ASSOCIATION STUDY OF VIREMIA LEVELS WITH CLINICAL CHARACTERISTICS IN THAI PATIENTS WITH DENGUE

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Abstract: A prospective observational study was conducted from June 2011 to March 2013 to investigate the association of dengue viremia levels with clinical characteristics and routine laboratory findings involving 121 dengue patients admitted to the Bangkok Hospital for Tropical Diseases, Thailand. In addition, a phylogenetic analysis of envelope (E) gene of dengue virus in circulation was carried out. There were 84 (69%) and 37 (31%) dengue fever (DF) and dengue hemorrhagic fever (DHF) patients respectively, with 89% being secondary dengue infection. Viremia level in DHF was slightly higher and more sustained than in DF patients, but there was no statistically significant association between viremia level and clinical characteristics. Dengue virus serotype 2 (DENV-2) was the most common serotype. E gene phylogenetic study revealed presence of DENV-1 genotype I, DENV-2 Asian genotype I, DENV-3 genotype II, and DENV-4 genotype I. Even though the study fails to discern any outstanding characteristics of the patients associated with dengue viremia level, it reveals the demographics and clinical characteristics of patients admitted to the Bangkok Hospital for Tropical Diseases, Bangkok, Thailand.

Keywords: clinical presentation, dengue infection, dengue virus E gene, viremia.

INTRODUCTION

Dengue virus (DENV) is an arbovirus in genus *Flavivirus*, family Flaviviridae (Henchal and Putnak, 1990), which poses an estimated 2.5 billion people to risk of infection worldwide (WHO SEARO, 2011). DENV consists of 4 serotypes (DENV1 - DENV4). The virus RNA encodes three structural (C, E and prM/M) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Zhang *et al*, 2003; Guzman *et al*, 2010).

Dengue infection may result in asymptomatic infection, dengue fever (DF), dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (WHO, 1997). Incidence of dengue has increased greatly between 1990 and 2013, with the number of cases more than doubling every decade, from 8.3 million to 58.4 million

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(Stanaway *et al*, 2016). Pathogenesis of disease severity is still not completely understood, with infection with one serotype causing life-long immunity against the original serotype and transient cross-protection to the other serotypes (Halstead, 1988). Secondary infection with a different serotype is a risk factor for severe dengue (Fried *et al*, 2010) that presents with severe thrombocytopenia and increased vascular permeability, the two major characteristics of DHF (Saito *et al*, 2004). Thrombocytopenia and hemorrhagic manifestations in severe dengue may also be associated with antibody-dependent enhancement (ADE) (Ito *et al*, 2010). In addition, individual factors, such as genetic background, gender and age, may influence immune response and dengue severity (Guzman *et al*, 2002; Stephens *et al*, 2002; Malavige *et al*, 2011; Carrasco *et al*, 2014), but other factors (virulent DENV genotype and high viremia level) have been suggested to play key roles in dengue severity (Vaughn *et al*, 2000; Cologna *et al*, 2005).

Here, the association between dengue viremia and patient's clinical characteristics were investigated. The result study would be able to predict the severity of the dengue infection by viremic level which would be helpful in patient management.

MATERIALS AND METHODS

Study design and recruitment of patients

This prospective observational study was conducted from June 2011 to March 2013 involving patients ($n = 127$) admitted to Bangkok Hospital for Tropical Diseases, Bangkok, Thailand. Inclusion criteria were (i) >2 years of age and (ii) having suspected dengue infection shown by positive dengue NS1 (SD BIOLINE, St Ingbert, Germany) or IgM/IgG (SD BIOLINE, St Ingbert, Germany) rapid test. Exclusion criteria were: (i) having fever of >5 days, (ii) having a history of receiving blood product within the prior six months, and (iii) being pregnant.

The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (Approval No. TMEC 11-007). Prior written consent was obtained from each participant and parent or legal guardian of a minor.

Data collection and IgM and IgG capture enzyme-linked immunosorbent assay (ELISA) procedures

