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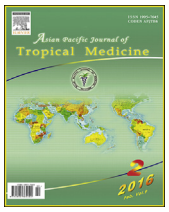

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Concurrent infections of dengue viruses serotype 2 and 3 in patient with severe dengue from Jakarta, Indonesia

Soroy Lardo[1], Yaldiera Utami[2], Benediktus Yohan[4], Seri MMU. Tarigan[2], Widayat Djoko Santoso[3], Leonard Nainggolan[3], R. Tedjo Sasmono[4*]

[1]*Division of Tropical Medicine and Infectious Diseases, Department of Internal Medicine, Gatot Soebroto Central Army Hospital, Jakarta, Indonesia*

[2]*Department of Internal Medicine, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia*

[3]*Division of Tropical Medicine and Infectious Diseases, Department of Internal Medicine, Cipto Mangunkusumo National Central Hospital, Jakarta, Indonesia*

[4]*Eijkman Institute for Molecular Biology, Jakarta, Indonesia*

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ABSTRACT

Objective: To describe the clinical manifestation of patient with severe dengue, to identify the serotypes and genotypes of dengue viruses (DENV) which concurrently infecting the patient, and to explore the possible relationship of severe dengue with the concurrent infection of DENV.

Methods: Dengue diagnosis was performed using NS1 antigen detection and IgG/IgM ELISA. Standard clinical and laboratory examinations were performed to obtain the clinical and hematological data. DENV concurrent infections were detected and confirmed using RT-PCR and DENV *Envelope* gene sequencing. Phylogenetic analyses were performed to determine the genotypes of the viruses.

Results: The patient was classified as having severe dengue characterized by severe plasma leakage, hemorrhage, and organ damage involving lung, liver, and kidney. Concurrent infection of DENV serotype 2 and 3 was observed. The infecting DENV-2 virus was grouped into Cosmopolitan genotype while DENV-3 virus was classified into Genotype I. Both viruses were closely related to isolates that were endemic in Jakarta. Viremia measurement was conducted and revealed a significantly higher virus titer of DENV-3 compared to DENV-2.

Conclusions: The occurrence of multi-serotype DENV infections was presented in a patient with severe clinical manifestation in Indonesia. The hyperendemicity of dengue in Indonesia may contribute to the DENV concurrent infections cases and may underlie the severity of the disease.

1. Introduction

Dengue is currently one of the most important arboviral disease in the world [1]. The disease is caused by infection of dengue virus (DENV), which is transmitted to human through the bites of *Aedes* spp. mosquito vectors. DENV consisted of four antigenically distinct but genetically similar serotypes, which are designated as DENV-1, DENV-2, DENV-3 and DENV-4 [2]. Dengue clinical manifestations vary from asymptomatic or mild flu-like syndrome known as classic dengue fever to more severe form known as dengue hemorrhagic fever (DHF) and the potentially fatal Dengue shock syndrome [3].

Dengue regularly occurred in tropical and sub-tropical regions around the world [4]. Co-circulation of multiple DENV serotypes is considered as one of the factors contributing to high cases of dengue infection. The circulation of multiple DENV serotypes in the same area has been reported since decades ago in countries in South East Asia, Central America, and South America [1]. In 2011, the entire tropical world is hyperendemic with multiple virus serotypes co-circulating in most large urban centers [1]. Indonesia is a tropical country that is

*Corresponding author: R. Tedjo Sasmono, Eijkman Institute for Molecular Biology, Jl. Diponegoro 69, Jakarta 10430, Indonesia.
Tel: +62 21 3917131
Fax: +62 21 3147982
E-mail: sasmono@eijkman.go.id
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hyperendemic to dengue, in which all four DENV serotypes circulating in the region. Dengue has become major public health problem in the country since it was first discovered in 1968 in Surabaya [5] and currently, the disease occurred in all 34 provinces in Indonesia [6]. Dengue cases occurred throughout the year and outbreaks occurred periodically such as those in 1998 [7] and 2004 [8].

Concurrent infections by more than one DENV serotypes have been suggested to influence clinical manifestation, and this was considered as one of the explanations for the emergence of DHF [9]. Living in dengue hyperendemic region, people in Indonesia have high probabilities of having concurrent DENV infections which may lead to severe dengue. Concurrent infections of DENV in Indonesia were first documented during 1975–1978 epidemics [10]. And then, more recent cases were also reported [11]. In this study, we reported a concurrent DENV-2 and DENV-3 infection case in patient with severe dengue. We examined the clinical, diagnostic, virological and molecular aspects of this DENV concurrent infections case.

