Original Article

Molecular surveillance of Dengue in Sukabumi, West Java province, Indonesia

Roy Nusa[1], Heni Prasetyowati[1], Febrina Meutiawati[2], Benediktus Yohan[2], Hidayat Trimarsanto[2], Tri Yuli Setianingsih[2], R Tedjo Sasmono[2]

[1] *Vector Borne Disease Control, Research and Development Council, Ministry of Health of the Republic of Indonesia, Ciamis, West Java, Indonesia*
[2] *Eijkman Institute for Molecular Biology, Jakarta, Indonesia*

Abstract

Introduction: Dengue is endemic and affects people in all Indonesian provinces. Increasing dengue cases have been observed every year in Sukabumi in West Java province. Despite the endemicity, limited data is available on the genetic of dengue viruses (DENV) circulating in the country. To understand the dynamics of dengue disease, we performed molecular and serological surveillance of dengue in Sukabumi.

Methodology: A total of 113 patients were recruited for this study. Serological data were obtained using anti-dengue IgM and IgG tests plus dengue NS1 antigen detection. Dengue detection and serotyping were performed using real-time RT-PCR. Viruses were isolated and the envelope genes were sequenced. Phylogenetic and evolutionary analyses were performed to determine the genotype of the viruses and their evolutionary rates.

Results: Real-time RT-PCR detected DENV in 25 (22%) of 113 samples. Serotyping revealed the predominance of DENV-2 (16 isolates, 64%), followed by DENV-1 (5 isolates, 20%), and DENV-4 (4 isolates, 16%). No DENV-3 was detected in the samples. Co-circulation of genotype I and IV of DENV-1 was observed. The DENV-2 isolates all belonged to the Cosmopolitan genotype, while DENV-4 isolates were grouped into genotype II. Overall, their evolutionary rates were similar to DENV from other countries.

Conclusions: We revealed the distribution of DENV serotypes and genotypes in Sukabumi. Compared to data obtained from other cities in Indonesia, we observed the differing predominance of DENV serotypes but similar genotype distribution, where the infecting viruses were closely related with Indonesian endemic viruses isolated previously.

Key words: dengue; surveillance; serotype; genotype

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Introduction

Dengue is the most important arthropod-borne viral infection of humans and has a large global burden. There are an estimated 50 million infections per year occurring across approximately 100 countries in tropical and sub-tropical regions in the world, and there is potential for further spread. The disease affects approximately 2.5 billion people living in Southeast Asia, the Pacific, and the Americas [1,2]. Dengue disease causes variable clinical manifestations, ranging from an undifferentiated fever (dengue fever, DF) to more severe forms of the disease, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [3].

Dengue disease is caused by dengue virus (DENV), a member of *Flaviviridae* family, with a substantial genetic diversity shown by the presence of four serotypes (DENV-1, -2, -3, and -4) and multiple genotypes (or subtypes) within each serotype [4,5]. DENV is transmitted to humans through the bites of *Aedes aegypti* and *Ae. albopictus* mosquito vectors. The genome consists of single-stranded positive-sense RNA, which encodes three structural (C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [1].

Indonesia is a vast archipelago country that is regularly affected by the disease. Dengue occurs in all 33 provinces in the country annually and periodic major outbreaks occur regularly. Sukabumi is a city located in the south central part of West Java province, Indonesia. The city is situated at the foot of Mounts Gede and Pangrango at an altitude of about 600 meters above sea level, with a maximum daytime temperature of 29°C. It is located 120 km south of Indonesia's capital city, Jakarta, and 96 km west of West Java's capital, Bandung. The population of the city was about

260,000 in 2011, residing in an area of 48.2 km^2. Dengue is reported in Sukabumi every year, and data from the Sukabumi Health Authority recorded an increase in the number of cases; 532 cases occurred in 2011, and 962 cases occurred in 2012 [6]. To understand the dynamic of dengue disease in Sukabumi, we performed a surveillance study. We report here information about the prevalence of the disease, demography, serological features, as well as the DENV serotype and genotype distribution in the city.

