CHARACTERIZATION OF DENGUE VIRUS SEROTYPE 4 INFECTION IN JAKARTA, INDONESIA

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Abstract. Dengue hemorrhagic fever has become a worldwide health issue. Heterologous infection by different serotypes may lead to severe forms of dengue infection and even death. In a cohort study in Jakarta from 2009 to 2010 with inclusion criteria of adults with fever of less than 48 hours, 72% were confirmed dengue infection from a total of suspected 190 dengue patients. Using RT-PCR, 16 patients were infected with DENV-4 with or without co-infection with other serotype(s). Dengue fever (DF) patients (73%) were infected with DENV-4 alone, while mixed infections were present in more severe clinical manifestations of DHF grade I and II. The nucleotide sequences of envelope (E) protein gene of 3 isolates of DENV-4 showed that they belonged to genotype II. There were 50 nucleotide substitutions, but only 3 amino acid changes were found at positions 130, 233 and 455, present in E protein isolated from DHF grade II patient.

Keywords: dengue virus serotype 4, genotype II, serotype mixed infection, Indonesia

INTRODUCTION

Mosquito-borne dengue viruses (DENV) are the cause of dengue disease, which commonly affect humans in tropical and sub-tropical areas of the world (Gubler and Meltzer, 1999). Over the decade of 2001-2010, there were 2.9 million dengue episodes and 5,906 deaths (Shepard *et al*, 2013). In 2003 alone, eight Southeast Asian Region countries (Bangladesh, India, Indonesia, Maldives, Myanmar, Sri Lanka, Thailand and Timor-Leste) reported dengue cases (WHO, 2011). Indonesia had the largest number of dengue infections among the South-East Asian countries with an estimate of 80,065 cases were reported in 2010 (WHO, 2011).

The pathological mechanisms of dengue hemorrhagic fever (DHF) are still unclear, although a number of hypotheses have been proposed, based on epidemiological and experimental studies to explain the pathogenesis of the severe form of the illness. One hypothesis is that

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cytokine storm is responsible for plasma leakage, the hallmark of DHF, which has been supported by many studies (Libraty *et al*, 2001; Bosch *et al*, 2002). Another hypothesis is that of antibody-dependent enhancement (ADE), which arose from the observation that secondary infection with heterotypic dengue virus is associated with an increased risk of DHF. This hypothesis was supported by experimental studies showing that sub-neutralizing concentrations of antibodies increase virus titers *in vivo* and *in vitro* (Halstead, 1979; Kliks *et al*, 1989). However, secondary infection by certain DENVs does not produce DHF/dengue shock syndrome (DSS) (Murgue *et al*, 2000; Prasetyo *et al*, 2011). DHF may also be due to the intrinsic biological properties of DENV strains (Rico-Hesse *et al*, 1997; Leitmeyer *et al*, 1999).

DENV genome is composed of approximately 10,600 nt single stranded positive sense RNA containing a single open reading frame (ORF) that is flanked by 2 un-translated regions (UTRs) at the 5′ and 3′ ends (Rice *et al*, 1985). DENV virion is composed of 3 structural proteins; a core protein (C), a membrane protein (M), an envelope (E) protein and 7 non-structural proteins (Chambers *et al*, 1990). There are 4 distinct serotypes of DENV, known as DENV-1, DENV-2, DENV-3, and DENV-4. The amino acid differences of DENVs have been implicated in the pathogenesis of DHF (Mangada and Igarashi, 1998; Pandey and Igarashi, 2000).

Most of the epitopes involved in neutralization of dengue viruses are located in the E protein (Roehrig *et al*, 1998). E protein is also important for virus attachment to host cells and for fusion of virions with host cell membrane (Crill and Rochrig, 2001), and the E protein sequence of DENV-4 is relatively conserved (Patil *et al*, 2012).

Although dengue epidemics occur at regular intervals in Indonesia, there are limited reports of the diversity of DENVs isolated in Indonesia, especially of DENV-4 (Personal observation: only 4 strains of DENV-4 have been deposited in GenBank). Analysis of the viruses isolated from DHF patients in Indonesia from 1975 to 1978 and from 2005 to 2010 demonstrated that all DENV serotypes are endemic in Jakarta, but DENV-3 is the most predominant (Gubler *et al*, 1979; Corwin *et al*, 2001; Prasetyo *et al*, 2011).