2. Materials and methods

2.1. Case report, sample collection and serological tests

A 38-years old female patient (ID JKT-AD001) was administered to Gatot Soebroto Central Army Hospital in Jakarta, Indonesia on 16 April 2013. Serum sample was collected at day five of fever and routine diagnosis for confirmation of dengue infection was performed. Written informed consent was obtained from the patient. Serology and DENV NS1 antigen detection were performed using the Panbio NS1 rapid test and IgG/IgM ELISA (Alere, Brisbane, Australia). The IgG/IgM ELISA results were used to determine primary versus secondary dengue infection, according to manufacturer's protocol. Briefly, primary dengue infection was indicated by positive IgM (>11 Panbio Units) and negative IgG (<22 Panbio Units) while secondary dengue infection was indicated by positive IgG (>22 Panbio Units), which may be accompanied by elevated IgM levels.

2.2. RNA extraction, DENV detection and serotyping

Strict controls were applied on RNA extraction and PCR preparation/reaction procedures to prevent cross-contamination between samples. All of activities were performed in separate areas/containments and using separate sets of equipment conducted within Good Clinical Laboratory Practice-certified laboratory at the Eijkman Institute. DENV genomic RNA was extracted from 140 μL of patient's serum using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany). DENV nucleic acid detection and serotyping were performed using two steps conventional RT-PCR according to protocol described by Lanciotti *et al.* [12], with modification according to Harris *et al.* [13]. Superscript III RT enzyme (Invitrogen-Life Technologies, Carlsbad, CA) was used in the reverse-transcription reaction. The resulting cDNA was used in subsequent PCR reactions using Taq DNA Polymerase (Roche, Mannheim, Germany).

2.3. Determination of viral load

Two steps quantitative RT-PCR (qRT-PCR) analysis was used to quantify the virus titer in serum according to protocol adapted from conventional RT-PCR method by Lanciotti *et al.* [12]. Extracted RNA was reverse-transcribed using D2 antisense primer. The resulting cDNA was used as template in qRT-PCR reaction performed using Power SYBR-Green PCR kit and 7500 RT-PCR System (Applied Biosystems-Life Technologies, Foster City, CA). Viral load determination was performed in separate reaction mixes using serotype-specific primers D1 and TS2 and D1 and TS3 for DENV-2 and DENV-3, respectively. The reaction was prepared in triplicate using 400 nM of primers final concentration and thermal cycling setup of 10 min initial denaturation step at 95 °C, followed by 35 cycles of 10 s denaturation step at 95 °C and 1 min annealing/extension step at 60 °C. An additional dissociation stage was used to verify the melting temperature of amplicons. A recombinant plasmid standard was generated by cloning DENV genome fragment covering the *C*, *prM/M*, and *Envelope* (*E*) genes into PCR-Blunt plasmid using Zero Blunt PCR Cloning Kit (Invitrogen-Life Technologies) according to the manufacturer's instructions. Known copy number of recombinant plasmid was serially diluted and used as genome copy number standards in RT-PCR analyses. The copy number standards were then used to quantify genome copy number of viral RNA transcripts from 10-fold dilutions of known DENV titer measured by plaque assay in BHK21 cells. The resulting copy number-titer equivalent standard curve was used to calculate viral load in sample determined as DENV plaque forming unit (PFU) equivalent/mL of serum.

2.4. DENV Envelope gene sequencing

Viral RNA was extracted directly from serum sample and used as template for DENV genotyping using *E* gene sequences according to protocol described previously in Ref. [11]. Capillary sequencing reactions were performed to obtain complete *E* gene sequences of DENV-2 (1485 nt) and DENV-3 (1479 nt), employing serotype-specific primers described elsewhere [14]. In order to prevent possible contamination from DENV reference strains/positive controls being used, we have performed *E* gene sequencing for all of the reference strains available in our laboratory and maintained a controlled virus archiving procedure. The *E* gene sequences obtained in this study have been deposited in GenBank with accession numbers KJ184316 (for isolate JKT-AD001A) and KJ184317 (for isolate JKT-AD001B).

