

# Detection of Multiple Dengue Infections by Rt-qPCR in West Sumatera, Indonesia

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## Detection of Multiple Dengue Infections by Rt-qPCR in West Sumatera, Indonesia

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### Abstract

**BACKGROUND:** Dengue is a disease caused by four distinct serotypes of dengue virus (DENV 1-4). DENV serotype differs from one another by 25–40% at the amino acid level. The detection of serotype is very important due to the fact that in secondary infection with heterologous serotype often leads to life threatening, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS); likewise, an infection caused by two serotypes or more in one individual can contribute to the severity of infection.

**AIMS:** The aims of the study were to detect the multiple dengue serotypes infection by quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) and to determine the viral load in dengue infection.

**MATERIALS AND METHODS:** This study applied the molecular examination for determining the serotype and viral load of DENV. The data were analyzed using Student's t-test.

**RESULTS:** A total of 119 samples, 91 samples showed positive dengue infection after amplification. The multiple dengue infection was found in 47 samples and 44 samples with single infection. There was a significant difference between the number of viral load DENV-2 and DENV-1 infection ( $p = 0.000$ ).

**CONCLUSION:** Two or more serotypes of dengue were found to infect a patient in West Sumatra. DENV-2 serotype was found predominantly in West Sumatra ( $n = 36$ , 39.56%) in patients with single infection. The molecular detection of dengue RNA by RT-PCR is a sensitive, rapid, and simple method. The RT-PCR method can detect the multiple dengue infection in clinical samples.

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## Introduction

Dengue is a disease caused by four distinct serotypes of dengue virus (DENV 1-4). Dengue virus is an ss-RNA virus, spherical particles with diameter of 50 nm, belongs to the genus Flavivirus. The RNA genome of the virus is approximately 10.7 kb in length and encodes three structural (C, prM/M, and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. They are antigenically diverse and only share about 60–75% identity at the amino acid level [1], [2], [3], [4].

Dengue virus is a mosquito-transmitted virus that infects millions humans each year [5]. This virus is transmitted to humans through the bites of infected *Aedes* mosquitoes primarily by *Aedes aegypti* and *Aedes albopictus* mosquitoes [6], [7]. Infected human is the main carrier and spreader of DENV, the virus then is transmitted to uninfected mosquitoes for subsequent transmission. Dengue fever is one of the major human infectious diseases in tropical and subtropical areas [8], [9], [10].

Indonesia is one of the largest countries in the region of endemic dengue. Indonesia has experienced

epidemic cycles of dengue since the first introduction in 1968. The frequent dengue cases are often followed by increasing number of infection which affected almost all provinces in Indonesia. Every year occurs the outbreak of dengue in several provinces with case fatality rate around 0.86–0.89%. Concurrent infection of multiple dengue serotypes has been reported in Indonesia, Mexico, and Puerto Rico as well as other countries [10], [11], [12], [13]. In 2010, Indonesia became the first ranked country in the Association of Southeast Asian Nations by the highest number cases of dengue hemorrhagic fever (DHF) and was predicated as a hyperendemic area because multiple serotypes were found in blood circulation of dengue patients [11], [14].

In dengue infection, detection of serotype is very important because the fact that in secondary infection with heterologous serotype often leads to life threatening, DHF and DSS [14]. Infection with one serotype does not confer cross-protection against the other serotypes, instead can cause a severe form of infection. Infection by one serotype provides the recovered patient with a lifelong immunity against specific serotype [15], [16]. Likewise, an infection caused by two serotypes or more in a single individual can contribute to the severity of infection, then

serotyping becomes very important in the management of patients with dengue virus infection [11].

At present, the previous methods that are used to diagnose DENV infection are virus isolation, detection of dengue specific antibodies and antigens, and amplification of viral RNA. To date, the molecular detection of dengue RNA by RT-PCR offers a sensitive, rapid, and simple. Several real-time PCR-based methods for the detection of DENV have been reported, these assays have targeted the 3' UTR, NS5, capsid, and the envelope gene sequences. The increased prevalence of dengue infection in recent decades and high mortality caused by DHF and DSS need for more sensitive and specific diagnostic assays such as RT-PCR for the detection and typing of DENV [4], [8], [16].

