に再度採血を実施してワクチン接種前後の中和抗体価を比較した。

2)中和抗体価測定

両血清について、JEV（Beijing−1株）と、WNV（NY 株）に対する中和抗体価を、50%フォーカス減少法（$FRNT_{50}$）を用いて測定した。このとき、血清希釈 10 倍以上で中和活性を示した血清を中和抗体陽性とした。幾何平均抗体価は、中和抗体価 10 倍未満を 0.5 として算出した。

なお、本研究は大阪府立公衆衛生研究所の倫理審査委員会で承認を受けており、検体提供者へはインフォームドコンセント及び検体の匿名化など倫理面への配慮がなされている。

3)国内ネットワークの確立

近畿ブロック地方衛生研究所のアルボウイルス担当者研修会を開催し、リアルタイム PCR と各種血清診断法について参加型実習を実施した。

C. 研究結果

1)日本脳炎ワクチンの効果

ワクチン被接種者 80 名のうち、29 名はワクチン接種前より JEV に対する中和抗体を保有しており、そのうち 2 名は WNV 中和抗体も保有していた。ワクチン接種により JEV 中和抗体価が上昇したのは 71 名(71/80, 88.8%)で、JEV に対する幾何平均抗体価は接種前 2.1 倍、接種後 49.3 倍と約 23 倍抗体価の上昇が認められた。 ワクチン接種前より抗体を保有していた 29 名は、接種により全員抗体価が上昇した。ワクチン接種によって抗体価が上昇しなかった 9 名のうち、3 名について追加接種を実施したところ、抗体価の上昇がみられた。ワクチン接種による副反応は、2 人から報告された（38℃の発熱、強い倦怠感：どちらも接種当日から約 2 日間、20 代女性）。

2) WNVに対する交差中和抗体の獲得

ワクチン接種により、WNV に対する中和抗体価が上昇したのは 28 名で、JEV 抗体陽性者の 39.4%(28/71)が WNV に対して抗体価 10 倍以上の交差中和を示し、WNV に対する幾何平均抗体価は接種前 0.6 倍、接種後 2.1 倍と約 3.9 倍上昇した。 JEV に対する中和抗体価が 40 倍以上ある場合、51.9%(27/52)に交差中和が見られた。JEV と WNV に対する中和抗体価の相関性はワクチン接種後に見られ、JEV に対する中和抗体価が高いほど WNV 交差中和抗体価が高い値となった。日本脳炎ワクチン接種による WNV 交差中和抗体の獲得には、年齢による傾向は認められなかった。

3) 国内ネットワークの確立

研修会に参加した 11 衛研 16 名に対し、実験室診断法の実習を行い国立感染症研究所より分与されたポジティブコントロールを配布した。また地方衛生研究所、検疫所、保健所、大阪府医師会を対象としてアルボウイルスに関する講演会を開催した。

D. 考察

日本脳炎ワクチン接種により、有意な抗体価の上昇が見られたことから、新しい日本脳炎ワクチンは成人に対する追加接種にも有効であると考えられた。ただし、一度の接種では抗体価が上昇しない場合が 1 割ほどあることが想定された。

また、JEV 中和抗体価が上昇した対象者の 4 割が WNV に対して交差中和を示し、JEV に対する中和抗体価が 40 倍以上の場合約半数に交差中和が見られたことから、WNV が JEV と同じく中和抗体価 10 倍以上で感染防御できると推論すると、日本脳炎ワクチン免疫によっても、JEV に対する中和抗体価を高く維持すれば、WNV を感染防御できる可能性が示唆された。

本結果では、昨年度よりも低い JEV 中和抗体価から WNV 交差中和抗体がみられた。この理由として、ワクチンの製造法の違いによる抗原性などの違いによって、獲得する抗体の反応性に差が生じていることなどが考えられる。

E. 結論

本研究結果から、日本脳炎ワクチン免疫により、WNV に対する交差中和抗体が誘導されることが示された。 また、追加接種後の JEV に対する中

和抗体価が40倍以上の場合、約半数にWNVに対する交差中和が見られたことから、日本脳炎ワクチンを追加接種することで、WNVを感染防御できる可能性があることが示唆された。