2.5. DENV genotype analyses

DENV genotype analyses were performed to generate classification of isolate sequences into genotypes. Isolate sequences were combined with the downloaded GenBank sequences according to sample's serotypes to create dataset for each genotypes grouping based on Twiddy [15] and Lanciotti [16] classifications for DENV-2, and DENV-3, respectively. Multiple sequence alignment for *E* gene was performed using MUSCLE [17]. Dataset for each serotype was prepared using BEAUti v.1.7.5 [18] and followed by phylogenetic reconstruction analysis using Bayesian Markov chain Monte Carlo method as implemented in BEAST v 1.7.5 [19] using GTR + Γ_4 model with codon model, relaxed uncorrelated lognormal molecular clock and Bayesian skyline prior, with 100 million generations and sampled for every 1000th iteration. All parameters showed effective sampling size values

of >100, measured using Tracer v.1.5.0. Maximum clade credibility (MCC) tree was created using TreeAnnotator v.1.7.5 and visualized in FigTree v.1.4.0, which are available inside the BEAST package.

3. Results

3.1. Clinical manifestation

A 38-year old female patient was administered to Gatot Soebroto Central Army Hospital in Jakarta, Indonesia after experiencing four days fever accompanied with myalgia, arthralgia, headache, vomiting, skin rash, epistaxis, and gum bleeding. Initial physical examination on the first day of hospitalization observed a normotensive blood pressure with 38.6 °C fever. Patient was fully conscious but presented a shortness of breath and bleeding gums. Lung examination presented smooth wet rhonchi, whereas the abdominal examination showed epigastric tenderness but no hepatomegaly observed. Petechiae were observed in both arms. Chest X-ray showed dextral pleural effusion. Normal electrocardiography was observed. Patient was diagnosed as DHF Grade III based on high fever with headache, myalgia, arthralgia, vomiting, abdominal pain, appetite loss, epistaxis and bleeding gums, wet rhonchi, mid-epigastric pain, petechiae and evidence of plasma leakage which include pleural effusion and hypoproteinemia. Thrombocytopenia and elevated level of transaminase enzymes were observed, however, hematocrit was still within normal level during acute phase. The patient condition on day five of hospitalization was worsened and then moved to ICU.

Further development in ICU, the patient showed stable vital signs and better breathiness. No fever, headache, abdominal pain, epistaxis and bleeding gums were observed. Appetite was gradually increased and nausea was absence. Vaginal bleeding was decreasing. At the tenth day of treatment in the ICU, a stable vital sign was observed and the patient was free of fever for approximately 3 d. Shortness of breath and hemorrhagic manifestations were not observed, although physical examination observed extensive hematoma in the arm region around the venipuncture area.

3.2. Clinical laboratory findings

Laboratory examination observed severe thrombocytopenia (13 000/μL) during febrile period, while final platelet count of 156 000/μL was observed when the patient has been fully recovered (convalescence phase at d 15 after hospitalization) (Table 1). Although thrombocytopenia was prominent in this patient, no significant hematocrit increment was observed during the course of hospitalization, although slight increase was observed during convalescence. The transaminase enzymes levels, markers for the liver function, showed gradual increment with the maximum level for SGOT/SGPT reached 5 641/1 807 IU/L. When the patient was fully recovered, the final levels of 82/134 IU/L were observed (Table 1). Leukocytosis was observed, with a maximum count of 22 300/μL. Leukocytosis was then improved to 8 000/μL at convalescence period (Table 1). We also examined the electrolyte level in the patient, in which hyponatremia, hypocalcemia, and hypoalbuminemia were observed (Table 1). Improvements of the above hematological findings were observed when patient in convalescence period. A sterile blood culture results was obtained. Chest X-ray result consistent with a sign of pneumonia. Patient was fully recovered.

3.3. Disease complication

We observed disease complications that may have effect to the clinical findings. Pulmonologist consultant observed the pulmonary edema with bronchopneumonia, and hemoptysis. The patient has a history of tuberculosis, and secondary bacterial infection was proposed based on fever, asphyxia, and coughing with white phlegm. Obstetrician diagnosed the patient with abnormal uterus bleeding caused by endometrium hyperplasia that had occurred within the last two months. This was confirmed by USG examination.

3.4. DENV serology, RT-PCR, and viral load determination

Patient serum collected on day five of fever was tested positive by anti-dengue NS1 Rapid Test. The anti-dengue IgM and

Table 1
Clinical laboratory findings of patient with concurrent DENV infections.