During the acute phase of primary infection, RNA can usually be detected using various platforms PCR. Although PCR is more sensitive during secondary infection, the window of viremia is very short. The level of DENV viremia in early infection might be predictive for the development of severe disease later. It has been suggested that a viral titer  $10^6$ – $10^8$  infectious units/mL during the febrile phase is predictive for the development of severe disease [3]. The high dengue viremia titer was associated with the increasing of disease severity. Peak viral titers were 100–1000-fold higher in patients with DSS than those with DHF in dengue infection. Although cases of DHF/DSS have been reported in individual without prior exposure to DENV, most of cases occurred in patients who have been sequentially infected by at least two DENV serotypes [17], [18]. The aims of this study were to detect of multiple dengue serotype infection by qRT-PCR and to determine the viral load in dengue infection patients.

## Materials and Methods

### Location and population of study

A total of 119 samples were used in this study, samples were collected from five clinical laboratories at public hospitals in West Sumatera (Dr. M. Djamil General Hospital, Padang; Dr. Ahmad Mughtar Regional Public Hospital, Bukit Tinggi; Regional Public Hospital, Padang Panjang, Regional Public Hospital, Pariaman, and Regional Public Hospital, Painan). This study was conducted on January 2018 to October 2019. This study used purposive sampling technique to investigate the multiple dengue infection in all subjects. All samples were collected within the first 5 days of illness. Samples were previously screened using NS1 and/or IgM and IgG anti-dengue detection. Molecular analysis was conducted in Biomedical Laboratory, Faculty of Medicine, Andalas University, Padang.

### Preparation of samples

Blood samples were collected from each patient who visited clinical laboratory to conduct serologic dengue test and whole blood examination (hematology analyzer). Blood was taken using an aseptic procedure from the *mediana cubiti* vein by trained personnel, using 3 cc syringe. Serum was separated and stored at  $-80^{\circ}\text{C}$ . All subjects were briefed on the study including the objectives, risks, and benefits of the study and inform consent was conducted through viva voce. The study was approved by Ethics Committee of Medical Faculty, Andalas University, Padang, Indonesia, No: 268/KEP/FK/2019.

### Viral RNA extraction and cDNA synthesis

Serum of patient's with dengue fever was collected from blood and followed by RNA viral extraction. RNA viral was extracted from 140  $\mu\text{L}$  serum samples using QIAamp Viral RNA Mini Kits (Qiagen, Germany) according to the manufacturer's instructions and then stored at  $-80^{\circ}\text{C}$  until further analysis.

The extracted RNA template was denatured at  $65^{\circ}\text{C}$  for 10 min with DNase Amp Grade (Invitrogen, USA) and then conversely transcribed to cDNA in 20  $\mu\text{L}$  reaction mixture comprising 11  $\mu\text{L}$  RNA, 4  $\mu\text{L}$  5 $\times$  trans Amp buffer, 1  $\mu\text{L}$  reverse transcriptase, and 4  $\mu\text{L}$  DNase/RNase free water (Bioline cat no. Bio 65053). The reaction was allowed to proceed at  $25^{\circ}\text{C}$  for 5 min,  $46^{\circ}\text{C}$  for 20 min, and followed by enzyme inactivation at  $95^{\circ}\text{C}$  for 1 min.