G. 研究発表

1.論文発表

なし（予定あり）

2.学会発表

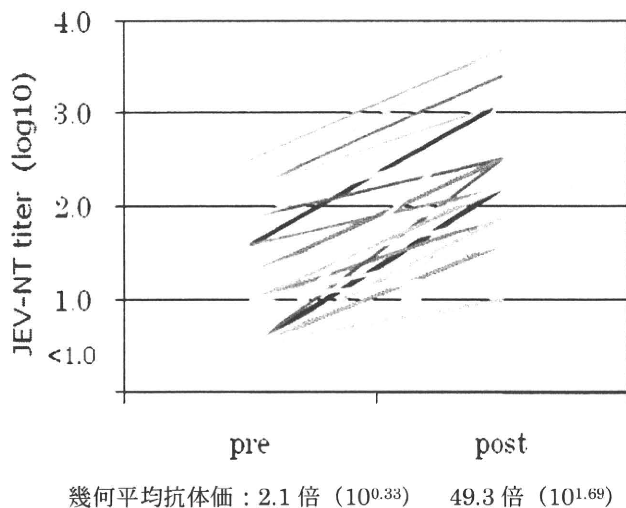
青山幾子、弓指孝博、高崎智彦、林昌宏、加瀬哲男、高橋和郎. 日本脳炎ワクチン接種によって獲得されるウエストナイルウイルスに対する交差中和抗体の検討. 第58回日本ウイルス学会学術集会 2010年10月（徳島）

H. 知的財産権の出願・登録状況　　なし

表1 ワクチン接種前後のJEVに対する年齢別中和抗体価

Age	all	pre JEV-NT							positive	post JEV-NT											positive
		<10	10	20	40	80	160	320	%	<10	10	20	40	80	160	320	640	1280	2560	5120	%
20-29	14	2	3	4			4	1	85.7				1	3	3	3		1	1	2	100
30-39	18	13		4	1				27.8	1		2	1	5	5	3		1			94.4
40-49	19	12	2	2	1	2			58.3	3	3	3	1	4	2	1	1	1			84.2
50-59	22	20	2						9.1	4	3	6	4	2	2		1				81.8
60-69	7	4	2	1					42.9	1		2	2		2						85.7
all	80（人）	51	9	11	2	2	4	1	36.3	9	6	13	9	14	14	7	2	3	1	2	88.8

pre：ワクチン接種前血清　post：ワクチン接種御血清　　JEV-NT：JEV neutralizing antibody titer by $FRNT_{50}$



幾何平均抗体価：2.1倍（$10^{0.33}$）　49.3倍（$10^{1.69}$）

図1 ワクチン接種前後のJEVに対する中和抗体価の推移

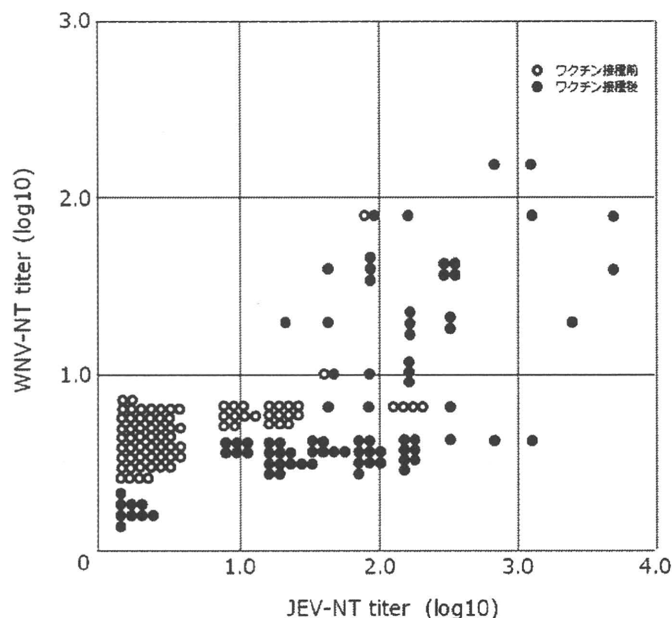


図2 ワクチン接種前後のJEVとWNVに対する中和抗体価の比較

表 2　ワクチン接種前後の JEV と WNV に対する中和抗体価の比較

a) ワクチン接種前

JEV-NT	all	WNV-NT <10	10	80	positive %
<10	51	51			0.0
10	9	9			0.0
20	11	11			0.0
40	2	1	1		50.0
80	2	1		1	50.0
160	4	4			0.0
320	1	1			0.0
all	80(人)	78	1	1	2.5