Blood samples were collected on day of enrollment (acute blood sample) and one week later (convalescent blood sample) for IgM and IgG capture enzyme-linked immunosorbent assay (ELISA). Acute blood samples also were used for determining DENV serotype and viremia level. Six patients who showed no rise in IgM/IgG capture ELISA values were excluded from the study. Patient's clinical presentation, symptoms and routine laboratory findings during illness were collected. Dengue-specific IgM and IgG capture ELISAs were performed to serologically diagnose infection using acute and convalescent samples (Innis *et al*, 1989). In brief, 96-well plates (MP Biomedicals, Illkirch, France) were coated with goat anti-human IgM or IgG (KPL, Gaithersburg, MD), then serum samples were added followed by incubation for 2 hours at room temperature. 96-well plates were washed prior to addition of a mixture of viral antigens of four dengue virus serotypes (DENV-1 strain Hawaii, DENV-2 strain 16681, DENV-3 strain H87, and DENV-4 strain C0036/06 in house propagate) and horseradish

peroxidase-conjugated human anti-flavivirus IgG (KPL) was added together with substrate *o*-phenylenediamine dihydrochloride (Calbiochem/Merck, Darmstadt, Germany). After incubation at room temperature for 30 minutes A492 nm of each well was measured (Microplate reader, Tecan, Männedorf, Switzerland) and transformed to binding index (BI) as previously described (Innis *et al*, 1989). Primary dengue infection is defined when anti-dengue IgM:IgG ratio ≥1.8 is obtained in either acute or convalescent sera, and defined as secondary infection when IgM:IgG ratio <1.8.

RT-PCR DENV serotype identification assay

RNA was extracted from serum samples of dengue patients using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and subjected to first round One-Step RT-PCR (Invitrogen, Carlsbad, CA) using D1 and D2 primers (Lanciotti *et al*, 1992). Product from the first round RT-PCR was then used in a multiplex nested-PCR with D1 primer and four internal dengue serotype-specific primers (TS1, TS2, TS3, and TS4) (Lanciotti *et al*, 1992). Amplicons were analyzed by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and amplicon of 482, 119, 290, and 392 bp designated presence of DENV-1, DENV-2, DENV-3, and DENV-4, respectively.

Quantitative (q)RT-PCR viral load assay

Reaction volume (50 μl) contained 1 μl of template RNA, 3.7 μl each of 20 μM forward primer and 20 μM reverse primer (Callahan *et al*, 2001) and 0.1 μl of 10 μM probe (AccuPower® RocketScript™ RT-qPCR Premix; Bioneer, Daejeon, Korea). Thermocycling was performed in CFX96 Touch™ Real time PCR (Bio-Rad, Hercules, CA) as follows: an initial step at 50°C for 15 minutes, 95°C for 5 minutes, 50 cycles of 95°C for 10 seconds, and 57.1°C for 45 seconds. Threshold cycle (C_t) was measured after extension step. A standard curve was generated from plot of C_ts versus solutions of dengue virus of known concentration (PFU/ml) prepared from DENV-1 strain Hawaii, DENV-2 strain 16681, DENV-3 strain H87, and DENV-4 strain C0036/06.

Table 1
PCR primers employed in amplification of dengue virus E gene.

Primer Name	Sequence (5´→ 3´)	Nucleotide position	Amplicon size (bp)
D1-855F	TAGCACATGCCATAGGAA	855-2425	1,569
D1-2425R	ATTTGAGTTCTCTGCCCTTCC		
D2-850F	GCAATCCTGGCATACACCAT	850-2493	1,642
D2-2493R	TTCTGTCCATGTRTGCACG		
D3-762F	CCT GGATGTCGGCTGAAGGAG	762-2519	1,756
D3-2519R	CACTCTTTTGGGGGAGTCTGC		
D4-821F	ACTCAGAAACCCAGGATTCGC	821-2457	1,635
D4-2457R	CCGCTTCCACACTTCAATTC		

R = A or G.

DENV E gene sequencing and phylogenetic analysis

RNA was reversely transcribed using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Rockford, IL) together with a random hexamer primer. E gene fragments of DENV-1-4 were amplified using Phusion Flash High-Fidelity PCR Master Mix kit (Thermo Scientific, Rockford, IL) with forward and reverse primers specific to each serotype (Table 1). Thermocycling was performed as described above using the following conditions: 98ºC for 5 minutes, 35 cycles of 98ºC for 10 seconds, 50ºC for 5 seconds and 72 ºC for 30 seconds with a final step of 72 ºC for 5 minutes. Amplicons (1.6 kb) were separated by 1% agarose gel-electrophoresis, lightly stained with ethidium bromide, purified using QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sequenced (Macrogen Co Ltd, Seoul, Korea). Sequences were aligned using ClustalW (Thompson *et al*, 1994) and a neighbor-joining method was used for phylogenetic tree construction carried out with MEGA software version 5 (Tamura *et al*, 2011). Bootstrap analysis (1,000 replicates) was performed and dengue genome sequences of each dengue serotype, obtained from GenBank database, were used as reference sequences for the phylogenetic analysis. DENV E gene sequences obtained in this study were submitted to GenBank database, accession numbers for DENV-1 KM501565-KM501579, DENV-2 KM501580-KM501591, DENV-3 KP100250-KP100257, and DENV-4 KP100258-KP100259.