Laboratory test	Value		Reference value
	Acute	Convalescence	
Blood count			
Leukocyte	22 300.0/μL	8 000.0/μL	4 500.0–10 000.0/μL
Thrombocytes	13 000.0/μL	156 000.0/μL	130 000.0–340 000.0/μL
Hematocrit	25.0%	41.0%	35.0%–50.0%
Hemoglobin	9.4 g/dL	10.6 g/dL	12.1–15.1 g/dL
APTT	2.3 × control	1.6 × control	Laboratory control
Biochemistry			
SGOT	5 641.0 U/L	82.0 U/L	5.0–40.0 U/L
SGPT	1 807.0 U/L	134.0 U/L	7.0–56.0 U/L
Sodium	131.0 mmol/L	138.0 mmol/L	134.0–145.0 mmol/L
Potassium	3.4 mmol/L	3.4 mmol/L	3.4–5.2 mmol/L
Albumin	2.6 g/dL	3.4 g/dL	3.5–5.3 mg/dL
Urea	21.0 mg/dL	67.0 mg/dL	15.0–45.0 mg/dL
Creatinine	0.8 mg/dL	1.5 mg/dL	<1.4 mg/dL

APTT: Activated partial thromboplastin time.
Acute phase was described as the day of hospitalization (day 0), while convalescence phase was d 15 after hospitalization.

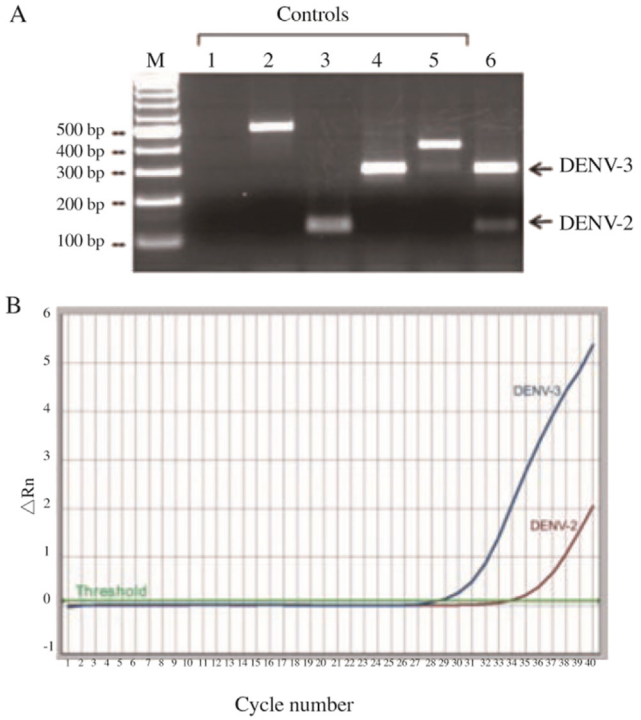

Figure 1. (A) Detection of DENV concurrent infections in JKT-AD001 patient's serum using conventional RT-PCR.
M: DNA molecular weight marker; lane 1: no-template control; lane 2–5: PCR amplicon positive controls for DENV-1 (strain WestPac), DENV-2 (strain NGC), DENV-3 (strain H87), and DENV-4 (strain H241) respectively; lane 6: PCR amplicons for patient serum.
(B) Confirmation of DENV detection and viral titer measurement using SYBR-Green qRT-PCR.
ΔRn is the fluorescence of the reporter dye divided by fluorescence of a passive reference dye, minus the baseline. Virus titers are depicted in the table below the RT-PCR amplification plot.

IgG ELISA were also performed on the serum and both were positive (data not shown). The infection status of the patient was secondary infection as determined by the IgM and IgG values, performed according to the assay method. Confirmation of DENV infection was further pursued using RT-PCR to detect and serotype the infecting virus. The RT-PCR detected the presence of two PCR amplicons which were corresponded to DENV-2 and DENV-3, albeit of fainter band for DENV-2 (Figure 1A). To quantitatively determine the titers of the infecting viruses, we performed qRT-PCR detection of viremia in the patient's serum. The presence of concurrent infections was further confirmed using this method (Figure 1B), and the DENV-2 virus titer was determined as 2.5×10^2 PFU equivalent/mL of serum, while DENV-3 viral load reached 6.8×10^4 PFU equivalent/mL (Figure 1B).

