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Full length Research Paper

Serotype infectivity and phylogenetic of dengue virus cause of dengue fever (DF), dengue hemorrhagic fever (DHF), and engue shock syndrome (DSS) in surabaya-Indonesia

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Infection with DENV causes a spectrum of clinical disease ranging. The aim of this study is to investigate the infectivity of DENV with degree of severity dengue infection in Surabaya. Dengue infection was established by IgM anti dengue, and two step multiplex RT PCR and Nucleotide sequence. Grading of degree severity infection follow the WHO criteria 2011. DSS cases found 3 from 36 patients caused by DENV 2. The most uninfective was DENV 1, and the most prevalence dengue infection caused by DENV 3. The infectivity of dengue infection shown 16 patients lead to severity with plasma leakage. All of sera patients detecting using multiplex RT-PCR were positive, but it were analyzed using Duo ELISA only 22 serum sera positive IgM and IgG from 36 sera.. The Phylogenetic analysis indicates that the isolates from 2011 to 2012 close related with dengue isolate from 1998 and belong to 2009 to 2020.In this study it indicates that DENV 2 predominantly is the cause of DSS.

Key words: Dengue virus**,** infectivity**,** envelop gene, phylogenetic analysis.

INTRODUCTION

The four serotypes of dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4) are closed related, but each serotype is sufficiently different that is, infection with one does not provide complete cross protection for the other three. Infection with DENV causes a spectrum of clinical disease ranging from the mild dengue fever (DF), or the more severe and potentially fatal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) which has a fatality rate as high as 10–15% depending on the availability of healthcare (Gubler, et al., 2002; Ong et al., 2008).

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The other hand, that the risk of DHF is increased when dengue is hyperendemic with the simultaneous circulation of multiple DENV serotypes within a population. Furthermore, the incidence and geographic distribution of dengue is growing alarmingly (Green and Rothman, 2006). Surabaya is a city with high density of population, and was first founding dengue virus serotype 2 (DENV-2) infected by humans at 1968 (Sumarmo et al., 1985) with DHF symptom, and becoming hyper endemic dengue area until now (Hariadi, 2005). Although was found DENV-1 from serum patients hospitalized at Dr. Soetomo Teaching Hospital at 2009 - 2010 (Yamanaka et al., 2011).. But after that, there was often outbreak of dengue virus with predominant DENV- 2 and has mostly caused DSS. Here, we report that dengue infection like

sport outbreak in Surabaya between April 2011 to April 2012 were identified using multiplex PCR shown that dengue cases were caused by all serotype.

MATERIALS AND METHODS

Collecting Samples

A total of 36 selected patients having febrile, grading severity of dengue infection followed by WHO criteria 2011.DHF grade I to grade IV (DSS), were enrolled in the study conducted from April 2011 to April 2012. Blood samples were collected from patients, aseptically processed for the separation of serum and immediately transferred to –70 °C deep freezer till processed further for serotyping using multiplex RT-PCR comply by Lanciotti et al., (1992). Detection of IgG, IgM using ELISA, Hematocrite and platelet count. Sera of positive samples were used for sequencing and finally for phylogenetic analysis.

Dengue Duo ELISA

IgM/IgG-ELISA was purchased from PanBio (product code DEC-400), and performed according to the manufacturer's instructions. Two microtiter plates were supplied, one containing stabilized dengue-1 through dengue-4 (antigen plate) and the other containing either anti-human IgM or anti-human IgG bound to separate wells (assay plate). Peroxidase-labeled anti-dengue virus monoclonal antibody (125µl/well) was added to the antigen plate to solubilize the antigens and form antibody-antigen complexes. Concurrently, 100µl of patient serum, diluted 1:100 in the diluent provided, was added to each well of the assay plate containing either bound anti-human IgM or bound anti-human IgG, and human IgM/IgG in the patient's serum which was collected. The plates were incubated for 1 h at room temperature (antigen plate) then the assay plate was washed, and 100µl of antibody-antigen complexes per well was transferred from the antigen plate to the assay plate. These complexes were captured by dengue virus-specific IgM/IgG during incubation for 1 h at 37°C. The plate was washed, and bound complexes were visualized through the addition of 100µl of tetramethylbenzidine substrate per well. After 10 min, the reaction was stopped by the addition of 100µl of 1 M phosphoric acid per well, and finally were read at 450 nm by ELISA reader. Positivity was determined by comparison to the IgM and IgG reference sera provided (cutoff calibrators). A positive sample was defined as having a sample/calibrator absorbance ratio of ≥1.0, and a negative sample was defined as having a ratio of <1.0. Dengue virus infection was characterized by the elevation of either IgM/IgG, with a negative sample being defined as having both IgM and IgG ratios of <1.0. The cut off value in the IgG ELISA represented a level used to distinguish secondary dengue virus infections from primary or past dengue virus infections, according to WHO classification by the HAI assay (WHO, 2000).