Data analysis

Categorical data are presented as frequency and percentage, viremia as geometric mean titer (GMT), and continuous data as mean ± SD and median plus interquartile range (IQR). Chi-square or Fisher's exact test are performed for analysis of categorical variables and Mann-Whitney *U* test or t-test for

Table 2
Characteristics and clinical presentations of dengue patients admitted to Bangkok Hospital for Tropical Diseases, Bangkok, Thailand (June 2011 to March 2013).

Characteristic/symptom	Number (%) (n = 121)
Gender	
Male	62 (51)
Female	59 (49)
Age range	
6-15 years	18 (15)
16-30 years	74 (61)
31-45 years	18 (15)
46-60 years	8 (7)
>60 years	3 (2)
Clinical presentation	
Headache	101 (83)
Myalgia	91 (75)
Anorexia	99 (82)
Nausea/vomiting	91 (75)
Abdominal pain	38 (31)
Joint pain	50 (41)
Rash	13 (11)
Rhinorrhea	13 (11)
Bleeding manifestation	
Petechial rash	7 (6)
Epistaxis	2 (2)
Gum bleeding	8 (7)
Other bleeding site	11 (9)
Clinical classification	
Dengue fever	84 (69)
Dengue hemorrhagic fever	37 (31)
Grade 1	20 (54)
Grade 2	12 (32)
Grade 3	5 (14)
Survival	121 (100)

DF: dengue fever; DHF: dengue hemorrhagic fever.

continuous variables using Predictive Analytics Software (PASW) Statistics 18 (SPSS Inc, Chicago, IL). A p-value <0.050 is considered statistically significant.

RESULTS

Demographic profiles and clinical severity of dengue patients

This study found that most of dengue patients aged between 16-30 years and the common clinical presentations were headache, myalgia, anorexia and abdominal pain. All dengue patients (n = 121) survived and length of hospitalization of DHF patients (median (IQR) = 4 (3-5) days) is not significantly different from that of DF patients (median (IQR) = 0 (0-1) day).

Serological diagnosis

As six patients missed the convalescent visit, 115 paired sera were available for serological diagnosis by IgM and IgG capture ELISAs. The majority of patients manifested secondary dengue infection (Table 3). Mean ratio of primary:secondary dengue infection was 0.06:1, with ratio in age group <15 years ten folds higher than that of age group >15 years (0.3:1 versus 0.03:1).

DENV serotype and daily rate of viral load

Out of 121 samples, we were able to determine dengue serotype in 114 (94%), revealing 31 (26%), 49 (40%), 27 (22%), and 7 (6%) cases of DENV-1, DENV-2, DENV-3, and DENV-4 infection, respectively. QRT-PCR indicated 105/121 (87%) samples had detectable viral load with a mean geometric mean titer of 1.84×10^3 plaque-forming units (PFU)/ml. Viremia level was highest in early period of illness and declined daily (Fig 1a). Mean viremia level in DHF patients (1.5×10^5 PFU/ml) was 2.7 times higher than that of DF patients and was sustained over a longer period than in DF patients (Fig 1b). Viremia levels were higher in DHF compared to DF patients infected with DENV-2 and -3 while the converse was obtained with DENV-4; however, the statistic significances could not be demonstrated (Fig 2).

Clinical presentation and viremia level

At a cut-off viremia level of 10^4

Table 3
Immunostatus and clinical classifications according to age range of dengue patients admitted to Bangkok Hospital for Tropical Diseases, Bangkok, Thailand (June 2011 to March 2013).

Age range (years)	Primary dengue infection		Secondary dengue infection	
	DF Number (%)	DHF Number (%)	DF Number (%)	DHF Number (%)
6-15 (n = 17)	3 (18)	1 (6) (grade III)	10 (58)	3 (18) (grade I = 2, grade III = 1)
16-30 (n = 69)	2 (3)	0 (0)	46 (67)	21 (30) (grade I = 10, grade II = 9, grade III = 2)
>30 (n = 29)	1 (4)	0 (0)	16 (55)	12 (41) (grade I = 8, grade II = 3, grade III = 1)

DF, dengue fever; DHF, dengue hemorrhagic fever.