In the present study, we determined the pathology of dengue with genetic relationship of DENV-4 E proteins isolated in Jakarta between 2009 and 2010.

MATERIALS AND METHODS

Patients

Study locations were in endemic dengue communities of Central, North, South, East and West Jakarta, Indonesia. Research design was a cohort study to determine severe diseases of the patients. Inclusion criteria were age ≥14 years with fever temperature >38.0°C of less than 48 hours. Clinical suspicions for dengue and disease severity were based on WHO 1997 criteria. The case definition for DHF lists the presence of four criteria: fever, hemorrhagic manifestations, thrombocytopenia (platelets ≤100,000 cells/mm^3), and evidence of plasma leakage (pleural effusion, ascites, hemoconcentration ≥20% or hypoproteinemia). DHF is divided into four grades (DHF I–IV). Grade I and grade II are non-shock DHF. In grade I the hemorrhagic manifestation is only a positive tourniquet test, while in grade II there is spontaneous bleeding usually in the form of petechiae, bleeding from the nose or gums, hematemesis and melena. Grade III and IV are cases of DHF with shock (DSS).

In grade III, a sign of shock may include cold clammy skin, restlessness, rapid and weak pulse, narrow pulse pressure (20mmHg or less) or hypotension. Grade IV case are those with profound shock with undetectable pulse and/or blood pressure. Occasionally, DF cases, particularly those with unusual hemorrhagic or with thrombocytopenia, are misclassified as DHF grade I or grade II.

After detection of fever, patients or their parents/guardians if <18 years old were asked for informed consent. Then, blood was drawn for dengue serologic test, culturing and other laboratory markers. NS1-antigen and IgM/IgG tests (Standard Diagnostic, Kyonggi, Korea) were performed for the first screening of dengue infection. Patents positive for putative dengue infection were hospitalized at Cipto Mangunkusumo General Hospital, Jakarta. The gold standard for dengue virus infection in this study was reverse transcriptase-polymerase chain reaction (RT-PCR), or virus isolation in C6/36 cell line, or IgG ELISA on acute and convalescence sera (Focus). A case was considered as having dengue infection when positive for one or more of these tests performed at the Department of Microbiology, Medical Faculty, Universitas Indonesia, Jakarta.

DENV serotyping

DENV serotypes were determined by semi-nested RT-PCR (Lanciotti *et al*, 1992). In brief, RNA was extracted from 140 l of plasma or culture supernatant of infected C6/36 cells using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA isolation and PCR were performed in a strict containment area in order to avoid sample to sample contamination. Negative controls were included in RNA isolation and in both steps of PCR.

PCR-I amplification reaction included 4 l of 10X PCR buffer containing 1.5 mM $MgCl_2$, 0.8 l of 10mM of each dNTPs, 0.4 l of Super Script II RTase (Invitrogen, Carlsbad, CA), 0.15 l of 5 U/ l Platinum *Taq* DNA polymerase (Invitrogen), 0.8 l of each D1 and D2 primer (10 M), and 8 l of RNA, in total reaction mixture of 40 l. PCR-1 thermocycling (Applied Biosystem Programmable 9700 Thermal Cycler; Applied Biosystem, Faster City, CA) was performed as follows: 53ºC for 30 minutes; 95ºC for 5 minutes; 30 cycles of 95ºC for 45 seconds, 60ºC for 30 seconds, and 72ºC for 90 seconds; and a final heating at 72ºC for 7 minutes. PCR-II included 2.5 l of 10X PCR buffer containing 1.5 mM $MgCl_2$, 0.5 l of 10 mM of each dNTPs, 0.15 l of 5 U/ l of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 1 l of each D1, TS1, TS2, TS3, and TS4 primers (10 M), 2 l of PCR- I product in total reaction mixture of 25 l. PCR-II thermocycling was as follows: 95ºC for 5 minutes; 35 cycles of 95ºC for 45 seconds, 60ºC for 30 seconds, and 72ºC for 60 seconds; with a final step at 72ºC for 7 minutes. PCR amplicons were analysed by 2% agarose gel-electrophoresis, stained with ethidium bromide, and visualized under UV light. For specimens with PCR results showing co-infection with more than one serotypes, we repeated PCR-II with primers for each serotype.