Hematocrite and platelet count

We used this step for comparing between serotype and the pathophysiology of dengue patients. Whole blood samples were analyzed with the total hematocrite and platelet by using Cell Dyne Ruby (BD).

Reverse Transcriptase-Polymerase chain reaction (RT-PCR)

RNA was extracted from the patient's serum using Trizol (Invitrogen) viral RNA purification kit according to the manufacturer's instructions (Qiagen). Synthesis of cDNA using reverse transcriptase and reverse primers. The sequence of specific primers were used for amplification of gen envelop (E) as below;

Reverse transcriptase-PCR was performed in a 12µl mixture composed of 2µl of RNA, 1µl of reverse primer, 2µl of 10mM dNTP mix, 7µl of DEPC treated water and incubate in 65^{o}C in 5 min, directly put on ice add 8µl of mix components of 4µl of 5x cDNA synthesis buffer, 1µl of 0, 1 M DTT, 0,5µl of RNAse out, 2µl of DEPC-treated water 0,5µl of thermo script RT (Invitrogen). Incubate 50^{o}C in 45 min, continued incubated in 85 ^{o}C in 5 min. 1 of µl of RNAse adding to the mixture components and continued incubated in 37^{o}C in 20 min. The PCR- amplification reaction was performed in a 21,2µl mixture composed of 15µl DEPC treated water, 2µl 10 × PCR reaction buffer, 0,6µl of 50$MgCl_2$, 0,4µl of dNTP, 0,5µl of primer forward, and 0,5µl of primer reverse for each D1,D2,D3, and D4 primer, *Taq* Polymerase (Invitrogen) 0,2µl, and 2µl synthetic cDNA. The PCR program was: 94°C for 4 min followed by 40 cycles of 94°C for 1 min the annealing temperature of each DENV are different for D1 61°C, for D2 59°C, for D3 and D4 55°C, and then 72°C for 2 min, and a final elongation step of 72°C for 10 min. Fragments of 400bp were obtained from the second amplification.

Nucleotide sequencing and phylogenetic analysis

Analysis of nucleotide sequencing whole genome just for DENV-2 were done like procedure and primers complied to Ong et al (2008). Product PCR were done at by Sasmono, Ph.D., Institute Molecular Biology, Eijkman, Jakarta Indonesia. Phylogenetic analysis of whole genome were done by Dr. David Lee, Environment National Institute, National University of Singapore, using genetic analysis Besar Program.

RESULTS

Infectivity and serotyping of DENV, clinical, hematology and serological identification and serotyping

Code	Sequence
RDen1/Env	5’-GTGCTCCACGGGCAGTTGTC-3’
RDen2/Env	5’-TCGCTCCTCTCATTGTTGTC-3’
RDen3/Env	5’-TTGCACCTCTGGCAGTGGCC-3’
RDen4/Env	5’-TTGCACCTCTGTATGTGGAC -3’

Envelope Forward Primers

Code	Sequence
FDen1/Env	5’-GGGGGCTTCAACATCCCAAG-3’
FDen2/Env	5’-CGGAGCAGACACACAAGGAT-3’
FDen3/Env	5’-AGGGGCTACAACAGAAACAC-3’
FDen4/Env	5’-AGGAGCAGACACATCAGAAG -3’

Envelope Revers Primers

Code	Sequence
RDen1/Env	5’-GTGCTCCACGGGCAGTTGTC-3’
RDen2/Env	5’-TCGCTCCTCTCATTGTTGTC-3’
RDen3/Env	5’-TTGCACCTCTGGCAGTGGCC-3’
RDen4/Env	5’-TTGCACCTCTGTATGTGGAC -3’

Figure 1. Multiplex RT-PCR analysis. Serotyping of dengue virus from sera samples. Lane 1: DENV-2 isolate DSS patient, Lane 2: sample positive of DENV-1, Lane 3: sample positive of DENV-3, and lane 4: sample positive of DEN-4., Lane 5: as positive control of DENV-2 (virus DENV-2 Lab) M: Marker.