### Detection of DENV RNA by qRT-PCR

The RT-PCR was performed using nested qPCR method, the capsid gene was amplified by PCR with first round of PCR used outer primer Dengue\_F (5' to 3'): GAGAAACCGCGTGTCAAC and Dengue R: TCCTGCTTGCTGACTATCATG; furthermore, the second round of PCR used four specific primers DENV-1 (5' to 3'): TTCTTTCTTGAACTCCGTAGC, DENV-2 (5' to 3'): GCGGGATTGTTAGGAAACGA, DENV-3 (5' to 3'): CTTTTCCGTCTGTTGATAATGC, and DENV-4 (5' to 3'): GACCTATCTCCTCCTGAATCCAA. The primers were derived from positive screening of DENV. The DENV was amplified and sequenced to obtain the whole sequences. Then, Basic Local Alignment Search Tool was needed to confirm the result of isolation. The primer was designed by Primer3 (version 0.4.0). The size of the nested PCR product was 205 bp for DENV-1, 125 bp for DENV-2, 244 bp for DENV-3, and 212 bp for DENV-4. DNA amplification was performed with reaction conditions as follows: Denaturation at  $95^{\circ}\text{C}$  for 30 s and five cycles of denaturation at  $95^{\circ}\text{C}$  for 5 s and annealing at  $65^{\circ}\text{C}$  for 5 s, and then 11 cycles touchdown ( $-1^{\circ}\text{C}/\text{cycle}$ ) denaturation at  $95^{\circ}\text{C}$  5 s and annealing at  $65^{\circ}\text{C}$  to  $55^{\circ}\text{C}$  for 5 s. Furthermore, 19

cycles of denaturation at 95°C for 5 s, annealing at 55°C for 5 s, and the melt curved at 65°–95° for 5 s (every 5 s the temperatures raised 0.5°C (Figure 1). For first round of PCR, 1 uL of cDNA added to 5 uL EvaGreen (Biorad, USA), 0.5 uL primer Dengue-F, 0.5 uL primer Dengue-R, and 3 uL of nuclease-free water in 10 uL reaction mixture. External PCR was followed by the second round of nested PCR using the primers Dengue-F and four serotype-specific primers (DEN-1, DENV-2, DENV-3, and DENV-4). The amplified product of the external PCR was diluted in ratio of 1:100. The 10 uL of nested PCR mixture was prepared by adding 1 uL of diluted external PCR product to 5 uL EvaGreen (Biorad, USA), 0.5 uL of primer Dengue-F, 0.5 uL of each primers DENV-1 or DENV-2, or DENV-3, or DENV-4, and 3 uL of nuclease-free water.

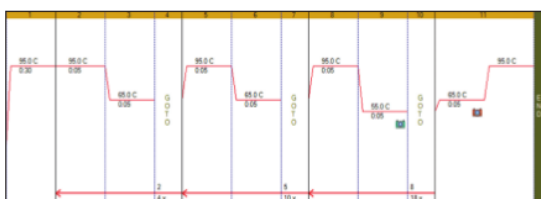


Figure 1. The profile of touchdown q-PCR

### Statistical analysis

Statistical analysis was performed using SPSS 15.0. Pooled data were presented as the average value. Data were compared using Student's t-test,  $p < 0.05$  was considered statistically significant.

## Results

### The serotype of dengue infection in West Sumatra

In this study, 119 samples were obtained from serologic reactive patients, 91 samples (76.47%) showed positive dengue infection after PCR amplification, and 28 samples (23.36%) were negative. Positive results consist of multiple dengue infection 47 samples of 91 positive samples (51.64%) and 44 samples with single serotype dengue infection (48.33%). From 47 samples with multiple serotype dengue infection, 30 samples (32.97%) were infected by DENV-1 and DENV-2, 13 samples (14.28%) were infected by DENV-1, DENV-2, and DENV-4, three samples (3.30%) were infected by DENV-2 and DENV-4, and one sample was (1.09%) infected by DENV-2 and DENV-3. The sample with single serotype of dengue virus infection was five samples with DENV-1 (5.49%), 36 samples with DENV-2 (39.56%), one sample with DENV-3 (1.09%), and two samples with DENV-4 (2.19%) (Table 1).