Methodology

Sample collection and serological tests

Clinical samples used in this study were collected from R. Syamsudin SH and Assyfa Hospitals in Sukabumi, West Java province, Indonesia, between March and December 2012. Ethical clearance was obtained from the National Institute of Health Research Development, Ministry of Health of the Republic of Indonesia. Dengue-suspected febrile patients with positive results for IgG/IgM and/or NS1 were included in the study after written informed consent was obtained. Febrile patients who were negative for the above tests or did not provide informed consent were excluded. Serology and DENV NS1 antigen detection were performed using IgG/IgM and NS1 rapid tests (CTK Biotech, San Diego, CA). All samples were collected during the acute phase, typically within the first six days of illness. Since this study focused on virus surveillance, the clinical manifestations of dengue in the patients were not specifically recorded.

RNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Virus RNA was extracted from serum samples using MagNA Pure LC Total Nucleic Acid Isolation Kit performed in automated MagNA Pure LC 2.0 Instrument (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 200 µL of serum samples were added into designated wells of the sample cartridge. Additional Simplexa Dengue Molecular Control (MC) (Focus Diagnostics, Cypress, USA) and No-Template Control (NTC) were included in the extraction as controls. In order to ensure optimum RNA extraction process, 5 µL of Simplexa RNA Internal Control (IC) was added into samples. The total NA variable elution volume purification protocol was used to isolate viral nucleic acid. During the set-up stage, the software listed the entire reagents and their necessary amounts for the extraction process. Reagents were prepared, filled into reagent tubs, and placed in the reagent reservoir rack. Following the sample and reagent preparation steps above, the sample cartridge and reagent reservoir rack were then transferred to the MagNA Pure LC 2.0 Instrument. The automated extraction process was started and the resulting nucleic acid was directly used as a template in real-time RT-PCR process or stored in a -80°C freezer until use.

Simplexa Dengue (Focus Diagnostics, Cypress, USA) was used to simultaneously detect and serotype DENV from extracted RNA samples. The assay is a real-time RT-PCR that discriminates DENV-1 and -4 in one reaction, and DENV-2 and -3 in another reaction. Bi-functional Scorpions-based fluorescent probe-primers together with reverse primers were used in this method to amplify NS5, NS3, NS5, and capsid genes of DENV-1, DENV-2, DENV-3, and DENV-4, respectively. An RNA internal control (RNA IC) was used to monitor the RNA extraction process and to detect RT-PCR inhibition. RT-PCR reactions were performed using the method described by the manufacturer. Samples were run using pre-programmed conditions set by the manufacturer. Data collection and analysis were performed using Integrated Cycler Studio Software version 4.2.

Virus isolation using cell culture

Serum samples with serological or RT-PCR-positive results were subjected to a maximum of two passages of inoculation in the C6/36 (*Aedes albopictus*, mid gut) cell line [7]. Briefly, a monolayer of cells was inoculated with 200 µL of sera in 2 mL of 1X RPMI medium supplemented with 2% of fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (all from Gibco-Life Technologies, Carlsbad, USA). Flasks were incubated for one hour at 28°C to allow virus attachment. Following the incubation period, the inoculation medium was discarded and the medium was replenished with 3 mL of fresh medium. Infected cells were incubated at 28°C for up to 14 days, during which viruses were harvested on day 9; the media were then replenished and followed by further five-day cultures for the final virus harvest.

DENV envelope gene sequencing

DENV genotyping was performed using the envelope (E) gene sequence (1,485 nt in length). DENV RNA extracted from the virus culture supernatant was reverse-transcribed into cDNA using Superscript III Reverse Transcriptase (RT)