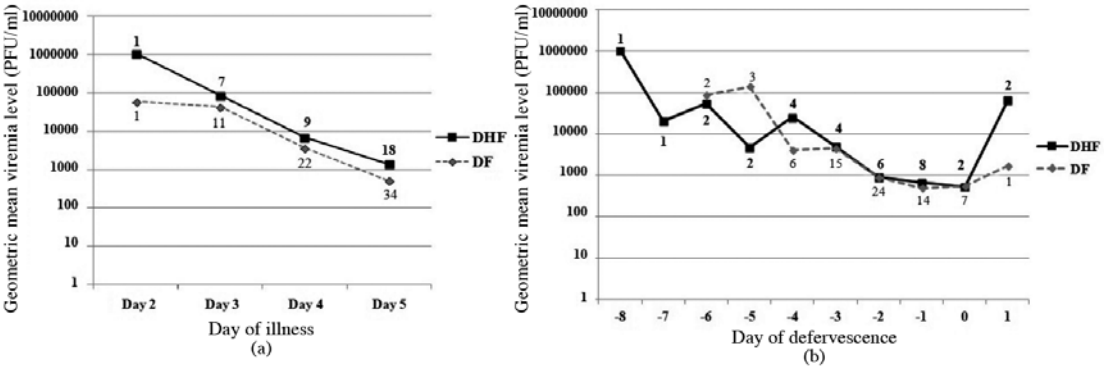

Fig 1-Viremia levels of patients with dengue fever (DF) and dengue hemorrhagic fever (DHF) according to days of illness (a) and days of defervescence (b).
Numbers indicate number of patients.

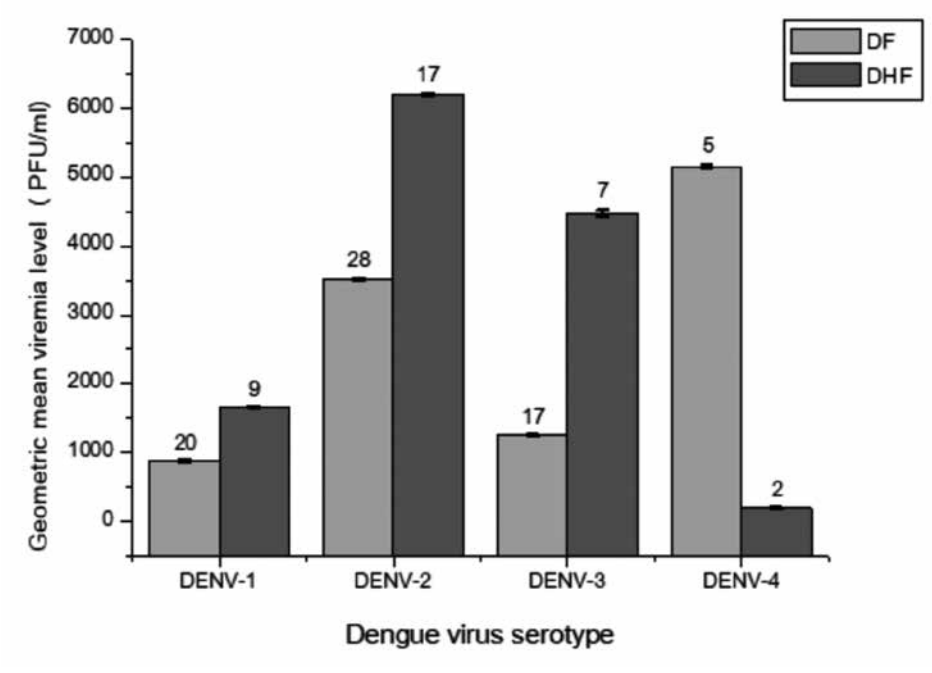

Fig 2-Viremia levels of patients with dengue fever (DF) and dengue hemorrhagic fever (DHF) according to dengue virus serotypes.
Numbers indicate number of patients.

PFU/ml, there is no statistical difference in clinical characteristics including clinical manifestations and clinical classifications (DF and DHF) (Table 4). Surprisingly, length of illness is significantly longer in patients with low compared to and high viremia level. The high viremia levels were found in the dengue patients with

rhinorrhea or bleeding but there were no statistical significances (Fig 3).

Genetic diversity of DENV E genes

E gene was successfully sequenced from 37/114 (32%) of RT-PCR positive samples, comprising 15 DENV-1, 12 DENV-2, 8 DENV-3, and 2 DENV-4 serotypes. Phylogenetic analysis showed all DENV-1 samples were of genotype I, all DENV-2 Asia genotype I, seven DENV-3 genotype II and 1 was genotype III, and all DENV-4 was genotype I (Fig 4). There was no difference in dengue genotypes between DENV from DF and DHF patients.