b) ワクチン接種後

JEV-NT	all	WNV-NT <10	10	20	40	80	160	positive %
<10	9	9						0.0
10	6	6						0.0
20	13	12		1				7.7
40	9	6	1	1	1			33.3
80	14	9	1		3	1		35.7
160	14	7	3	3		1		50.0
320	7	1		2	4			85.7
640	2	1					1	50.0
1280	3	1				1	1	66.7
2560	1			1				100.0
5120	2				1	1		100.0
all	80(人)	52	5	8	9	4	2	35.0

表 3　ワクチン接種前後における幾何平均抗体価の年代別比較

年齢	人数	JEV-NT 幾何平均抗体価 pre	JEV-NT 幾何平均抗体価 post	JEV-NT 抗体価上昇率 post/pre	WNV-NT 幾何平均抗体価 pre	WNV-NT 幾何平均抗体価 post	WNV-NT 抗体価上昇率 post/pre
20 代	14	22.5	336.2*	15.0	0.5	5.3*	10.5
30 代	18	1.4	88.7*	61.3	0.5	3.8*	7.7
40 代	19	2.3	30.4*	12.9	0.7	2.7*	3.6
50 代	22	0.7	16.9*	25.8	0.5	0.6	1.3
60 代	7	1.5	24.3*	15.9	0.5	1.7	3.4
ALL	80(人)	2.1	49.3*	23.1(倍)	0.6	2.1*	3.8(倍)

*P<0.05　Wilcoxon signed-rank test

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, 2010

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Summary:

We conducted laboratory-based surveillance on mosquito-borne viral diseases in Taiwan. In 2010, a total of 304 imported dengue cases and 13 imported chikungunya cases were identified. From the acute phase serum samples of all imported dengue cases, 62 DENV-1, 52 DENV-2, 24 DENV-3, and 14 DENV-4 strains were isolated. Local dengue outbreaks were circulated mainly in Kaohsiung City, Tainan City, Tainan County and Kaohsiung County with 1591 laboratory confirmed indigenous dengue cases including 18 dengue hemorrhagic fever and 2 deaths. Sequence analyses from DENV isolates obtained from indigenous cases showed that a total of 13 DENV strains, which were most likely introduced from the Philippines, Indonesia, Vietnam, Thailand and Cambodia were responsible for local outbreaks. Among these strains, a DENV-3 genotype I strain was found to be an overwinter strain causing major dengue outbreak in Kaohsiung City during August 2009 and February 2010, and reemerged in Kaohsiung City on August 2010. Surveillance results of Japanese encephalitis virus (JEV) in Taiwan during 2005-2010 showed that JEV strains in Taiwan is rapidly shifting from GIII to GI, similar to Japan and Vietnam, where the GIII strains have been replaced by GI strains. Phylogenetic analyses of 10 isolated chikungunya viruses (CHIKVs) from Indonesia showed that 5 strains belong to East/Central/South African genotype, whereas the other 5 strains belong to Asian genotype. Interesting, all African genotype strains were found to have E1-A226V mutations.

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. Dengue and Chikungunya surveillance

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, Centers for Disease Control, Taiwan (Taiwan CDC), Department of Health, for laboratory diagnosis. A confirmed dengue or chikungunya case was defined as febrile illness associated with a positive real-time reverse transcription (RT)-PCR test, isolation of DENV or CHIKV, or the detection of DENV- or CHIKV-specific IgM and IgG antibodies. A multiplex one-step real-time RT-PCR was used 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 flavivirus/alphavirus-specific capture ELISA was used to detect and differentiate various flavivirus/alphavirus infections.

