands	2	0	0	0	1	2
India	1	0	0	0	0	1
Bangladesh	1	0	0	1	0	0
Laos	1	0	0	0	0	1
Total	170	41	33	23	5	68

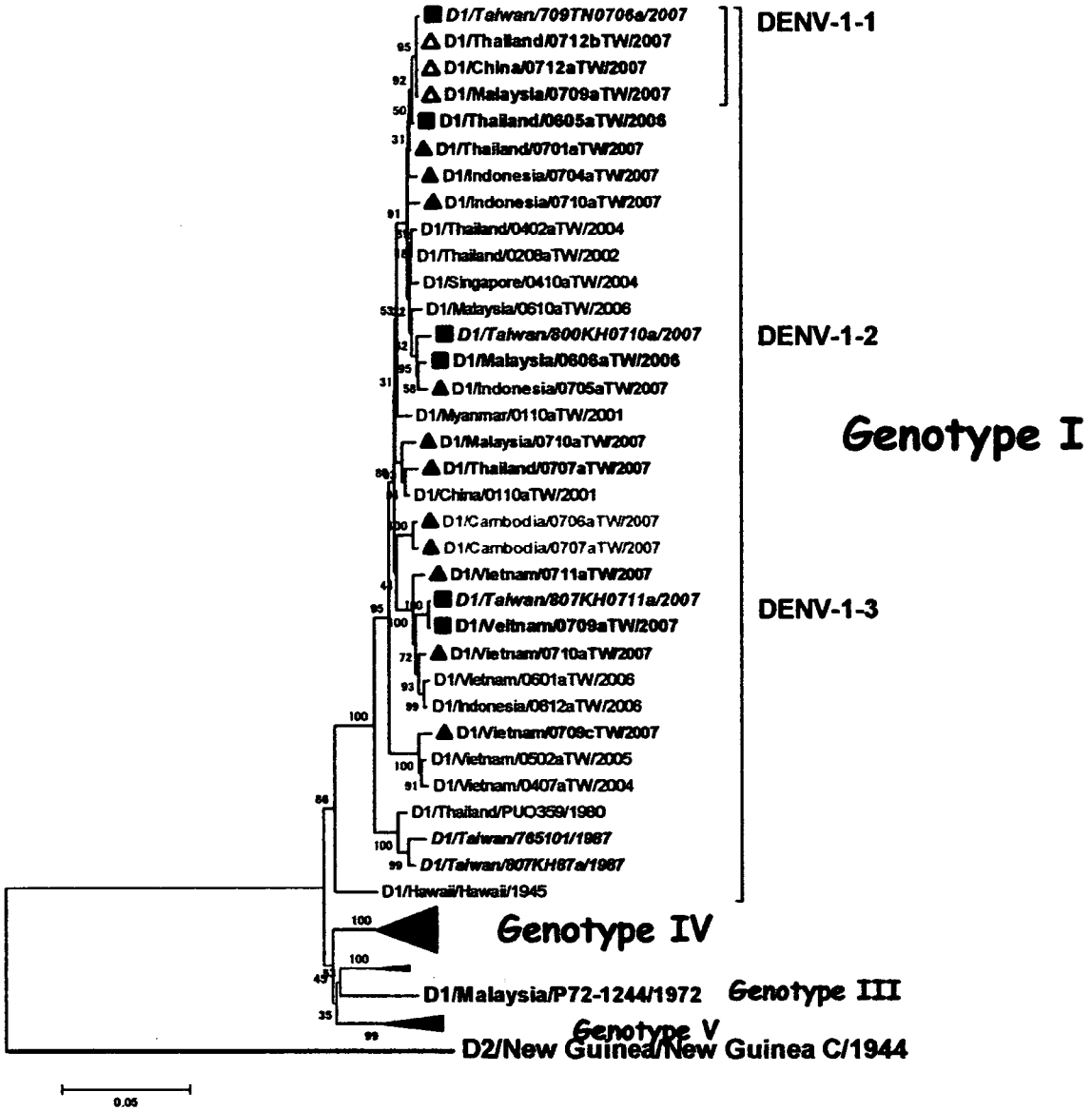


Figure 1. Phylogenetic tree derived from full-length E gene sequences (1485 b.p.) of DENV-1 strains using Neighbor-Joining method.