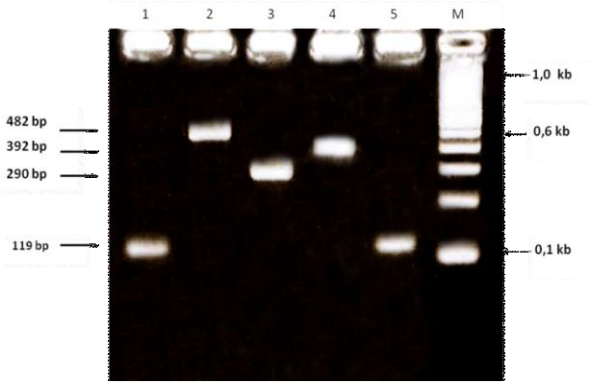


Table 1. Serotypes and grading severity of dengue infection.

DENV Serotype	Grading Severity Dengue fever (DF)	Dengue hemorrhagic fever grad I (DHF I)	Dengue hemorrhagic fever grad II (DHF II)	Dengue hemorrhagic fever grad II (DHF III)	Dengue shock syndrome (DSS)	Total cases
DENV-1	0	1	1	0	0	2
DENV-2	1	1	5	0	3	10
DENV-3	1	2	8	0	0	11
DENV-4	0	0	6	0	0	6
DENV-mix infection	0	1	6	0	0	7
Total	2	5	26	0	3	**36**

Table 2. Grading severity analysis base on total count of hematocrit and platelet.

Blood Analysis	Grading Severity					DENV Serotype
	Dengue fever (DF)	DHF grad I (DHF I)	DHF grad II (DHF II)	DHF grad II (DHF III)	Dengue shock syndrome (DSS)	
Hematocrit*	-	1 D-mix 2,3	6 D-4;2;3;1	-	2 D-2	D-1(1), D-2 (2), D-3 (3), (D-4(2), mix-2,3 (1)
Platelet**	-	4 D-mix 2,3;D-3,2,1	11 D-mix 2,3;D-3,4,2	-	2 D-2	D-1 (2), D-2 (4), D-3 (6), D-4 (4), mix-2,3 (2)
Total	-	5	17	-	4	26

*)Positive when increasing HCT≥20%, **) positive when platelet ≤100.000, HCT: hematocrite, PLT: platelet.

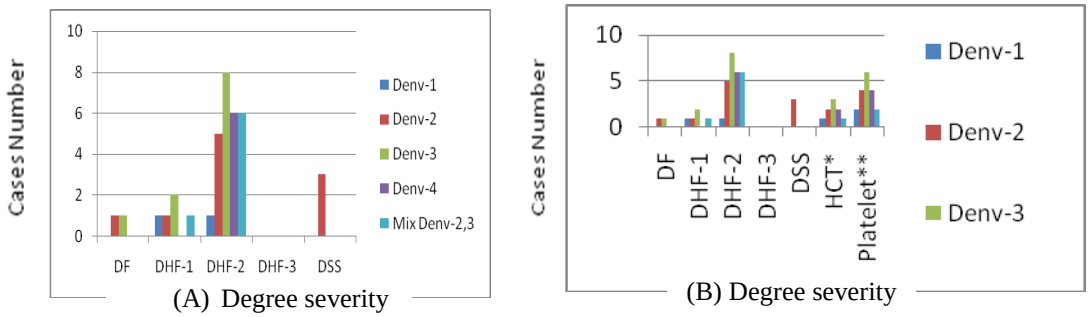


Figure 2. A, B. Severity and serotype of dengue virus caused DF, DHF-1, DHF-2, DHF-3, and DSS**. (A)** , *)Positive when increasing HCT≥20%, **) positive when platelet ≤100.000. **(B).**