(Invitrogen-Life Technologies, Carlsbad, USA) and DENV-specific primers as described elsewhere [8]. The resulting cDNA was then used as a template for PCR amplification using *Pfu* Turbo Polymerase (Stratagene-Agilent Technologies, La Jolla, USA). PCR products were purified from 0.8% agarose gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and used in cycle sequencing reaction using six overlapping primers from both strands and BigDye Dideoxy Terminator Sequencing Kits v3.1 (Applied Biosystems-Life Technologies, Carlsbad, USA), following the manufacturer's instructions. Purified DNA was subjected to capillary sequencing performed on 3130xl Genetic Analyzer (Applied Biosystems-Life Technologies, Carlsbad, USA) at the Eijkman Institute sequencing facility. Primers used in genotyping have been described elsewhere [8]. Sequence reads were assembled using SeqScape v.2.5 software (Applied Biosystems-Life Technologies, Carlsbad, USA) with additional manual adjustment performed when manual inspection of the assembly showed some discrepancies. The E protein gene sequences obtained in this study were deposited in GenBank; accession numbers are shown in Table 1.

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 the sample's serotypes to create datasets for each genotype grouping based on the classifications of Goncalvez *et al*. [9], Twiddy *et al*. [10], and Lanciotti *et al*. [11] for DENV-1, DENV-2, and DENV-4, respectively. Multiple sequence alignment was performed using MUSCLE [12] to generate sequence alignment representing the E protein segment (1,485 nt). Datasets for each serotype were prepared using BEAUti v.1.7.5 [13] followed by phylogenetic reconstruction and evolutionary rate analysis using the Bayesian Markov chain Monte Carlo (MCMC) method as implemented in BEAST v 1.7.5 [14] 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 1,000th iteration. 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.

Results

Patients' characteristics

A total of 113 dengue-suspected patients were involved in this study. The patients were recruited based on dengue clinical and serological/antigen (IgG/IgM and NS1) diagnoses. Dengue cases were observed in all months during the course of the study. From March 2012, the number of cases increased, with the peaks observed in June and September (Figure 1A). Of the 113 patients recruited, 61 (54%) were female and 52 (46%) were male. The cases occurred in patients with ages ranging from 12 to 77 years (mean ± SD = 38.5±18.6 years old). Most cases (21.4%) were observed in patients between 10 and 20 years of age (Figure 1B). All samples were confirmed for dengue infection by dengue NS1 antigen detection and/or dengue IgG and IgM rapid tests, in which 103 (91.2%) samples were positive for IgM. Molecular detection using Simplexa dengue real-time RT-PCR assay detected the presence of DENV nucleic acids in 25 (22.1%) samples. Virus culture of the RT-PCR-positive samples successfully isolated 22 viruses (Table 1), which were then used in the DENV genotyping study described below.

Figure 1. Characteristics of dengue cases in Sukabumi in the year of 2012. Dengue cases distribution by month (A) and patient's age (B) were grouped according to their hospital admission date and ages, respectively. Distribution of DENV serotype (C) was determined by Simplexa dengue real-time RT-PCR as described in the Methodology section.