Determination of DENV-4 E protein nucleotide sequence

We performed DNA sequencing of DENV-4 E protein gene obtained from plasma of patients with complementary (c)DNA as a templete. cDNA strands were reverse-transcribed using Super Script II First Strand Synthesis System with random hexanucleotide primers (Invitrogen, Carlsbad, CA) according to

Table 1
Name and nucleotide sequence of primers to applified envelope gene of DENV-4.

Primer Name	Sequence 5′-3′
DEN4-602s	5′-CCTACTGGTCAATACCGAAC-3′
DEN4-1097c	5′-CACTTCCTTGGCTGTTGTCT-3′
DEN4-979s	5′-GAGTCTCAGGTGGAGCATGG-3′
DEN4-1719c	5′-GCATGGCTCCTTCCTGAGAT-3′
DEN4-1660s	5′-AGGTTCCTCATGCCAAGAGA-3′
DEN4-2113c	5′-AACCAATGGAGTGTTAATGC-3′
DEN4-2009s	5′-GGCTGAGAATACCAACAGTG-3′
DEN4-2517c	5′-ACTGTTCTGTCCAAGTGTGC-3′
DEN4-2411s	5′-CACAGTTCAAGCAGACATGG-3′
DEN4-2966c	5′-GTGGTCACACACTTCTGAAC-3′

the manufacturer's instructions. PCR amplification of the E protein gene was performed using 2 l of cDNA, 3 l of 10 X PCR buffer containing 1.5 M $MgCl_2$, 1.5 l of 0.2 mM each dNTPs, 0.3 l of 2.5 U Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA), 0.5 l of 10 pM each primer (Table 1) in a total reaction mixture of 30 l. Thermocycling (Applied Biosystem Programmable 9700 Thermal Cycler; Applied Biosystem, Foster City, CA) was performed as follows: 94ºC for 1 minute; 30 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds, and 72ºC for 60 seconds; with a final step at 72ºC for 2 minutes. PCR amplicons were separated by electrophoresis as described above and purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) for direct sequencing. Primer sequences were designed from the published data of DENV-4 (GenBank accession no. GQ 398256) using a PRIMER design program, and DNA sequencing was conducted at the Eijkman Institute for Molecular Biology, Jakarta. Sequencing results were analysed by BioEdit software.

Phylogenetic analysis

DENV-4 E protein sequence from GenBank was used to develop a phylogenetic tree. Analysis of homology and phylogenetic tree was performed using Genetyx-Win version 6.1 and MEGA 5.1 Beta 4 program, respectively.

RESULTS

Dengue serotype

From 190 dengue-suspected patients fulfilling the inclusion criteria, 72% were confirmed as having dengue infection, with DENV-2 being the most dominant (Dewi *et al*, unpubished). Only 16 patients were infected with DENV-4, of whom 11 cases (69%) were infected with DENV-4 alone and 5 cases were co-infected with other serotypes, *ie*, DENV-1 (3 cases), DENV-2 (1 case) an DENV-1 plus DENV-3 (1 case) (Fig 1). Eight (72.7%) patients

Table 2
Severity of disease of patients infected with DENV-4.

Serotype	Disease severities (*n*, %)		
	DF	DHF I	DHFII
4 only	8 (72.7)	1 (9.1)	2 (18.2)
1 and 4	2 (66.7)	1 (33.3)	0
2 and 4	0	1 (100)	0
1,3 and 4	0	0	1 (100)

Table 3
Nucleotide sequences and amino acid subtitutions of DENV-4 strain IDS 44/10 and IDS 63/10 from DF patients and of IDS 96/10 from a DHF grade II patient. Amino acid position number corresponds to positions in the respective proteins.

	IDS 44/10	IDS 63/10	96/10
IDS 44/10		99.50%	96.50%
IDS 63/10	100%		96.20%
IDS 96/10	99.30%[a]	99.30%[a]	

[a]Amino acid substitutions were V130I, H233Y and I455V.

infected with DENV-4 alone showed mild disease (DF), whereas those with co-infection with other DENV serotype (s) showed more severe disease (Table 2). However, 2 cases (67%) with co-infection of DENV-4 with DENV-1 had DF, but co-infection of DENV-4 and DENV-2 showed clinical manifestation of DHF grade I and co-infection with 3 serotypes (DENV-1, DENV-3 and DENV-4) caused clinical manifestation of DHF grade II. All cases of mixed DENV infections were from secondary infections by HI test.