3.5. DENV E gene sequencing and phylogenetic analysis

Given the presence of two DENV serotypes in RT-PCR detection and serotyping result, we sought to further confirm the concurrent DENV infections by using *E* gene sequencing. Amplification of *E* gene in RNA directly extracted from serum sample successfully obtained both DENV-2 and DENV-3 *E* gene amplicons (data not shown). The *E* gene fragments were then subjected to DNA sequencing. The sequences of the *E* genes confirmed the serotypes of the infecting viruses as DENV-2 and DENV-3. To determine the genotype of the viruses, we performed phylogenetic analyses of both DENVs. Our phylogenetic analysis of DENV-2 grouped the infecting virus into Cosmopolitan genotype according to Twiddy *et al* [15] (Figure 2). The isolate (JKT-AD001A) was closely related to viruses we recently isolated in Sukabumi, West Java province, Indonesia in 2012 [20] and grouped together with isolates from

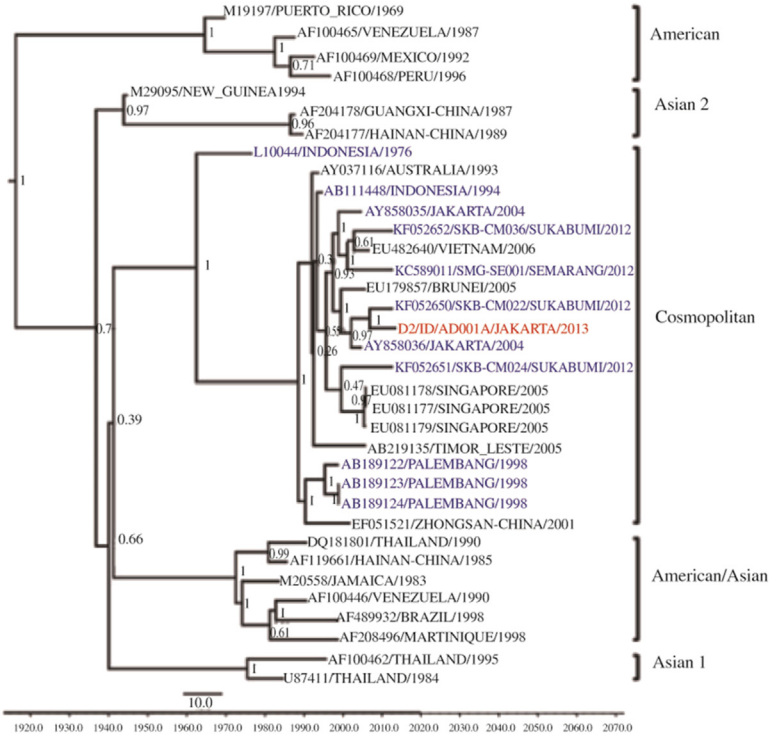

Figure 2. MCC tree of DENV-2 genotypes grouping generated by Bayesian inference method as implemented in BEAST using GTR evolution model and gamma parameter rates from the *E* gene sequences.
The Jakarta JKT-AD001A isolate (denoted with red label) was grouped into the Cosmopolitan genotype based on classification by Twiddy *et al.* Isolates from Indonesian cities were denoted with blue labels. The number in the node indicated the posterior probability of that particular cluster.