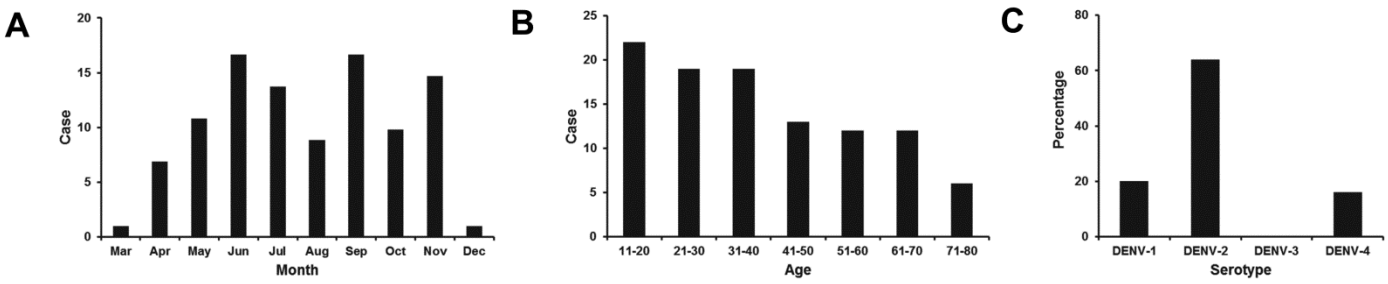


Table 1. Characteristics of dengue patients with DENV envelope gene sequenced

No.	Sample ID	Gender	Age	Serotype	Genotype	GenBank Accession No.
1	SKB-CM002	F	35	DENV-1	Genotype I	KF052647
2	SKB-CM013	F	70	DENV-1	Genotype IV	KF052648
3	SKB-CM046	M	54	DENV-1	Genotype IV	KF052649
4	SKB-CM022	F	74	DENV-2	Cosmopolitan	KF052650
5	SKB-CM024	F	67	DENV-2	Cosmopolitan	KF052651
6	SKB-CM036	M	28	DENV-2	Cosmopolitan	KF052652
7	SKB-CM052	F	12	DENV-2	Cosmopolitan	KF052653
8	SKB-CM057	F	24	DENV-2	Cosmopolitan	KF052654
9	SKB-CM062	M	51	DENV-2	Cosmopolitan	KF052655
10	SKB-CM072	M	41	DENV-2	Cosmopolitan	KF052656
11	SKB-CM075	F	38	DENV-2	Cosmopolitan	KF052657
12	SKB-CM081	M	12	DENV-2	Cosmopolitan	KF052658
13	SKB-CM103	M	18	DENV-2	Cosmopolitan	KF052659
14	SKB-CM105	M	48	DENV-2	Cosmopolitan	KF052660
15	SKB-CM109	M	16	DENV-2	Cosmopolitan	KF052661
16	SKB-CM110	F	-	DENV-2	Cosmopolitan	KF052662
17	SKB-CM112	M	17	DENV-2	Cosmopolitan	KF052663
18	SKB-CM113	M	-	DENV-2	Cosmopolitan	KF052664
19	SKB-CM116	F	50	DENV-2	Cosmopolitan	KF052665
20	SKB-CM028	F	77	DENV-4	Genotype II	KF052666
21	SKB-CM048	F	44	DENV-4	Genotype II	KF052667
22	SKB-CM084	F	29	DENV-4	Genotype II	KF052668

Table 2. Bayesian Markov chain Monte Carlo (MCMC) estimates of substitution rate in DENV isolated in Sukabumi in 2012

Serotype	Genotype	N	Mean substitution rate (X 10^{-4} subs/site/year)	95% CIs (X 10^{-4} subs/site/year)	Published CIs[d] (X 10^{-4} subs/site/year)
DENV-1	I[a]	1	6.89	NA	3.08 – 7.16
DENV-1	IV[a]	2	6.77	5.69 – 7.85	
DENV-2	Cosmopolitan[b]	16	8.90	7.99 – 9.81	4.00 – 8.45
DENV-4	II[c]	3	10.19	6.19 – 14.19	6.32 – 11.79

[a]Goncalvez *et al*. classification [8]; [b]Twiddy *et al*. classification [9]; [c]Lanciotti, *et al*. classification [10]; [d]Maximum likelihood (ML); estimates provided by Twiddy *et al*. [14]; CIs, confidence intervals; NA, not applicable