of dengue serotype shows that about 9 patients have got positive HCT and 16 patients with positive PLT. All cases have the increase of HCT more than 20% and platelet count below of 100.000, interestingly NS1 assay was all sera negative but all patients had symptoms of positive dengue virus infection, due to the long incubation period and NS1 became more sensitive in early infection (data not shown). Thirty six sera samples from suspected dengue patients were identified by using multiplex RT-PCR complay to Lanciotti, et al., 1992 were all positive. Data showed that serotypes of dengue virus from the outbreak area during April 2012 in Surabaya like in Table 1. The role of Dengue cases this period were caused by dengue infection of different serotype, most were found which are DENV-2 and DENV-3 and then DENV-4 and DENV-1, and the clinical manifestation was leading to DSS especially infected by DENV-2. The highest prevalence was caused by DENV-3 and the most rare case was DENV-1. The result of serotyping while using multiplex RT-PCR analysis is like Figure. 1

Figure 1. Multiplex RT-PCR analysis

Serotyping of dengue virus from sera samples. Lane 1: DENV-2 isolate DSS patient, Lane 2: sample positive of DENV-1, Lane 3: sample positive of DENV-3 and lane 4: sample positive of DEN-4., Lane 5: as positive control of DENV-2 (virus DENV-2 Lab) M: MarkerIn, this study four serotypes of dengue virus which was found from all (36) sample with any degree of severity, but mostly that of DENV-3. Grading severity of dengue infection could see in Table 1.

Severity and serotype of dengue virus

Many cases of dengue infection lead to dengue fever, dengue hemorrhagic fever has different grades including dengue shock syndrome, and the data can be shown in Figure. 2 A, B. We have analyzed severity manifestation of dengue infection, and the data shows that serotypes of DENV-2 and DENV-3 have predominant infection than DENV-1 and DENV-4 during outbreak in Surabaya, all serotypes have been manifested in DHF II.

Stability of genetic analysis

DENV-2 has predominance as the cause has expanded dengue syndrome and DSS and which usually ends in death. This way we have continued nucleotide sequence analysis and then it was compared to nucleotide sequence from Gene Bank to perform phylogenetic like

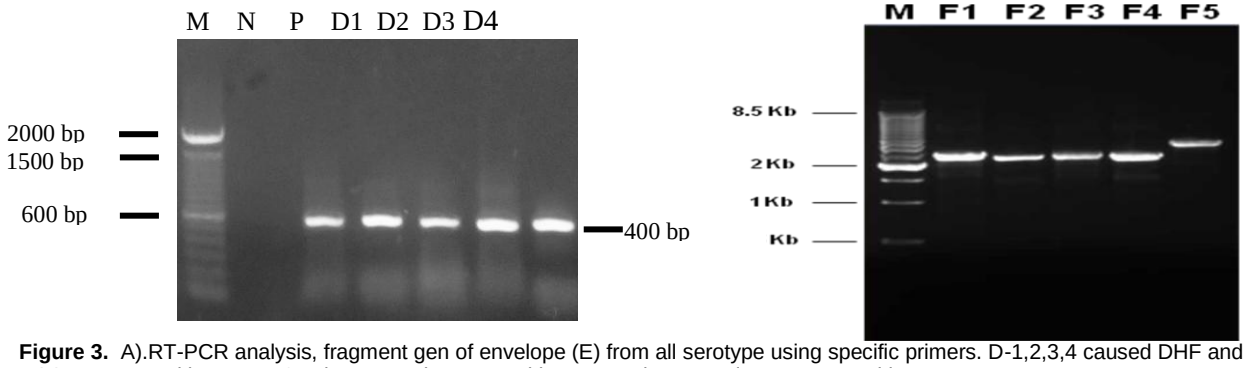


Figure 3. A).RT-PCR analysis, fragment gen of envelope (E) from all serotype using specific primers. D-1,2,3,4 caused DHF and DSS was caused by DENV-2 only. M: Marker DNA 1 kb, N: negative control serum, P: positive.