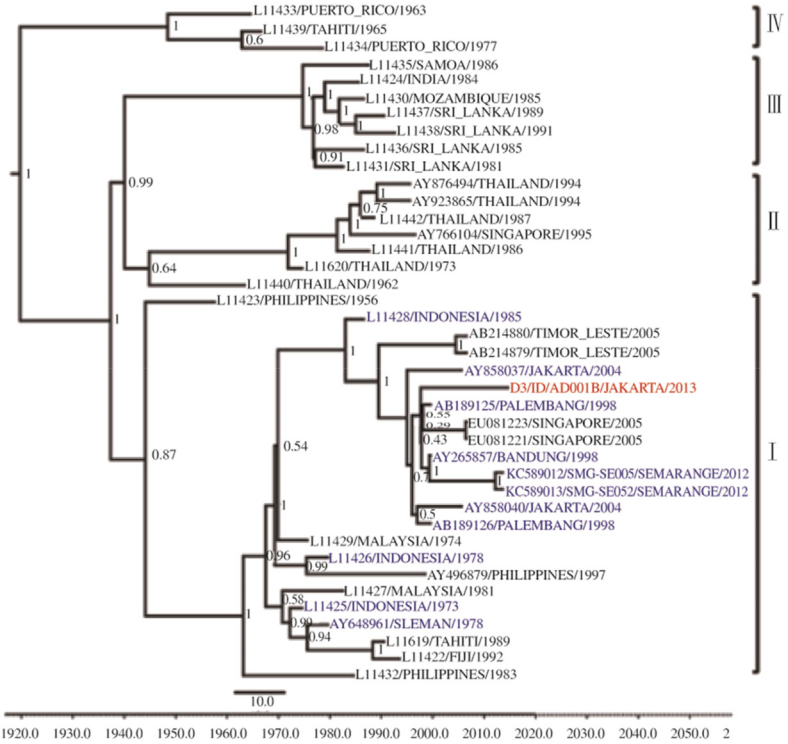

Figure 3. MCC tree of DENV-3 genotypes grouping generated by Bayesian inference method as implemented in BEAST using GTR evolution model and gamma parameter rates from the *E* gene sequences.
The Jakarta JKT-AD001B isolate (denoted with red label) was grouped into genotype I based on classification by Lanciotti *et al.* Isolates from Indonesian cities were denoted with blue labels. The number in the node indicated the posterior probability of that particular cluster.

other cities in Indonesia such as Jakarta isolated in 1994 and 2004, Semarang (isolated in 2012 [11]), and Palembang (isolated in 1998) (Figure 2). Meanwhile, phylogenetic analysis of DENV-3 *E* protein gene revealed the genotype of the infecting virus as Genotype I based on Lanciotti classification [16].

As seen in Figure 3, the virus isolate (JKT-AD001B) was closely related with viruses from Jakarta (isolated in 2004), Palembang (isolated in 1998), Bandung (isolated in 1998), and Semarang (isolated in 2012).

4. Discussion

Dengue has become a health problem in many tropical countries including Indonesia, in which hyperendemicity contributes to the increasing dengue cases. Coupled with high vector index, the occurrence of concurrent infections with multiple DENV serotypes is highly possible. With the advancement of DENV detection technologies, more and more concurrent dengue infections cases were reported. Concurrent infections cases have been reported in Mexico, Puerto Rico, Taiwan, China, Brazil, Thailand, and India [10,21–28]. For Indonesia, the concurrent infections were first reported during outbreaks in 1976–1978, which was accounted for 11.1% of the cases [10]. We have also recently reported other concurrent infections cases in Semarang city, Indonesia [11] and other cities in Indonesia [29]. In this study, we reported a concurrent infections case in patient from Jakarta, a city with highest dengue incidence rate in Indonesia [6]. We believe that concurrent infections cases may also occurred in other cities in Indonesia. However, because of the limited data on DENV surveillance in Indonesia, the available data may be underreported. This study was the first to report the concurrent infections case in Indonesia in detail, in which the clinical, diagnostic, molecular and virological aspects of the infection were presented.

Concurrent infections have been proposed as one of contributing factors to severe dengue [9]. However, several reports described the absence of relationship between concurrent infections with severe disease [10,26]. Unlike those reports, the patient reported in this study presented a severe dengue. The patient exhibited abdominal pain and persistent vomiting, clinical warning signs of severe dengue [4]. Leukocytosis was also observed, which may indicate a warning sign of severe dengue [30]. The atypical manifestations of dengue are uncommon, but there have been increasing reports of DF and DHF with unusual manifestation [4,31]. In this patient, atypical manifestations of dengue were observed and involved the hepatic, pulmonary, and renal manifestations. It has been reported that DHF may cause mild to moderate liver dysfunction in most cases, however, only some patients may suffer from acute liver failure [32]. The hepatic manifestation in this patient was characterized by increasing level of aminotransferase enzymes. The pulmonary involvement included shortness of breath, pleural effusion, cough, and hemoptysis. Although renal failure is a rare complication in dengue infections, the deranged serum urea and creatinine levels during the course of illness in this patient were indicative of renal dysfunction. Altogether, these are consistent with severe dengue.

As mentioned above, although reports indicated that concurrent infections did not related with severe dengue [10,26], other studies observed the higher percentage of cases with concurrent infections had severe disease [25,27]. Thus, our study is in accordance with those reports.