E protein of DENV-4 isolates

From all DENV-4 found in this study, we chose to randomly selected 3 isolates of DENV-4 for sequencing of the E protein gene: IDS 44/10 (infected with DENV-4 only) and IDS 63/10 (co-infected with DENV-1) from DF patients, and IDS 96/10 (co-infected with DENV-1 and DENV-3) from a patient with DHF grade II. The nucleotide sequences of envelope gene of IDS 44/10 and IDS 63/10 were 99.5% identical but that of IDS 96/10 was only 96.2% (Table 3). The deduced amino acid sequence was 100% identical between IDS 44/10 and IDS 63/10, but was only 99.3% identical (difference of 3 amino acids) with that of IDS 96/10 (Table 3). However, the amino acid sequence of envelope Domain III was identical in all 3 DENV-4 samples.

Phylogenetic analysis

In order to determine the genetic relationship among DENV-4 E genes, the nucleotide sequences were compared along with other published DENV E gene sequences available in GenBank. A phylogenetic tree was constructed on the basis of the full-length E gene, which cor-

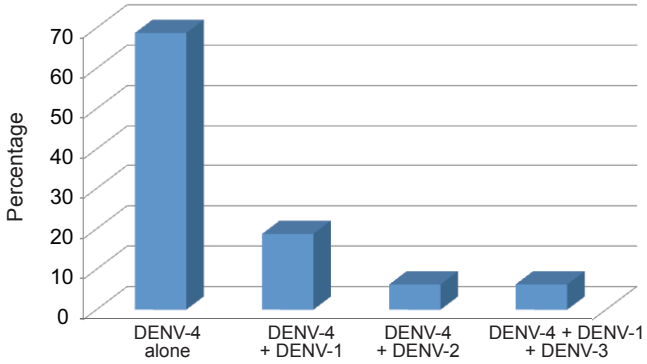

Fig 1–Prevalence of DENV-4 and co-infection in dengue patients in Jakarta, Indonesia during 2009 to 2010.