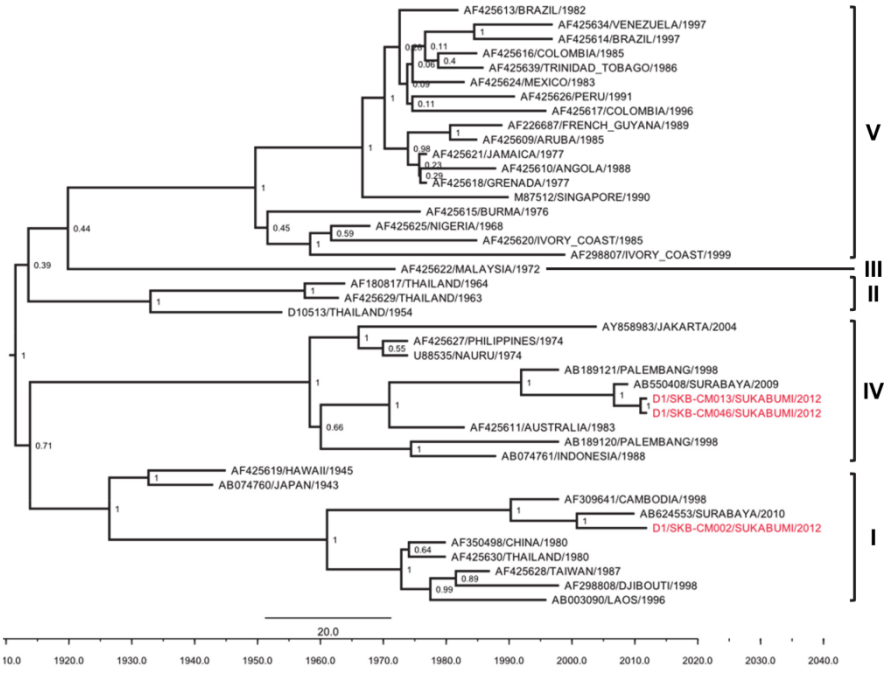


Figure 2. Maximum clade credibility (MCC) tree of DENV-1 genotypes grouping generated by Bayesian inference method as implemented in BEAST using GTR evolution model and gamma parameter rates from the E gene sequences. The Sukabumi isolates (red font) were grouped into genotype I (SKB-CM002) and genotype IV (SKB-CM013 and SKB-CM046) based on classification by Goncalvez *et al*. [9]. The number in the node indicated the posterior probability of that particular cluster.

Figure 3. 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 Sukabumi isolates (red font) were grouped into the Cosmopolitan genotype based on classification by Twiddy *et al*. [10]. The number in the node indicated the posterior probability of that particular cluster.