2. JEV surveillance

To understand the genetic variation of JEV strains currently circulating in Taiwan, we conducted a surveillance program in the following areas: northern (Taipei, Taoyuan, and Yilan counties and Taipei City), central (Taichung and Changhua counties), southern (Tainan and Kaohsiung counties), and eastern (Hualien County) during 2005–2010. Real-time reverse transcription–PCR (RT-PCR) was used to screen JEV in mosquito pools, pig serum specimens, and human cerebrospinal fluid. Mosquitoes were pooled by species, location, and collection date in groups of 30–50 mosquitoes. Mosquito pools were homogenized and clarified by centrifugation, and the supernatants were sterilized by filtration and removed for real-time RT-PCR and virus isolation. We used 3 sets of primers for real-time RT-PCR: flavivirus-specific (FL-F1: 5′-GCCATATGG TACATGTGGCTGGGAGC-3′; FL-R3: 5′-GTKATTCTTGTGTCCCAWCCGGCTGTGTCATC-3′; FL-R4: 5′-GTGATGCGRGTGTCCCAGCCRGCKGTGTCATC-3′), JEV-specific (JE3F1: 5′-CCCTCAGAACCGTCTCGGAA-3′ and JE3R1: 5′-CTATTCCCAGGTGTCAATATGCTGT-3′), and JEV GIII–specific (E12F: 5′-CTGGGAATGGGCAATCGTG-3′ and E325R: 5′-TGTCAATGCTTCCCTTCCC-3′) (1). Samples with positive results by RT-PCR were subjected to virus isolation by using a mosquito C6/36 cell line.

3. 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 C6/36. 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 flavivirus and alphavirus

The diagnostic tests for flavivirus infection from fevered patients on the basis of the results of one-step SYBR Green I-based real-time RT-PCR and envelope/membrane-specific capture IgM and IgG ELISA had been described previously (2-4). To screen viremic fever patients with alphavirus infection, a multiplex one-step SYBR Green I-based real-time RT-PCR was developed (5-6). 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. 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 (7). 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 4.0 software package. 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, 2010

A total of 304 imported dengue cases were identified in Taiwan, 2010. The majority of these imported cases were infected in Southeast Asian countries with exceptions of India, Bangladesh, Peru, Saint Vincent and Maldives. Similar to our previous report, Indonesia, Vietnam, Thailand and the Philippines are on the top list of importing countries (8). From acute phase serum samples of these imported dengue cases, a total of 152 DENV strains were isolated including 62 DENV-1, 52 DENV-2, 24 DENV-3, and 14 DENV-4 strains. 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. Table 1 showed the summary of serotype and genotype distributions of 146 DENV isolates. The data reveals that genotype I strains of DENV-1 and cosmopolitan genotype strains of DENV-2 were the most epidemic strains circulating in Southeast Asian countries.

2. Multiple dengue epidemics in southern Taiwan, 2010

For local dengue outbreaks in Taiwan, a total of 1591 indigenous dengue cases were laboratory confirmed in 2010. The nucleotide sequences of partial NS5 gene fragment were routinely determined from the acute phase serum samples of confirmed cases. The full-length structural 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 of 108 DENV isolates obtained from acute phase serum samples of indigenous cases showed that a total of 13 DENV strains, which were most likely introduced from the Philippines, Indonesia, Vietnam, Thailand and Cambodia were responsible for local outbreaks. Among these strains, 4 caused major dengue outbreaks; a genotype I strain of DENV-3 and a Cosmopolitan genotype strain of DENV-2 in Kaohsiung City/County, a genotype II strain of DENV-4 in Tainan City, and a genotype I strain of DENV-1 in Tainan County. Phylogenetic analysis showed that these DENVs were imported from the Philippines, the Philippines, Indonesia, and Vietnam, respectively. Most important, the genotype I strain of DENV-3 was

found to be an overwinter strain causing major dengue outbreak in Kaohsiung City resulting more than 750 confirmed dengue cases during August 2009 and February 2010, and reemerged in Kaohsiung City on August 2010.