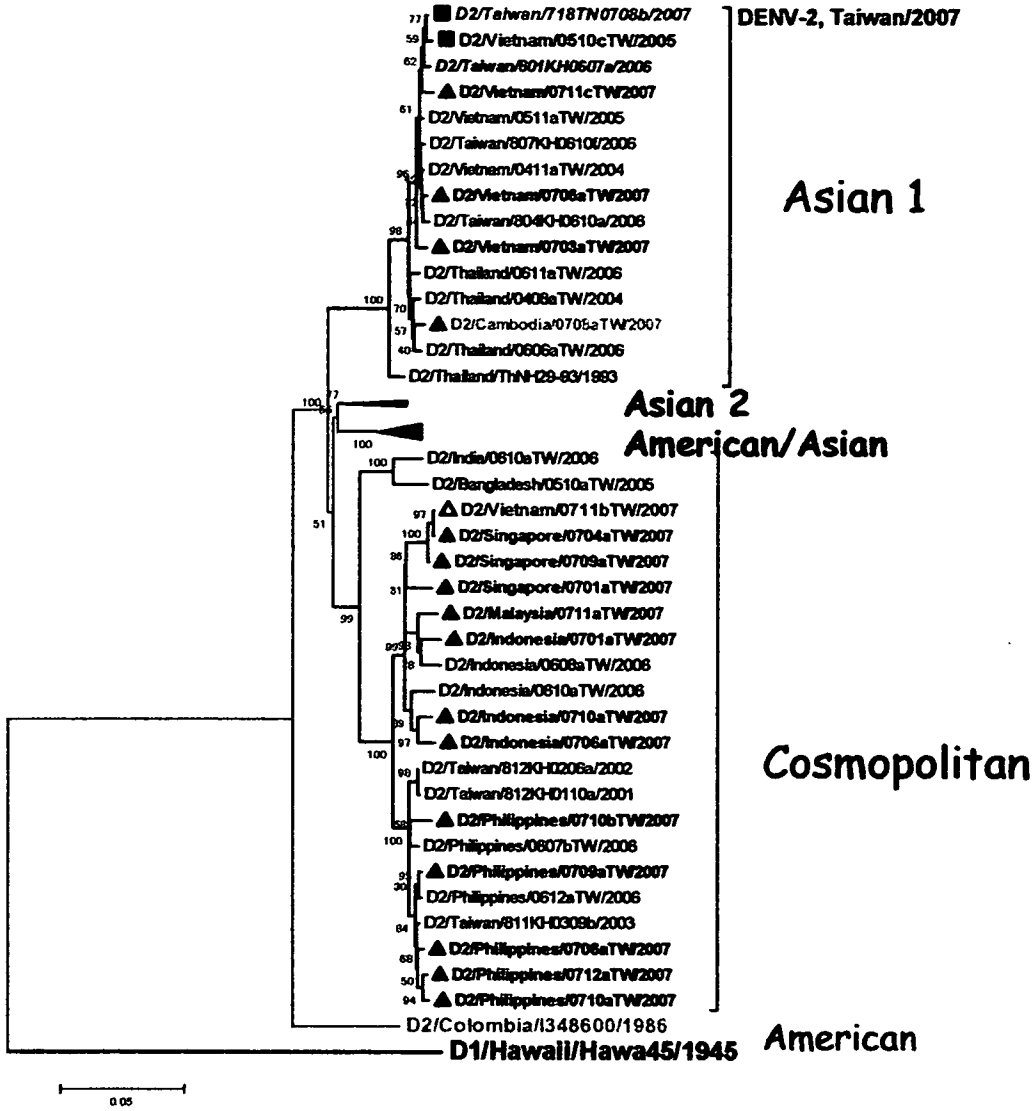
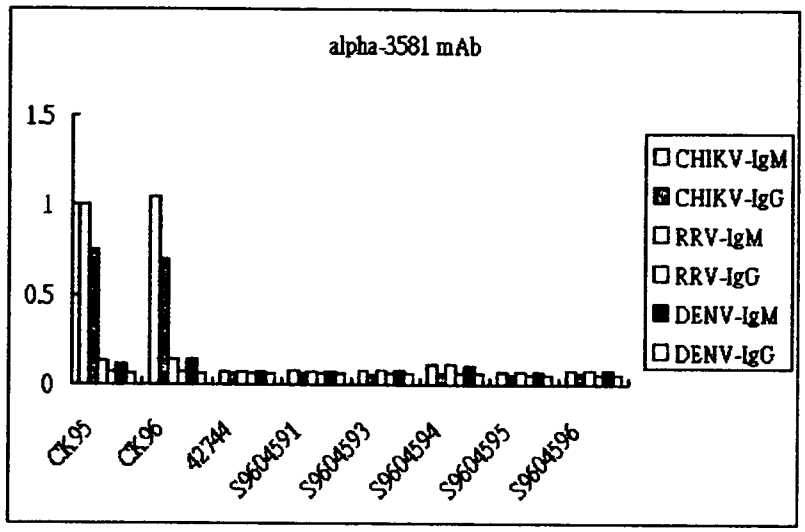


Figure 2. Phylogenetic tree derived from full-length E gene sequences (1485 b.p.) of DENV-2 strains using Neighbor-Joining method.