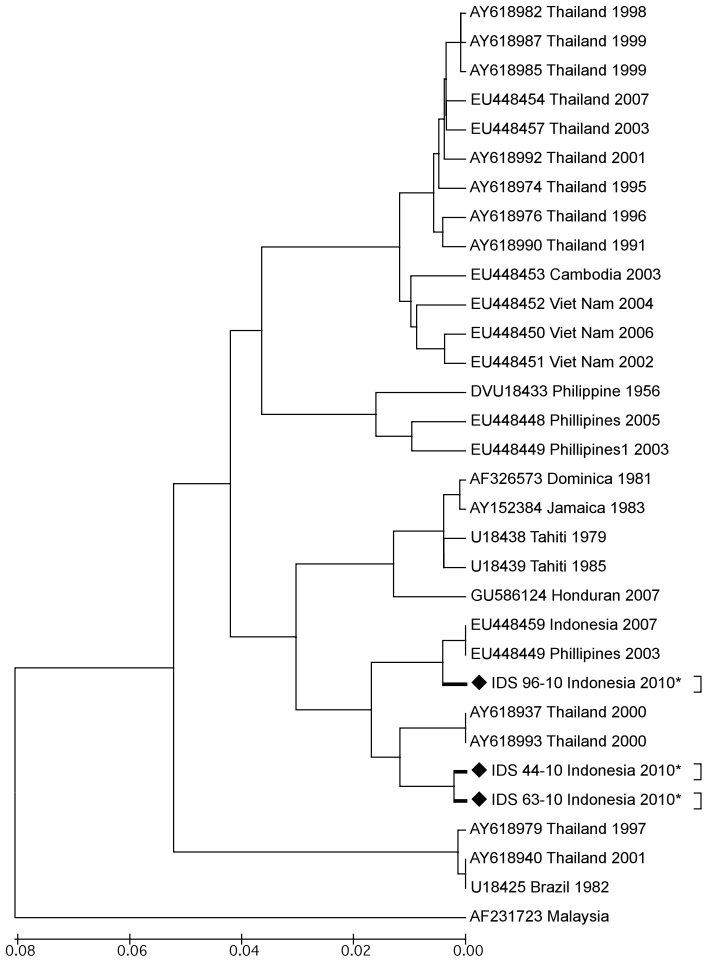

Fig 2–Phylogenetic tree of DENV-4 based on E protein gene sequence. Genotype clusters are labeled according to the scheme of Wu *et al* (2011). Names of isolates refer to country of origin and year of isolation. *Strain sequenced in this study.

responds to nt 916 to 2499 of DENV-4 genome. DENV-4 E genes in this study were that of genotype II together with DENV-4 from Dominica, Jamaica, Tahiti, Honduran, Thailand and other regions of Indonesia; Genotype I from Phillipines, Thailand and Malaysia in 1993 and genotype III in Thailand isolated in 1997 and Brazil (Fig 2) (Wu *et al*, 2011; Patil *et al*, 2012).

DISCUSSION

More recently, Indonesia is one of the countries with the highest cases of dengue infection, mostly notified in Jakarta. Among dengue patients in Jakarta during 2009 to 2010 infected with DENV-4, 69% were infected by DENV-4 alone and 72% of these patients manifested clinically mild disease. Study in India in 2009-2010 reported detection of 2 cases with DENV-4 infection in Pune, Maharashtra after an absence of almost 30 years, both were recorded as severe dengue requiring intensive care treatment, with one fatality (Cecilia *et al*, 2011). However in 2012, DENV-4 infection in central India showed a less severe clinical manifestation (Barde *et al*, 2012). In this study we found that co-infection of DENV-4 with DENV-1, DENV-2 or

with DENV-1 and DENV-3 showed severe clinical manifestations.

Based on E gene sequence, DENV-4 associated with DF and DHF in this study belonged to genotype II, the same genotype identified in Indonesia in 1973, 1977 and 2002 (Wu *et al*, 2011; Patil *et al*, 2012). DENV-4 isolated from India in 2009-2010 that cause severe clinical manifestations also belong to genotype I, the same as in Sri Lanka (Neeraja *et al*, 2013). The nucleotide and amino acid sequence diversity of the E gene of the Indian isolates increased from 1996 to 2009 (Neeraj *et al*, 2013). Genetic evolution among the DENV preM and E proteins has occurred independently within geographical regions where the viruses are endemic (Lanciotti *et al*, 1994). Phylogenetic studies have shown that DENVs can move short distances between neighboring countries (Kobayashi *et al*, 1999) as well as long distances between continents (Rico-Hesse *et al*, 1997).

As there were no differences in genotype among DF and DHF DENV-4 strains, we evaluated the sequences of DENV-4 E protein gene from 3 strains as a preliminary study. DF-associated DENV-4 E protein sequences were 100% identical although one sample came from a co-infection, whereas that from a DHF patient had 3 amino acid subtitutions (at positions 130, 233 and 455). It is possible that severe clinical manifestations of dengue could be due to amino acid subtitutions, and not due to mixed infection, but further studies are needed to evaluate this notion.

One hypothesis regarding pathogenesis of DHF is that secondary infection by another serotype is a risk factor (Rothman and Ennis, 1999; Morens and Fauci, 2008). Primary infection with one DENV serotypes induces an immune response that protects against secondary infection by the same serotype. Patients with primary infection are either negative for both IgM and IgG or positive for IgM only, whereas patients with secondary infection are positive for IgG only (Chanama *et al*, 2004; Sa-Ngasang *et al*, 2006). In this study, severe disease observed in all mixed DEN-4 infections were secondary infections.

The mechanisms involved in the pathogenesis of DHF are complicated and likely include multiple viral and host determinants. In this study we found DHF to be associated with mixed secondary DENV-4 infections and that a DENV-4 E protein from a DHF patient had 3 amino acid substitutions compared with 2 samples from DF subjects. We did not analyze the entire DENV-4 genome of the isolates, but in future studies we plan to sequence the whole DENV-4 genome in order to obtain a better understanding of the involvement of amino acid muations in the pathogenesis of DHF.

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