3. JEV surveillance

To understand the genetic variation of JEV strains currently circulating in Taiwan, we conducted a surveillance program in northern, central, southern, and eastern areas during 2005–2010. Real-time RT-PCR assay was used to screen JEV in mosquito pools, pig sera and human cerebrospinal fluid. Samples with positive RT-PCR were subjected to virus isolation using a mosquito C6/36 cell line. The surveillance data in 2010 showed that most of the JEV strains isolated or detected belong to GI (Taipei City, Hualien County, Taichung County, Kaohsiung County, Tainan County), and only a few JEV strains belong to the GIII (Yilan County, Taichung County, Kaohsiung County). This is in contrast to JEV isolates during 2005-2007, when all belong to GIII, and during 2008-2009, when a few JEV strains belong to the GI (Beitou District, Taipei City Guandu Nature Park; pig farms in Wujie Township, Yilan County, and Wufeng Township, Taichung County). Overall, the results show that JEV strains in Taiwan is rapidly shifting from GIII to GI, similar to Japan and Vietnam, where the GIII strains have been replaced by GI strains. Figure 1 showed the phylogenetic tree of GI strain sequences obtained in Taiwan and other reference sequences retrieved from GenBank.

4. CHIKV surveillance

For chikungunya surveillance, we identified 13 imported chikungunya cases in 2010. Most of these travelers were returned from Indonesia (12 cases) and only one from Malaysia. A total of 10 CHIKVs were isolated from the acute phase serum samples and the partial nucleotide sequences of envelope protein 1 (E1) gene (836 bp) were determined. Table 2 showed the summary of 10 isolated viruses. The results showed that 5 strains belong to East/Central/South African genotype, whereas the other 5 strains belong to Asian genotype. Interesting, all African genotype strains were found to have E1-A226V mutations. Figure 2 showed the phylogenetic tree of partial E1 gene of CHIKV East/Central/South African genotype.

Discussion:

Studies on returned travelers have provided valuable information regarding the geographic distribution and global movement of DENV strains. Mainly through fever screening surveillance at airports, we identified 304 imported dengue cases and 13 imported chikungunya cases in Taiwan, 2010. The results witness the growing global threat of dengue in the past 6 years. However, the chikungunya transmission was declining in most of the epidemic areas except Indonesia in 2010. It is interesting to note that half of the CHIKV strains belong to East/Central/South African genotype, and all these strains were found to have E1-A226V mutations.

The increasing trend of E1-A226V East/Central/South African genotype is in agreement with the hypothesis that CHIKV strains with E1-A226V mutation has replication advantage in Aedes albopictus species. The genetic database generated from these isolated virus strains can provide us useful information for the understanding of global distributions and movements of various DENV and CHIKV strains grouped in various serotypes and genotypes.

Surveillance results of JEV in Taiwan during 2005-2010 revealed that JEV strains in Taiwan is rapidly shifting from GIII to GI. Therefore, continuous monitoring of dynamic change of JEV strains isolated in Taiwan is important for better understanding of the epidemiological, virological and immunological features of JEV infection in Taiwan and other areas.

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Table 1. Summary of genotype distributions of DENV strains isolated from imported cases in Taiwan, 2010

Serotype	DENV-1			DENV-2				DENV-3			DENV-4		Total
Genotype	I	II	III	Asian1	Asian2	Asian/ American	Cosmopo litan	I	II	III	I	II	
Indonesia	14*	8					17	7				7	53
Vietnam	17			3					2		3		25
Thailand	6			9					2	3*			20
Philippines		3					8	4				1*	16
Malaysia	4						4	3					11
India			2				1			2	1		6
Cambodia	3												3
Singapore	1						2						3
Myanmar				1							1		2
Lao				2									2
Peru												1	1
Saint Vincent						1							1
Unknown	1			1			1						3
Total	46	11	2	16	0	1	33	14	4	5	5	9	146

Table 2. Imported chikungunya viruses in Taiwan, 2010

No.	Year	Seq name	Location	E1-226	Genotype
CK9900002	2010	1002aTW	Indonesia	V	East/Central/South African
CK9900003	2010	1002bTW	Indonesia	A	Asian
CK9900004	2010	1002cTW	Indonesia	V	East/Central/South African
CK9900010	2010	1002dTW	Indonesia	V	East/Central/South African
CK9900013	2010	1003aTW	Indonesia	V	East/Central/South African
CK9900173	2010	1005aTW	Indonesia	A	Asian
CK9900176	2010	1005bTW	Indonesia	A	Asian
CK9900182	2010	1006aTW	Indonesia	V	East/Central/South African
CK9900183	2010	1006bTW	Indonesia	A	Asian
KSD9910743	2010	1011aTW	Indonesia	A	Asian