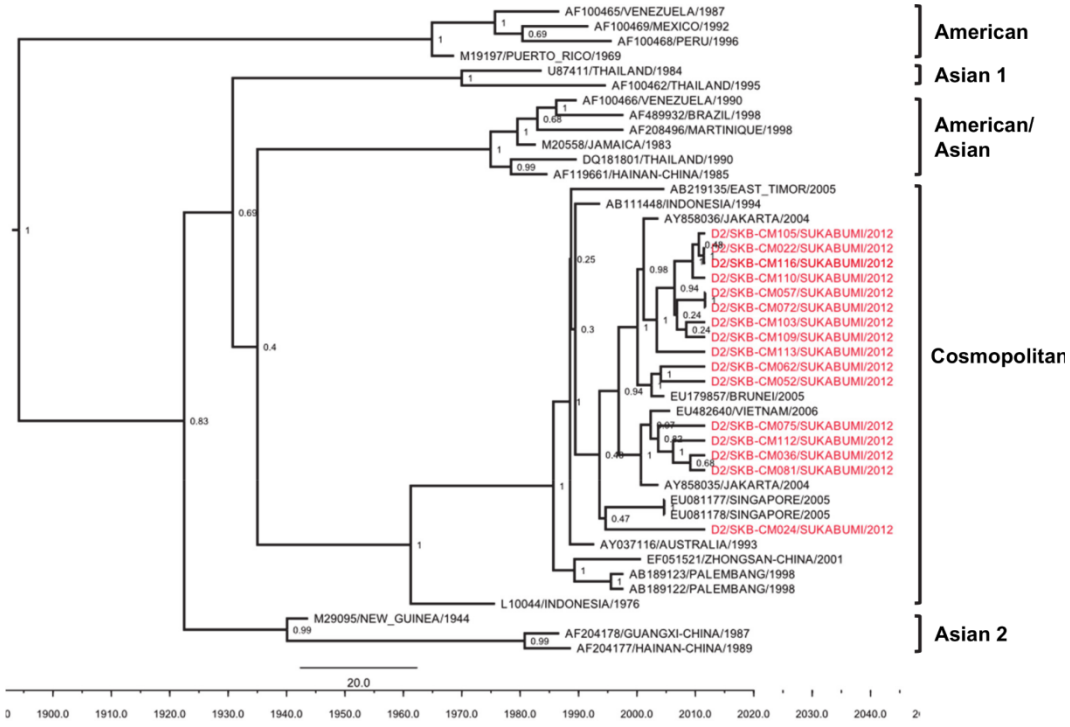
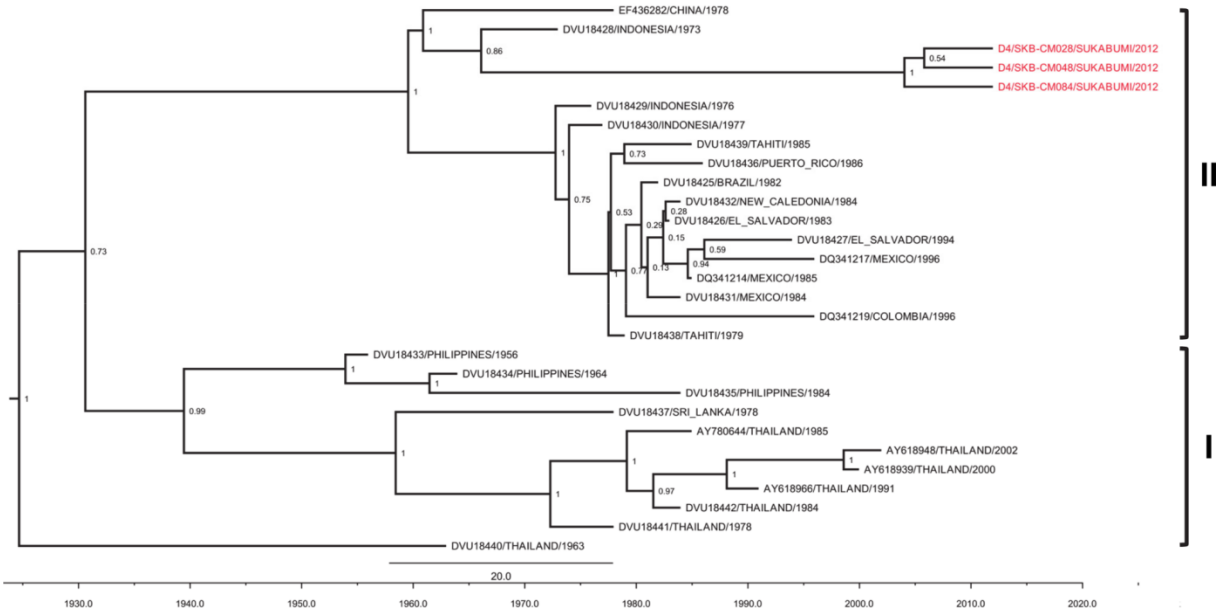


Figure 4. MCC tree of DENV-4 genotypes grouping generated by Bayesian inference method as implemented in BEAST using GTR evolution model and gamma parameter rates from the E gene sequences. The Sukabumi isolates (red font) were grouped into genotype II based on classification by Lanciotti *et al*. [11]. The number in the node indicated the posterior probability of that particular cluster.



DENV serotype and genotype distribution

To determine the circulating DENV serotype in Sukabumi during the course of the study, serotyping was performed using Simplexa dengue real-time RT-PCR assay. Using this method, 25 samples were successfully serotyped. The predominant serotype circulating in Sukabumi was DENV-2 (64%), followed by DENV-1 (20%), and DENV-4 (16%). In this study, the DENV-3 serotype was not detected (Figure 1C and Table 1).

The genotypes of the infecting viruses were then determined by sequencing of envelope genes of the DENV followed by phylogenetic analysis. For DENV-1 serotype, co-circulation of genotype I and IV according to genotype grouping by Goncalvez *et al*. [9] was observed (Figure 2 and Table 1). The genotype of 16 DENV-2 isolates were successfully determined; all of them were grouped into the Cosmopolitan genotype described by Twiddy *et al*. [10] (Figure 3). Although all of the viruses were grouped into one genotype, the presence of several clades was observed, which may indicate their genetic variability. Meanwhile, three DENV-4 isolates obtained in this study were grouped into genotype II according to classification described by Lanciotti *et al*. [11] (Figure 4).