DISCUSSION

This prospective study was conducted at Bangkok Hospital for Tropical Disease, Bangkok, a tertiary care center located in a region with a high dengue endemicity, averaging an incidence of some 16,000 cases/years during the study period (MoPH, 2013).

Table 4
Clinical symptoms and laboratory results between patients with low and high dengue viral load, Bangkok Hospital for Tropical Diseases, Bangkok, Thailand (June 2011 to March 2013).

Clinical symptom/laboratory finding	Low viral load ($<10^4$ PFU/ml) Number (%) ($n = 73$)	High viral load ($>10^4$ PFU/ml) Number (%) ($n = 32$)	p-value*
Duration of illness, mean (SD) days	4.6 (0.6)	3.6 (0.9)	<0.001**
Symptom			
Headache	59 (81)	28 (87)	0.575
Myalgia	57 (78)	24 (75)	0.802
Anorexia	59 (81)	26 (81)	1.000
Nausea/vomiting	53 (73)	25 (78)	0.633
Abdominal pain	19 (26)	10 (31)	0.638
Joint pain	35 (48)	10 (31)	0.163
Rhinorrhea	4 (5)	2 (6)	1.000
Rash	7 (10)	5 (16)	0.506
Petechial rash	6 (8)	2 (6)	1.000
Other bleeding site	5 (7)	4 (12)	0.450
Complete blood count, median (IQR)			
Highest hematocrit (%)	42.9 (40.6-46.5)	45.2 (41.0-47.0)	0.594
Lowest platelet count ($10^3/\mu$l)	33.0 (19.0-62.5)	36.5 (21.5-59.5)	0.515
Lowest white blood cell count (/μl)	2,100 (1,750-2,800)	2,150 (1,425-2,675)	0.689
Liver enzyme, median (IQR)			
Aspartate aminotransferase (U/l)	180 (83-300)	225 (91-421)	0.298
Alanine aminotransferase (U/l)	101 (47-210)	140 (48-252)	0.271
Clinical classification			
Dengue fever	49 (67)	21 (66)	1.000
Dengue hemorrhagic fever	24 (33)	11 (34)	

*Significance at <0.050; ** Comparison made by t-test.

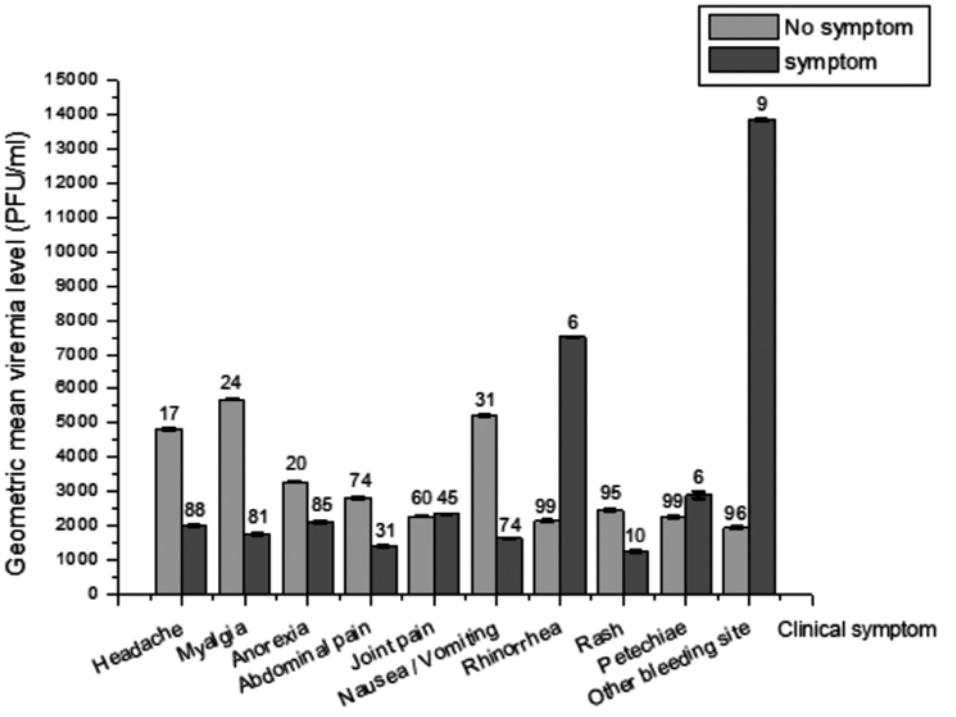

Fig 3-Viremia levels and clinical symptoms of dengue patients with symptoms and no symptom. Numbers indicate number of patients.