Phylogenetic tree of partial E gene (1144 bp) in JEV

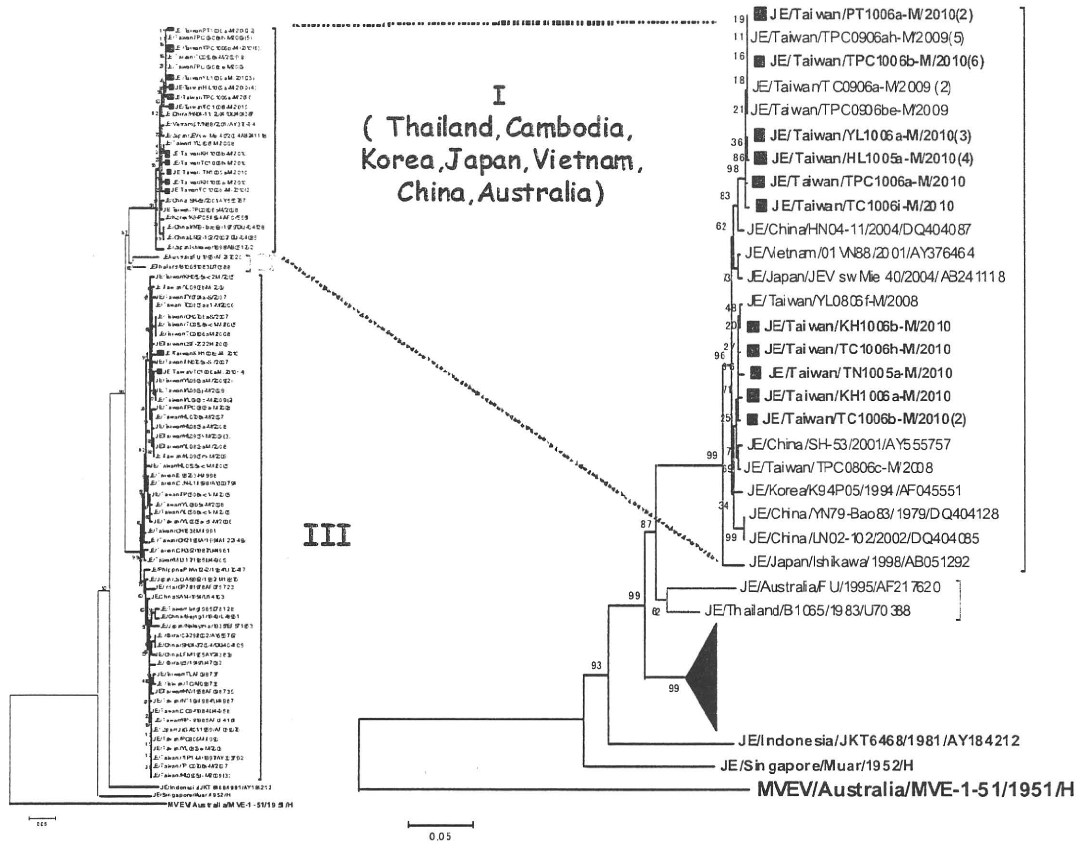

Figure 1. Phylogenetic relationships of genotype I Japanese encephalitis virus (JEV) isolated or detected from different geographic regions in Taiwan, 2008-2010. Partial E gene (1144 bp) of JEV isolates or RT-PCR positive mosquito pools were sequenced to determine the genetic relationship of these local strains.