Given the co-circulation of two genotypes of DENV-1 and the presence of clades within the DENV-2 Cosmopolitan genotype, the evolutionary rate of DENV circulating in Sukabumi in 2012 was investigated. Table 2 shows the estimated rate of nucleotide substitution in each serotype of DENV isolated in this study. As shown in Table 2, the confidence intervals (CIs) for the DENV-1, DENV-2, and DENV-4 data sets overlapped considerably, indicating similar rates among these serotypes. The CIs were also relatively similar to maximum likelihood (ML) estimates generated by Twiddy *et al*. [15].

Discussion

Dengue is an endemic disease and a major health problem in Indonesia [16]. Sukabumi, a small city in the West Java province, is also afflicted by the disease annually. Sukabumi Health Authority reported 962 dengue cases in 2012. In this study, we investigated 113 cases for their serological and virological characteristics. During the surveillance period, dengue cases occurred throughout the months of March through December 2012. The peak numbers were observed in June and September (Figure 1A). June is post-monsoon season in Indonesia, while September is categorized as the dry season. The high number of cases recorded in September may not be directly related to the seasonal breeding of the mosquito vectors, but instead is likely to be associated with the abundance of artificial water storages that facilitate mosquito breeding during dry season, as recently reported in Yogyakarta, Indonesia and the Philippines [17].

All samples were positive for dengue based on at least one of the dengue detection methods – by NS1 antigen and/or IgM and IgG serology. Most cases (103, 91.2%) were positive for dengue IgM. Dengue diagnosis based on IgM detection is useful for surveillance studies [18]. The high detection rate based on IgM antibodies is reasonable, since all patients were in the acute phase and mostly admitted to hospitals at a late stage of illness. The IgM antibodies are detectable by days three through five after the onset of illness [16]. The high proportion of serologically-confirmed dengue patient suggests that dengue still puts a considerable burden on the community.

In term of patients' ages, we observed that the high number of dengue patients were in the age range of 10 to 20 years (Figure 1B). This data is understandable, as our patients were mostly recruited from adult wards. However, if this age distribution really reflects the actual cases in the community, this finding is different from that of dengue cases described in other cities in Indonesia, *i.e.*, in Jayapura in 1993 [19] and in Palembang in 1998 [20], in which the largest proportions of dengue cases were found in the under-15 years of age group. Interestingly, a similar age distribution was observed during an outbreak in Jakarta in 2004 [21]. The changing proportion of dengue cases in young adults and adolescents has also been described in other places in Indonesia [22].

In this study, we successfully determined the serotypes of 25 DENV isolates (Figure 1C). The most predominant serotype was DENV-2 (64%), followed by DENV-1 (20%), and DENV-4 (16%). The predominance of DENV-2 has been described previously in other cities in Indonesia, namely Yogyakarta in 1995 [23] and Bandung in 2002 [24]. This DENV-2 predominance is different from the data from a more recent survey in Jakarta in 2004, in which DENV-3 was found to be the predominant serotype [21]. The interesting finding was the absence of DENV-3 in this study. Indonesia is hyperendemic for dengue, with all serotypes of DENV circulating. Historical data on the serotype distribution in Indonesia also recorded the circulation of DENV-3 in

most regions surveyed [22]. Our previous survey in several cities in West Java, including Sukabumi, in 2008 revealed the circulation of DENV-3 [25]. Furthermore, our recent surveys in other cities in Indonesia such as Semarang in 2012 [26] and Surabaya in 2012 (Wardhani *et al*., in preparation) also detected the presence of DENV-3. Therefore, given the absence of DENV-3 in Sukabumi, our data may reflect the dynamic of DENV serotype circulation in Indonesia, where serotype predominance is changing over the time. As the number of samples involved in this study was about 12% of hospitalized dengue cases in Sukabumi in 2012, we suggest that this serotype distribution was quite representative of the actual condition in the city. Further surveillance activities will be useful in determining the dynamics of dengue disease in Sukabumi.