DF:DHF incidence ratio was slightly lower than that from other community surveys (Thomas *et al*, 2010; Sabchareon *et al*, 2012). This might be due to high number of severe dengue cases referred to the study hospital. Percent adult patients reflected the increasing trend of dengue among the older population observed in the country (Temprasertrudee *et al*, 2018). This might account for the higher proportion of secondary infection found in the study.

The study identified an 11-year old patient with DHF grade III caused by a primary infection with DENV-1 (acute IgM/IgG = 41/14, convalescent IgM/IgG = 115/59). This emphasizes the severity of dengue infection could be influenced by other factors apart from ADE, such as genetic factor, gender and age (Guzman *et al*, 2002; Loke *et al*, 2002; Stephens *et al*, 2002; Hammond *et al*, 2005; Carrasco *et al*, 2014).

The present study did not demonstrate any association between dengue viremia level and clinical characteristics, although there was a trend a patient with higher viremia level having higher liver enzymes levels. This highlights degree of liver damage and hepatic viscerotropism might be associated with viremia level. Higher levels of liver enzymes were observed in DHF compared to DF patients (Chongsrisawat *et al*, 2009; Trung *et al*, 2010).

All four dengue serotypes were identified in patients' blood samples, DENV-2 being the most common serotype found in both DF and DHF samples. In Thailand, all four dengue serotypes