Table 1: Frequency of dengue serotype

Serotype	Number of samples	Percentage (%)
Multiple DENV:		
DENV-1 and DENV-2	30	32.97
DENV-1, DENV-2, and DENV-4	13	14.28
DENV-2 and DENV-4	3	3.30
DENV-2 and DENV-3	1	1.09
Total	47	51.64
Single DENV:		
DENV-1	5	5.49
DENV-2	36	39.56
DENV-3	1	1.09
DENV-4	2	2.19
Total	44	48.33

### Viral load of dengue infection in West

#### Sumatra

The viral load count showed that DENV-2 ( $8.01 \pm 0.79$ ) log/mL was the highest serotype number than DENV-4 and DENV-1 ( $7.89 \pm 1.01$  and  $7.57 \pm 0.61$ , respectively). Data of viral load for DENV-3 were not analyzed because only two samples were positive result. The number of viral load for DENV-2 and DENV-1 showed significant difference ( $p < 0.05$ ), while DENV-4 was not significantly different. Based on gender, this study showed that most infected patients were male of 54 samples (59.34 %) and female of 37 samples (40.66%) (Table 2).

Table 2: The average of several parameters on dengue patients

Parameter	n (%)	Mean (SD)	Min.	Max.	p-value
Gender					
Male	54 (59.34)				
Female	37 (40.66)				
Hematology parameters					
Platelet (/uL)		$61,000 \pm 30,490$	15,000	132,000	
Hematocrit (%)		$39.62 \pm 5.76$	21	49	
Leukocyte (/uL)		$4364.71 \pm 1483.05$	2400	8400	
Viral load (log mL <sup>-1</sup> )					
DENV-2		$8.01 \pm 0.79$	6.93	11.59	.000
DENV-1		$7.57 \pm 0.61$	7.00	11.00	.138
DENV-4		$7.89 \pm 1.01$	7.14	11.15	

The results showed that hematology parameter such as thrombocyte was to decrease with the average 61,000 per uL  $\pm$  30,490. Eleven samples (12.1%) with the number of thrombocyte were above 100.000/uL but <150.000/uL. The average of leukocyte count also decreased  $4364.71 \pm 1483$ , and hematocrit count was less than normal which found in 12 samples) (13.2%).

The curves of melt peak in Figure 2 showed specific result for DENV1, DENV2, DENV3, and DENV4. All serotypes showed one peak result with uniform melt peak temperature. The optimization temperature of the fourth serotypes for annealing was at 55°C and the melt curved at 65°–95°C.

## Discussion

The dengue infection in West Sumatra was high, 91 samples (76.47%) showed positive result of 119 samples. The serotype of dengue infection showed

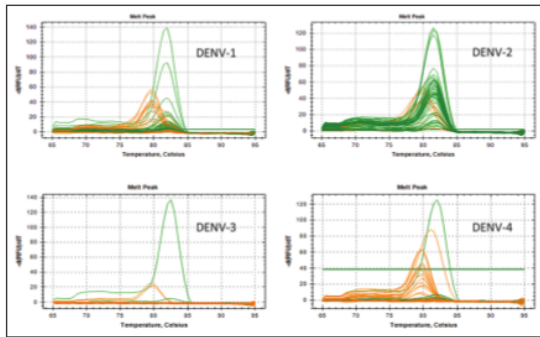


Figure 2: Melting curves of DENV-1 to DENV-4 real-time RT-PCR products from patients who infected by dengue virus

that DENV-2 was found predominantly in West Sumatra ( $n = 36, 39.56\%$ ) in patients with single infection. Whereas, DENV1 and DENV2 dominated in multiple infection. In parameter of gender, it was found that male was slightly more affected by dengue virus than female.

In Indonesia, DENV-2 serotype dominated, followed by DEN-3 in 2003–2005. In 2008–2009, Surabaya was also dominated by DENV-2. Most of isolated DENV in Semarang was DENV-1, while the most predominant serotype in Purwokerto was DENV-3 and other cities such as Jakarta, Palembang, and Bali in 2015, whereas DENV-4 was found in Bandung. These findings described that the differences of DENV serotype were found in different cities. Thus, it demonstrated the spatial and temporal dynamics of DENV distribution in Indonesia [11], [12].