Table 3. Nucleotide sequence analysis and compare to Dengue virus Indonesia isolate in GenBank, and nucleotide changing

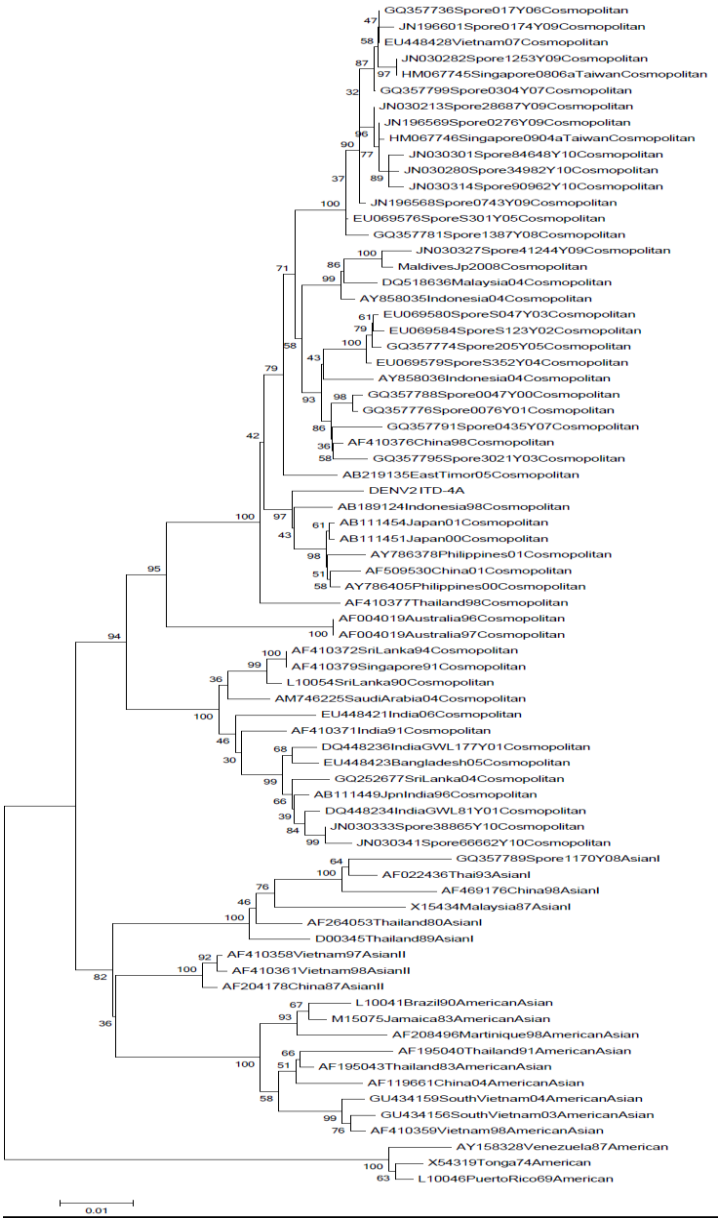
DENV-2 Serotype Indonesia isolate accessed from GenBank	**DENV-2 ITD- Isolate 2011** Nucleotide Changing of gen E	Nucleotide position
Denv-2 nucleotide sequence consensus were constructed (isolate from 1975 to 2009): DENV 2 strain 98900666 DSS 2009, DENV 2 strain 98900663 DHF 2009, DENV 2 strain 98900665 DF 2009, DENV 2 strain TB16i2008, DENV 2 strain BAO05i2008, DENV2/ID/1017DN/1976, DENV2/ID/1046DN/1976, DENV2/ID/1127DN/1976, DENV2/ID/1070DN/1976, DENV2/ID/1172DN/1976, DENV2/ID/1016DN/1975, DENV2/ID/1022DN/1975, DENV2/ID/1183DN/1977	C T → G A → A T → A T → C T → A G → C T → A G → G A → C T → G A →	1003 1042 1081 1093 1159 1171 1176 1185 1193 1198 1322