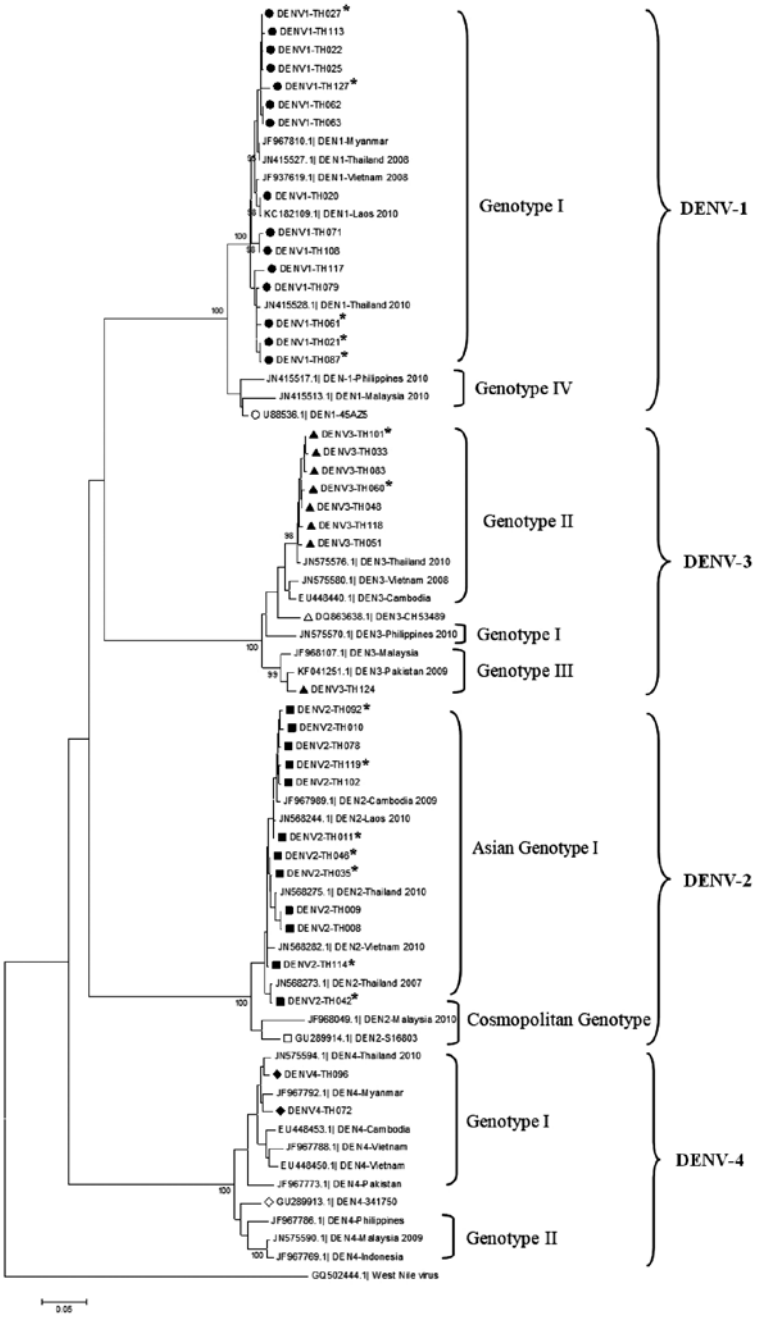

Fig 4 - Phylogenetic tree of DENV1-4 isolated from patients at Bangkok Hospital for Tropical Diseases, Bangkok, Thailand (June 2011 to March 2013).
Sequences of DENV E protein gene (600 bp) were analyzed using a neighbor-joining algorithm. Percent similarity is shown at node of branch site. A West Nile virus strain is used as an outlier. Scale bar indicates 5% nucleotide sequence divergence.
Black circle: DENV-1 strain; Black square: DENV-2 strain; Black triangle: DENV-3 strain; Black diamond: DENV-4 strain; Open symbol: DENV vaccine strain: * From patient with dengue hemorrhagic fever.

have been circulating since 1973 and DENV-2 was the most prevalent in 2010-2012 (Nisalak *et al*, 2016). Several studies reported an association of dengue serotype and severity. For example, secondary DENV-2 infection is associated with DHF although DENV-3 is related to a large outbreak in Bangkok (Nisalak *et al*, 2003). On the other hand, an association of DENV-1 with DHF and DENV-2 with DF was reported from Singapore (Yung *et al*, 2015).

Although an earlier study noted association of high viremia level with increased disease severity (Vaughn *et al*, 2000), a more recent later report showed viremia levels were higher in DF than DHF patients (de la Cruz-Hernandez *et al*, 2013). The present study found higher viremia levels in DHF compared to DF patients infected with DENV-2 and -3 and the opposite with DENV-4 infection. Patients infected with DENV-2 tended to have higher viremia level compared to those infected by other serotypes, but the small number of patients precluded any meaningful statistical analysis. Furthermore, viremia level during febrile phase could be used to differentiate between DF and DHF. Following nadir on defervescent day, the mean geometric viremia in DHF rose slightly higher than in DF, possibly reflecting an association of dengue viremia level and severity of the disease; however, further research is needed to confirm this finding.

DENV-2 Southeast Asian genotype has been reported to cause DHF while American genotype causes DF only (Cologna *et al*, 2005). Due to the small number of genotypes observed in the present study, no association of dengue genotype and dengue severity could be demonstrated. However, the genotypes detected in the DENV samples were consistent with an epidemiologic study in Myanmar in 2013, reporting DENV-1 belonged to genotype I, DENV-2 to Asian genotype I and DENV-4 to genotype I (Ngwe *et al*, 2016); there is no correlation between DENV genotype and clinical severity.

In conclusion the study fails to discern any outstanding characteristics of the patients associated with dengue viremia level. This might be due to the limited number of patients recruited and future investigations will need to be conducted on a larger cohort. Discovery of clinical characteristics related to viremia level will lead to a better understanding of pathogenesis and to prediction of disease severity of dengue infection.

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REFERENCES

Callahan JD, Wu SJ, Dion-Schultz A, *et al*. Development and evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. *J Clin Microbiol* 2001; 39: 4119-24.

Carrasco LR, Leo YS, Cook AR, *et al*. Predictive tools for severe dengue conforming to World Health Organization 2009 criteria. *PLoS Negl Trop Dis* 2014; 8: e2972.

Chongsrisawat V, Hutagalung Y, Poovorawan Y. Liver function test results and outcomes in children with acute liver failure due to

dengue infection. *Southeast Asian J Trop Med Public Health* 2009; 40: 47-53.

Cologna R, Armstrong PM, Rico-Hesse R. Selection for virulent dengue viruses occurs in humans and mosquitoes. *J Virol* 2005; 79: 853-9.

de la Cruz-Hernandez SI, Flores-Aguilar H, Gonzalez-Mateos S, *et al*. Determination of viremia and concentration of circulating nonstructural protein 1 in patients infected with dengue virus in Mexico. *Am J Trop Med Hyg* 2013; 88: 446-54.

Fried JR, Gibbons RV, Kalayanarooj S, *et al*. Serotype-specific differences in the risk of dengue hemorrhagic fever: an analysis of data collected in Bangkok, Thailand from 1994 to 2006. *PLoS Negl Trop Dis* 2010; 4: e617.

Guzman MG, Halstead SB, Artsob H, *et al*. Dengue: a continuing global threat. *Nat Rev Microbiol* 2010; 8(12 Suppl): S7-16.

Guzman MG, Kouri G, Bravo J, Valdes L, Vazquez S, Halstead SB. Effect of age on outcome of secondary dengue 2 infections. *Int J Infect Dis* 2002; 6: 118-24.

Halstead SB. Pathogenesis of dengue: challenges to molecular biology. *Science* 1988; 239: 476-81.