In terms of the genetic aspects of DENV in Sukabumi, we successfully genotyped 22 DENV isolates (Table 1). Phylogenetic analyses using DENV envelope gene sequences were employed to determine the genotypes of the virus and their closeness with other strains previously isolated in Indonesia and its surrounding countries. For DENV-1, we genotyped three isolates, which were grouped into two genotypes, namely genotype I and IV, according to Goncalvez *et al*.'s classification [9] (Figure 2). This co-circulation of two DENV-1 genotypes was similar to the co-circulation observed in other cities in Indonesia, namely Surabaya [27]. The Sukabumi isolates were closely related to viruses isolated in Surabaya in 2009 and 2010 (Figure 2), which suggests that the Sukabumi viruses were endemic to Indonesia. Genotype I has been described as replacing the old genotype IV in Indonesia [27], but it may be that these two genotypes are actually able to be present together and maintain their stable circulation rates in Indonesia. Continuous surveillance will be useful in monitoring this co-circulation. This finding also suggests the diverse genetic distribution of DENV in Indonesia.

A total of 16 DENV-2 isolates were genotyped in this study. This predominant serotype was grouped together as the Cosmopolitan genotype, according to classification by Twiddy *et al*. [10]. Although all isolates were grouped into a single genotype, clustering was also observed (Figure 3). This suggests that the DENV-2 virus from Sukabumi possess diverse genetic characteristics. The Sukabumi isolates were closely related to the virus isolated in Jakarta in 2004 that was associated with a fatal outbreak [8]. This close relationship suggests that the isolates have been quite long established in the area within the last decade and that the predominant serotype that was responsible for high dengue prevalence in Sukabumi was not imported from other countries in the region. Other isolates that were clustered together with DENV-2 from Sukabumi were viruses from Singapore and Brunei isolated in 2005. As reflected by its name, the Cosmopolitan genotype is widely distributed in areas such as India, South East Asia, Africa, the Middle East, and Australia [10].

On the genotype distribution of DENV-4, we managed to sequence the E gene of three DENV-4 isolates and analyzed their genotype. All the isolates were grouped into genotype II (SE Asia and America), according to classification described by Lanciotti *et al*. [11] (Figure 4). All the viruses were closely related to a virus sampled in Indonesia in 1973. This genotype was also similar to a virus isolated in Jakarta in 2004 [8]. Overall, this suggests that the viruses were endemic to Indonesia and have been circulating for more than three decades.

The genetic diversity of DENV in this study was represented by the presence of multiple genotypes of DENV-1 and the grouping of the DENV-2 Cosmopolitan genotype into several clades. Phenotypic implications of this diversity may appear as viruses with different virulence, altered antigenicity, or tissue tropism [4]. Estimation of nucleotide substitution rates among viruses has been used to gain information about the evolutionary rate, which may be useful in understanding the epidemiological aspects of DENV [15]. In general, the DENV isolates in this study possessed nucleotide substitution rates similar to previous reports (Table 2), indicating the common evolutionary rate and that they had not undergone significant genetic changes over time. The information about the DENV evolutionary rate is a useful tool in monitoring whether particular strain(s) of DENV have undergone peculiar evolution, as in the case of our recent finding of the DENV-1 strain, which has undergone a very slow evolutionary rate circulating in Semarang, Indonesia [26].

In conclusion, this study has provided the first molecular data of DENV in Sukabumi, West Java, Indonesia. Although different serotype predominance from other cities in Indonesia was observed, the genotypes of the infecting virus were apparently similar to ones observed in other cities in Indonesia, suggesting the endemic nature of the infecting virus that caused a high prevalence of dengue in Sukabumi in 2012. Continuous surveillance will be useful to

further understand the dynamic of dengue disease in Indonesia.

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Corresponding author

R. Tedjo Sasmono
Eijkman Institute for Molecular Biology
Jl. Diponegoro 69, Jakarta 10430, Indonesia
Phone: +62-21-3917131
Fax: +62-21-3147982
Email: sasmono@eijkman.go.id

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