Ong, et al. (2008) procedure, and the results as seen in **Figure 4**. But we have first amplified the fragment of E gen before, and the result in **Figure.3** control serum, D1: DENV-1, D2: DENV-2, D3: DENV-3 and D4: DENV-4. Visualization of fragment gen E using agarose gel 0, 8% in buffer TBE. The purification of cDNA where sequences were compared to the whole genome of dengue Indonesia isolated access from Gene Bank. **B)**. PCR product amplifies used by Ong et al., 2008 primers for sequence and phylogenetic analyzed Nucleotides code E protein which was found changing in several region. Mostly changes from cytosine be Thymine (C□T) and the rare was from Adenine to Guanine (A□G), but the changes could explain the correlation with grading severity, because E protein has a role in mechanism of pathogenecity and stability properties. The complete changes as seen in **Table. 3**. In **Figure 4.** DNA Analysis of DENV2-ITD-4 a whole genome was one of DENV-2 isolated at Institute of Tropical Disease, Airlangga University, during outbreak DENV in Surabaya at 2012, and then compared to dengue serotype which was accessed from Gene Bank from different countries. We analyzed especially from the whole genome, one of three isolate DENV-2 that causes DSS and leads the patient to death. This isolate have stable biological properties and inoculated into vero cell (given from Dr. Yamanaka, CRC-ERID Kobe University) shows virulence (data not shown). Nucleotide sequence analysis shows that this DENV-2 inside the genotype cosmopolitan and has no findings of sequence of nucleotide shifting if compared to the other isolate across the world (data not shown).

DISCUSSION

The findings of these studies indicates that there are many kinds of manifestation on the new spot outbreak of dengue infection in Surabaya, and contrary symptoms were found in these cases if compared to the result of study at 2009-2010 about shifting of DENV-2 to DENV-1 (Yamanaka, et al., 2011). In these studies we have found DENV-2 has predominant infection although following DENV-3, DENV-1 and DENV-4. We found some kind of manifestation by patients of dengue like Dengue fever

Figure 4. DNA Analysis of DENV2-ITD-4A whole genome was one of DENV-2 isolate at Institute of Tropical Disease, Airlangga University, during outbreak DENV in Surabaya at 2012, and then compared to dengue serotype was accessed from GenBank with different countries.



(DF), Dengue hemorrhagic fever (DHF), Dengue shock syndrome (DSS), although these epidemics were not occurring simultaneously. The cause of dengue infection in this period outbreak were DENV-1, DENV-2, DENV 3, DENV-4 and the most important things was found in mix infection about 20% between DENV-2 and DENV-3. All of dengue mix infection occurs in children under 5 years old. This phenomena although like cases by children at 1999 in Surabaya at Dr. Soetomo Teaching Hospital, Airlangga University Surabaya using multiplex RT-PCR. (Rantam, et al 2005) data was not published. Evidence of dengue infection based on the serotypes are 37% of DENV-2 , 37% of DENV-3, 20% of DENV-1, and 17% of DENV-4, but mix infection was 1% DENV-2 and DENV-3 about 1%. These phenomena may have a correlation between immune system development and genetic sensitivity. Beside this argument is the possibility of geographic or endemic area and nutrition factors and then antibody dependent enhancement (ADE) (Morens, 1991).In this study DENV-1 had been found causing only DHF grade 1 and 2, DENV-2 causing DHF grade 2 and DSS, DENV-4 causing DHF grade 2. Mix infection (DENV-2&3) causing DHF grade 1 and grade 2. DSS caused only by DENV-2 because DENV-2 has high virulence and could cause 60 times replication than others and could interfer with cells causing modulated replication of dengue virus in the cell (Goncalves et al., 2007, Mukherjee and Hanley, 2010).All serotypes can induce loss of platelet and could increase haematocrite that were to lead to DHF grade 2 mostly but DENV -2 leads to DSS. The data as in **Table**. **2** and **Figure. 2**. The results of serological studies confirm clinical observations based on IgG and IgM that dengue fever and dengue hemorrhagic fever are found mostly in endemic area. Moreover, it could also be true that many self-limiting fevers in the resettlement area have not been diagnosed with dengue infections. Also we have designed specific primers of fragment gen E for detection of all DENV with the product PCR about 400 bp. It is very important to use molecular surveillance of dengue because gen E as the role of pathogenesis especially the first attachment to the cell surface membrane, which can be included to analyze genotype variants. The changes in nucleotides DENV-2 ITD isolate compared with consensus sequence from DENV-2 from Gene Bank indicates that cytosine, guanine, thymine have unstable purine base which could be hipervariable and influence infectivity. In addition to the analysis of nucleotide evolution in the whole genome, we have sequenced and we also examined the dynamics of DENV-2 evolution through phylogenetic analysis. The DENV-2 isolate from outbreak area during 2012 is closely related with dengue virus Indonesia isolate from 1998.

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