Previous study has demonstrated that higher viremia titer was associated with more severe disease [33]. In this report, we measured the viremia using qRT-PCR. To our knowledge, our study is the first to present data on the viral loads of concurrent DENV infections. We observed a significantly higher titer of DENV-3 (*ie*. 272 times) compared to the DENV-2. This data suggest that, compared to DENV-2, the DENV-3 might possessed better replication rate in the patient and might predominantly replicated in the body and thus affect the immune system. However, we are not sure whether the higher viral loads of DENV-3 compared to DENV-2 was occurred since the early days of infection since we only measured the viral load in a single time point (day five of fever). As far as we aware, there is no previous study that describe the comparative fitness of DENV in patient with concurrent infections. Further, it may be that the severity of disease in concurrent infections is not caused by the synergistic action of both serotypes, but more likely caused by one of the virus that have higher virus titer, just as in single infection cases. As this study only observed the difference in serotype-specific viral load in one patient, we cannot confirm that the finding is generalizable to other concurrent infections cases. Determination of viral loads in larger number of concurrent infections cases will be beneficial to confirm this observation.

With respect to the infecting DENV, we determined the genotypes of both DENV-2 and DENV-3 viruses using phylogenetic analyses based on *E* protein gene sequences. The DENV-2 isolate was grouped into Cosmopolitan genotype according to Twiddy *et al.* [15]. This genotype is widely distributed in many countries such as in India, countries in South East Asia, Africa, the Middle East, and Australia [15]. In term of genetic relationship with other DENV-2 isolates, this isolate was closely related and grouped together with DENV-2 viruses from Jakarta isolated in 2004, and from other cities in Indonesia such as Sukabumi (isolated in 2012), and Palembang (isolated in 1998). This suggested that the infecting virus was endemic in the area, and has been circulated for a long time. Similarly, the genotype of infecting DENV-3, which was grouped into Genotype I according to Lanciotti *et al.* [16], was closely related to DENV-3 viruses from Jakarta and other cities in Indonesia such as Bandung, Palembang, and Semarang. Altogether, our phylogenetic data demonstrated the sustainable circulation of endemic strains of DENV in Indonesia which regularly infecting people and actively transmitted in the community.

In summary, our study presented the occurrence of multi-serotype DENV infections in a patient with severe clinical manifestation in Indonesia. The hyperendemicity of dengue in Indonesia may contribute to the DENV concurrent infections cases and may underlie the severity of the disease.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Gubler DJ. Dengue, urbanization and globalization: the unholy trinity of the 21st century. *Trop Med Health* 2011; **39**(4): 3-11.
[2] Simmons CP, Farrar JJ, van Nguyen VC, Wills B. Dengue. *N Engl J Med* 2012; **366**(10): 1423-1432.
[3] Martina BEE, Koraka P, Osterhaus ADME. Dengue virus pathogenesis: an integrated view. *Clin Microbiol Rev* 2009; **22**(10): 564-581.
[4] WHO. Comprehensive guidelines for prevention and control of dengue and dengue haemorrhagic fever. World Health Organization; 2011.
[5] Sumarmo. Dengue haemorrhagic fever in Indonesia, southeast Asian. *J Trop Med Public Health* 1987; **18**(3): 269-274.
[6] Ministry of Health Republic of Indonesia. *Indonesia health profile 2010*. 2011. Jakarta.
[7] Corwin AL, Larasati RP, Bangs MJ, Wuryadi S, Arjoso S, Sukri N, et al. Epidemic dengue transmission in southern Sumatra, Indonesia. *Trans R Soc Trop Med Hyg* 2001; **95**(3): 257-265.
[8] Suwandono A, Kosasih H, Nurhayati, Kusriastuti R, Harun S, Ma'roef C, et al. Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004. *Trans R Soc Trop Med Hyg* 2006; **100**(9): 855-862.
[9] Hammon WM. Dengue hemorrhagic fever-do we know its cause? *Am J Trop Med Hyg* 1973; **22**(1): 82-91.
[10] Loroño-Pino MA, Cropp CB, Farfán JA, Vorndam AV, Rodríguez-Angulo EM, Rosado-Paredes EP, et al. Common occurrence of concurrent infections by multiple dengue virus serotypes. *Am J Trop Med Hyg* 1999; **61**(5): 725-730.
[11] Fahri S, Yohan B, Trimarsanto H, Sayono S, Hadisaputro S, Dharmana E, et al. Molecular surveillance of dengue in Semarang, Indonesia revealed the circulation of an old genotype of dengue virus serotype-1. *PLoS Negl Trop Dis* 2013; **7**(8): e2354.
[12] 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**(3): 545-551.
[13] Harris E, Roberts TG, Smith L, Selle J, Kramer LD, Valle S, et al. Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *J Clin Microbiol* 1998; **36**(9): 2634-2639.
[14] Ong SH, Yip JT, Chen YL, Liu W, Harun S, Lystiyaningsih E, et al. Periodic re-emergence of endemic strains with strong epidemic potential-a proposed explanation for the 2004 Indonesian dengue epidemic. *Infect Genet Evol* 2008; **8**(2): 191-204.
[15] Twiddy SS, Farrar JJ, Vinh Chau N, Wills B, Gould EA, Gritsun T, et al. Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. *Virology* 2002; **298**(1): 63-72.
[16] Lanciotti RS, Lewis JG, Gubler DJ, Trent DW. Molecular evolution and epidemiology of dengue-3 viruses. *J Gen Virol* 1994; **75**(Pt1): 65-75.
[17] Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004; **32**(5): 1792-1797.
[18] Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol* 2012; **29**(8): 1969-1973.
[19] Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007; **7**: 214.
[20] Nusa R, Prasetyowati H, Meutiawati F, Yohan B, Trimarsanto H, Setianingsih TY, et al. Molecular surveillance of dengue in Sukabumi, west Java province, Indonesia. *J Infect Dev Ctries* 2014; **8**(6): 733-741.
[21] Laille M, Deubel V, Sainte-Marie FF. Demonstration of concurrent dengue 1 and dengue 3 infection in six patients by the polymerase chain reaction. *J Med Virol* 1991; **34**(1): 51-54.
[22] de Carvalho Araújo FM, Nogueira RMR, de Araújo JMG, Ramalho ILC, de Sá Roriz MLF, de Melo MEL, et al. Concurrent infection with dengue virus type-2 and DENV-3 in a patient from Ceará, Brazil. *Mem Inst Oswaldo Cruz* 2006; **101**(8): 925-928.