3A



3B

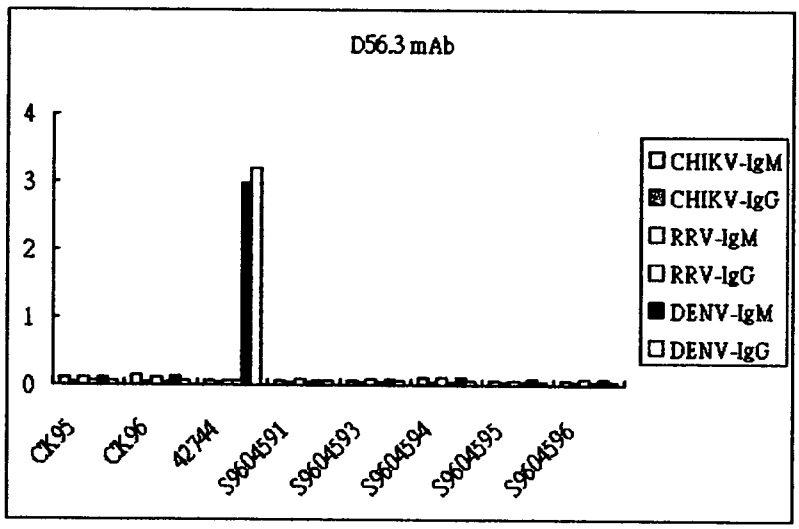


Figure 3. Chikungunya virus- and Flavivirus-specific capture IgM and IgG ELISA. Figure 3A showed the chikungunya virus-specific IgM and IgG ELISA. The convalescent-phase serum samples from the first two patients with chikungunya virus infections (CK95 and CK96) showed positive IgM and IgG responses to chikungunya virus but not to dengue virus and Ross River virus. Figure 3B showed the dengue-specific IgM and IgG ELISA. The convalescent-phase serum sample from the dengue patients (42744) showed positive IgM and IgG responses to dengue virus but not to chikungunya virus and Ross River virus.

Sequence analysis of the envelope genes of dengue viruses type 1, 2, and 3 and NS-1 gene of dengue virus type 3 isolated in Jakarta

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ABSTRACT

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. In this study, from March 2006 to April 2008 a total of 238 plasma specimen from children and adults at the Cipto Mangunkusumo Central Hospital in Jakarta were examined for dengue viruses. We sequenced one DENV-1, two DENV-2 and four DENV-3. We found that among year 2006 isolates of DENV-1 belongs to genotype IV, DENV-2 belong to genotype cosmopolitan and DENV-3 sequenced in this study belong to genotype I . Sequencing of year 2007 isolates is now in progress.

INTRODUCTION

Dengue viruses are serious cause of mortality and morbidity in tropical and sub-tropical countries of the world. More than 2.5 billion people live in the area with a risk of dengue virus infection (WHO, 2002). Advances in technology of gene cloning, nucleotide sequencing and gene expression have facilitated the understanding of the molecular biology of dengue viruses. Dengue virus genome is single stranded, positive sense RNA and composed of approximately 10,600 nucleotides (Rice et al., 1985). It contains a single open reading frame that is flanked by two un-translated region; 5` and 3` un-translated region (UTR). Dengue virus virion is composed of 3 structural proteins; core protein (C), membrane protein (M), and envelope protein (E). Seven non-structural proteins are also present; NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Chambers et al., 1990). The amino acid differences of dengue viruses have been implicated to the pathogenesis of DHF (Mangada and Igarashi, 1998; Pandey and Igarashi, 2000).

Dengue epidemic occurred at regular intervals in Indonesia, since it was first recognized in Java (Pratana et al., 1970). However, there have been limited reports of the diversity of dengue viruses isolated in Indonesia. Analysis of the viruses isolated from DHF patients in Indonesia from 1975 to 1978 demonstrated that all 4 dengue viruses were endemic in Jakarta, but DV-3 was the dominant (Gubler et al., 1979; Lee et al., 1993). Dengue 3 virus was also the most frequently isolated virus outside Jakarta and had the widest distribution in Indonesia (Gubler et al., 1979; Corwin et al., 2001).

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). The