Phylogenetic tree of partial E1 gene (836 bp) in CHIKV

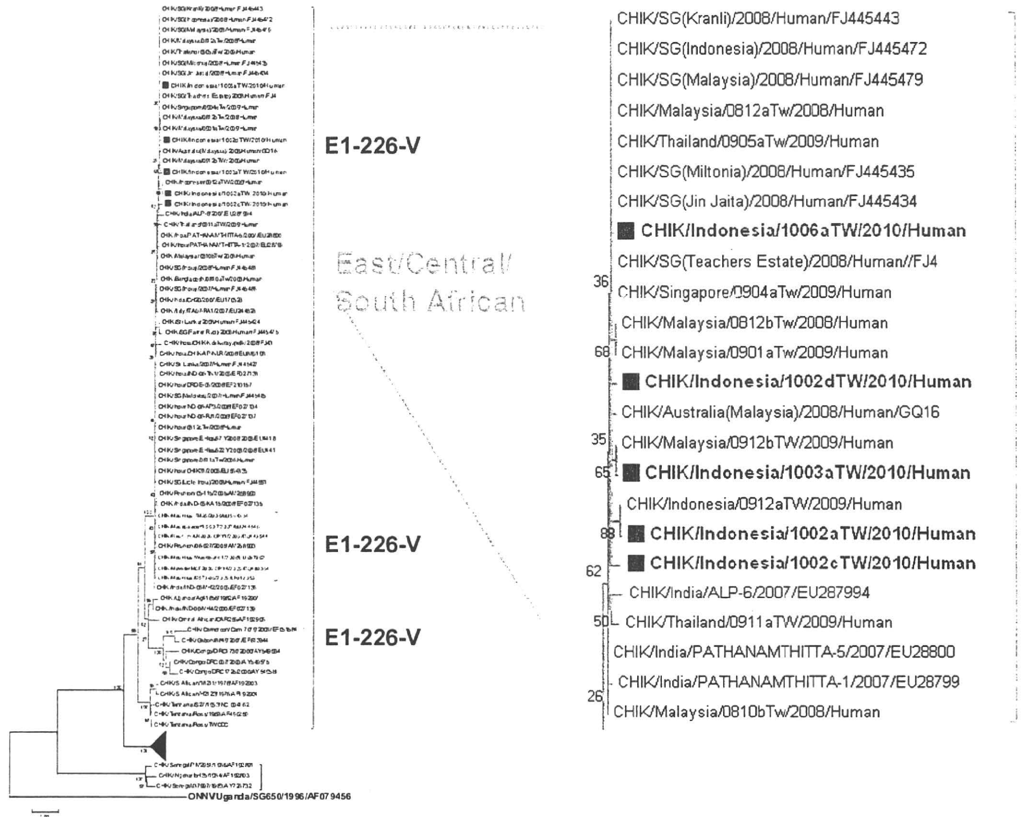

Figure 2. Phylogenetic tree of CHIKV strains of East/Central/South African genotype isolated from imported cases in Taiwan, 2010. The tree was constructed by the neighbor-joining method using partial nucleotide sequences of envelope protein 1 (E1) gene (836 bp) of various CHIKV strains. O'nyong-nyong (ONN) virus sequence was used as the outgroup virus. 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.

GENOTYPE ANALYSIS OF DENGUE VIRUSES IN JAKARTA

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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, 2007). In Indonesia all serotypes are endemic. DHF have been reported in all provinces. (Setiati, 2007). Dengue virus infection continues to increase in tropical and sub-tropical countries of the world and more than 2.5 billion people live in the regions with the risk of dengue virus infection (WHO, 2002). In Jakarta, a capital city of Indonesia hold the highest cases of DHF about 28,000 cases anually. The pathological mechanisms of DHF are still poorly understood. A number of models have been proposed, based on epidemiological and experimental data, to explain the pathogenesis of severe dengue illness, and among them is the role of intrinsic biological properties of dengue virus strains (Rico Hesse *et al.*, 1997) and the serotype of infecting virus in the secondary infection (Fried et al., 2010). Several study also showed the dynamic of circulating dengue globally and evolution of dengue viruses (Goncalves, 2002; Zhang et al, 2005; Shu, et al., 2009; Hang, et al., 2010). Such studies are important for basic data for understanding the disease and the development of laboratory diagnosis and vaccine.

As mentioned in previous report, in this study we were more focus to continuing molecular epidemiology study based on env genes. Genotype analysis of the four serotypes of dengue viruses will be described.