Hammond SN, Balmaseda A, Perez L, *et al*. Differences in dengue severity in infants, children, and adults in a 3-year hospital-based study in Nicaragua. *Am J Trop Med Hyg* 2005; 73: 1063-70.

Henchal EA, Putnak JR. The dengue viruses. *Clin Microbiol Rev* 1990; 3: 376-96.

Innis BL, Nisalak A, Nimmannitya S, *et al*. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg* 1989; 40: 418-27.

Ito M, Mukai RZ, Takasaki T, Kotaki A, Kurane I. Antibody-dependent enhancement of dengue virus infection in vitro by undiluted sera from monkeys infected with heterotypic dengue virus. *Arch Virol* 2010; 155: 1617-24.

Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 1992; 30: 545-51.

Loke H, Bethell D, Phuong CX, *et al*. Susceptibility to dengue hemorrhagic fever in Vietnam: evidence of an association with variation in the vitamin d receptor and Fc gamma receptor IIa genes. *Am J Trop Med Hyg* 2002; 67: 102-6.

Malavige GN, Rostron T, Rohanachandra LT, *et al*. HLA class I and class II associations in dengue viral infections in a Sri Lankan population. *PLoS One* 2011; 6: e20581.

Ministry of Public Health (MoPH). Annual Epidemiological Surveillance Report (AESR) 2013 [cited 2019 Aug 30]. Available from: URL: http://www.boe.moph.go.th/Annual/AESR2013/index.php [in Thai]

Ngwe Tun MM, Kyaw AK, Makki N, *et al*. Characterization of the 2013 dengue epidemic in Myanmar with dengue virus 1 as the dominant serotype. *Infect Genet Evol* 2016; 43: 31-7.

Nisalak A, Clapham HE, Kalayanarooj S, *et al*. Forty years of dengue surveillance at a tertiary pediatric hospital in Bangkok, Thailand, 1973-2012. *Am J Trop Med Hyg* 2016; 94: 1342-7.

Nisalak A, Endy TP, Nimmannitya S, *et al*. Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. *Am J Trop Med Hyg* 2003; 68: 191-202.

Sabchareon A, Sirivichayakul C, Limkittikul K, *et al*. Dengue infection in children in Ratchaburi, Thailand: a cohort study. I. Epidemiology of symptomatic acute dengue infection in children, 2006-2009. *PLoS Negl Trop Dis* 2012; 6: e1732.

Saito M, Oishi K, Inoue S, *et al*. Association of increased platelet-associated immunoglobulins with thrombocytopenia and the severity of disease in secondary dengue virus infections. *Clin Exp Immunol*

2004; 138: 299-303.

Stanaway JD, Shepard DS, Undurraga EA, *et al*. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. *Lancet Infect Dis* 2016; 16: 712-23.

Stephens HA1, Klaythong R, Sirikong M, *et al*. HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. *Tissue Antigens* 2002; 60: 309-18.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; 28: 2731-9.

Temprasertrudee S, Thanachartwet V, Desakorn V, Keatkla J, Chantratita W, Kiertiburanakul S. A multicenter study of clinical presentations and predictive factors for severe manifestation of dengue in adults. *Jpn J Infect Dis* 2018; 71: 239-43.

Thomas L, Brouste Y, Najioullah F, *et al*. Predictors of severe manifestations in a cohort of adult dengue patients. *J Clin Virol* 2010; 48: 96-9.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; 22: 4673-80.

Trung DT, Thao TT, Hien TT, *et al*. Liver involvement associated with dengue infection in adults in Vietnam. *Am J Trop Med Hyg* 2010; 83: 774-80.

Vaughn DW, Green S, Kalayanarooj S, *et al*. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis* 2000; 181: 2-9.

World Health Organization (WHO). Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Geneva: WHO; 1997. Available from: URL: http://apps.who.int/iris/bitstream/10665/41988/1/9241545003_eng.pdf

World Health Organization, Regional Office for South-East Asia (WHO SEARO). Comprehensive guideline for prevention and control of dengue and dengue haemorrhagic fever. Revised and expanded edition 2011 [cited 2018 Jun 30]. Available from: URL: https://apps.who.int/iris/handle/10665/204894

Yung CF, Lee KS, Thein TL, *et al*. Dengue serotype-specific differences in clinical manifestation, laboratory parameters and risk of severe disease in adults, Singapore. *Am J Trop Med Hyg* 2015; 92: 999-1005.

Zhang Y, Corver J, Chipman PR, *et al*. Structures of immature flavivirus particles. *EMBO J* 2003; 22: 2604-13.