[23] Chinnawirotpisan P, Mammen MP Jr, Nisalak A, Thaisomboonsuk B, Narupiti S, Thirawuth V, et al. Detection of concurrent infection with multiple dengue virus serotypes in Thai children by ELISA and nested RT-PCR assay. *Arch Virol* 2008; **153**(12): 2225-2232.

[24] Wenming P, Man Y, Baochang F, Yongqiang D, Tao J, Hongyuan D, et al. Simultaneous infection with dengue 2 and 3 viruses in a Chinese patient return from Sri Lanka. *J Clin Virol* 2005; **32**: 194-198.

[25] Bharaj P, Chahar HS, Pandey A, Diddi K, Dar L, Guleria R, et al. Concurrent infections by all four dengue virus serotypes during an outbreak of dengue in 2006 in Delhi, India. *Virol J* 2008; **5**(1); http://dx.doi.org/10.1186/1743-422X-5-1.

[26] Gubler DJ, Kuno G, Sather GE, Waterman SH. A case of natural concurrent human infection with two dengue viruses. *Am J Trop Med Hyg* 1985; **34**(1): 170-173.

[27] Vinodkumar CS, Kalapannavar NK, Basavarajappa KG, Sanjay D, Gowli C, Nadig NG, et al. Episode of coexisting infections with multiple dengue virus serotypes in central Karnataka, India. *J Infect Public Health* 2013; **6**(4): 302-306.

[28] Wang WK, Chao DY, Lin SR, King CC, Chang SC. Concurrent infections by two dengue virus serotypes among dengue patients in Taiwan. *J Microbiol Immunol Infect* 2003; **36**(2): 89-95.

[29] Aryati A, Trimarsanto H, Yohan B, Wardhani P, Fahri S, Sasmono RT. Performance of commercial dengue NS1 ELISA and molecular analysis of *NS1* gene of dengue viruses obtained during surveillance in Indonesia. *BMC Infect Dis* 2013; **13**: 611.

[30] Lee IK, Liu JW, Yang KD. Fatal dengue hemorrhagic fever in adults: emphasizing the evolutionary pre-fatal clinical and laboratory manifestations. *PLoS Negl Trop Dis* 2012; **6**(2): e1532.

[31] Gulati S, Maheshwari A. Atypical manifestations of dengue. *Trop Med Int Health* 2007; **12**(9): 1087-1095.

[32] Nguyen TL, Nguyen TH, Tieu NT. The impact of dengue haemorrhagic fever on liver function. *Res Virol* 1997; **148**(4): 273-277.

[33] Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis* 2000; **18**(1): 2-9.