METHODS:

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

Specimens were collected from hospitalized patients with suspected dengue infection in Dr. Cipto Mangunkusumo Central Hospital, Jakarta within the period of 2006 to 2010. 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 2010 specimens. The sera were also tested by RT-PCR (Lanciotti, 1992; Reynes J-M, 2003) to see the presence and type of virus. To the year 2009-2010 samples, NS-1 ELISA (Panbio Inc, Brisbane, Australia) and/or NS-1 rapid dengue test were also done. In adult patients we used NS-1 rapid dengue test (SD Duo, Korea) to screen suspected dengue infected patients in a community study in East Jakarta. NS-1 positive patients would be hospitalized. Then, to confirm dengue infected patients we used RT-PCR or virus isolation in C6/36 or Vero sel line

2. Determination of viral nucleotide sequences

RT-PCR and DNA sequencing

RNA were extracted from 140 □l 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 to primers used (Table 1) and length of the expected product. After amplification and purification, the DNA was sent to the DNA sequencing facility at the Eijkman Institute, Jakarta or Microbiology Laboratory of the Faculty of Sciences, University of Indonesia. The sequences were determined using Taq Big Dye Deoxy Terminator Cycle sequencing kits. Table 1 shows the primers used for PCR and sequencing. The nucleotide sequences were analysed using software Genetyx-Win version 5.1.

RESULTS AND DISCUSSION

Specimen characterization

During 2010, 124 samples, consisting of 69 samples from adults and 55 samples from children. Of those, 34 samples from children and 51 from adults were positive RT-PCR (Table 1). Detection rate of dengue viral RNA were 61.8% and 73.9% in children and adult respectively.

Dengue serotypes

As shown in table 1, all four serotypes could be found in Jakarta, with the dominance of DENV-2 and DENV-3. DENV-4 was still least to be found. This pattern was similar to our study from 2006 to 2009. This is also in agreement with previous report by Setyati et al. DENV-4 was rarely found.

Table 1. Dengue serotypes in patients samples collected in 2010

	Children (< 14 y.o.)	Adult ≥ 14 y.o.	Total
DENV-1	3	12	15
DENV-2	11	19	32
DENV-3	11	15	26
DENV-4	5	5	10
Mix infection		0	
D1+D2	1		1
D1+D3	3		3
Percentage of positive samples	34/55 (61.8%)	51/69 (73.9%)	85/124

Virus culture

Serum samples were inoculated to C6/36 or Vero cell lines. At 2 to 5 passage, tissue culture supernatants were tested by hemaglutination assay (HA) and to some of them by

plaque assay. We could get virus isolates of 5 DENV1, 7 DENV2, 5 DENV3, 3 DENV4 (Table 3).

Table 3. Isolation of four serotypes of DENV.

Dengue serotype	DENV1	DENV2	DENV3	DENV4
Positive HA	5	7	5	3
Positive plaque assay	1 of 5	5 of 7	5 of 5	1 of 3
Not tested by plaque assay	4	0	0	2

Sequencing of envelope gen

During 2010 we sequenced *env* gene from three DENV-1, one DENV-2, three DENV-3 strains and three DENV-4; as well as prM gene from three DENV-1, 2 DENV-2, three DENV-3 and three DENV-4 strains isolated in 2009 and 2010 (table 2).

For genotype analysis, the sequencing results of env genes from 2009 and 2010 were analysed together with sequencing results in 2006 to 2009 and other seuences in the Genbank. As reported previously, the genotype of DENV-2 sequenced in this study were identified as cosmopolitan genotype and and DENV-3 belong to genotype I (Zhang, 2005). Among DENV-1 isolated we found ... strains to be genotype I as previously reported and 1 strain belonged to genotype II (according Shu et al 2009; Zhang 2005) or genotype IV (according to Goncalves, 2002). DENV-4 was found to be genotype II (Shu et al 2009).

Table 2 : Dengue virus strains sequenced in this study

No	Strain	Year isolated	Serotype	Genes sequenced
1.	DS 06/09	2009	DENV-1	prM, Env
2	DSA 37/09	2009	DENV-1	Env
3	RDS 53/09	2009	DENV-1	prM, Env
4	DS 18/09	2009	DENV-2	prM, Env
5	IDS 39/10	2010	DENV-3	prM, Env
6.	DSA 05/10	2010	DENV-3	prM
7	DSA 07/09	2009	DENV-3	prM, Env
9.	RDS 98/09	2009	DENV-4	prM, Env
10.	IDS 63/10	2010	DENV-4	prM, Env
11.	IDS 44/10	2010	DENV-4	prM, Env

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