Over the past few decades, the number of people infected with DENV has risen steadily due to the expansion of urban population, global travel and commerce, and paucity of mosquito control program [5].

There are many complex factors that may have contributed to the increase in dengue cases such as the increasing number of people migration, population growth, poverty, health inequality, and climate warming which have increased mosquito density and expanded the geographic distribution and seasonal distribution of *Aedes* mosquitoes [1]. Hot and wet environmental conditions increase the number of vector mosquito. Controlling of main vector is essential to prevent epidemics caused by the virus.

The number of multiple dengue case found has increased. In 2010, Indonesia became the first ranked country in with the highest number cases of DHF and hyperendemic predicate [11]. Dengue hyperendemic regions refer to locations with two or more simultaneously circulating dengue serotypes [19]. In many dengue hyperendemic countries, multiple DENV of all four serotypes are cocirculate [20]. Detection of dengue virus serotypes is very important because secondary infection with a different serotype may impact more severe. Likewise, an infection caused by two serotypes or more in a single individual can contribute

to the severity of the infection. Then, serotyping is very important in the management of patients with dengue virus infection [11].

The dramatic increase in global dengue burden has promoted social interest in improving dengue diagnosis. The current methods for confirming dengue in the laboratory are serology test, viral isolation, and nucleotide detection [21]. Early diagnosis is important for the clinical management of dengue and may prevent unsatisfactory outcomes. Dengue virus and dengue viral products are detected in serum at the early illness period; consequently, the sensitivity of dengue diagnostic methods as real-time PCR, virus isolation, or NS1 detection is higher at the 1<sup>st</sup> day of illness [22].

Infection by one serotype provides the recovered patient with a lifelong immunity against the specific serotype. The previous studies have showed that if a recovered patient is infected with another serotype, the preexisting heterologous antibodies from previous infection complicate with novel serotype of dengue virus, but the pathogenesis not neutralized by the immune system. Instead, the complex of antigen antibody seems to facilitate the virus entry to the cells, which can cause an uncontrolled virus replication, higher peak viral titers, and more severe dengue disease [19].

The infection begins with an infected mosquito injects dengue virus, disseminates and infects multiple lymphoid and non-lymphoid tissues. A viremia is presumably representing the underlying severity of tissue infection. Viremia peaks shortly after fever onset and then becomes flat for 1–2 days before gradually declining [23]. Melt curve analyses is often used to confirm the fidelity of the reaction. Viremia was found to be higher in the initial days of illness decreasing gradually during the later stages of the infection [16]. Circulation of DENV remains detectable in blood during febrile period and is rapidly cleared with appearance of specific antibody [18]. In routine assay, two steps method with a RT-PCR followed by a nested PCR is used for serotyping of dengue viruses [14].

In this study, high multiple infection was detected in West Sumatra (51.64%) because every sample was amplified for 5 times, amplified with outer primer and with specific serotype (DENV1, DENV2, DENV3, and DENV4). Technically, this procedure could prevent mismatch between primer and target, as it occurs in PCR multiplex. Peripheral blood parameters change during the course of illness. Dengue fever is characterized by leukopenia  $<5000/uL$ , rising hematocrit up to 5–10%, and thrombocytopenia  $<150.000/uL$ . In dengue hemorrhagic fever, peripheral blood parameters characterized by thrombocytopenia  $<100.000/uL$  and hematocrit rise  $>20\%$ . Thrombocyte contributes to increase vascular permeability by inflammation, depends on releasing of IL-1 $\beta$ . A rapid decrease in thrombocyte count, concomitant with a rising of hematocrit, leads the progression of plasma leakage[24]. As thrombocytopenia implicates higher

risk for menorrhagia [25]. Thrombocytopenia occurs commonly in laboratory-confirmed dengue cases on days 2–7. A patient with dengue had more severe thrombocytopenia on days 4–7, and the platelet count reached the lowest number on day 5 [26]. The pathogenesis of thrombocytopenia and leukopenia is suggested that dengue virus induced bone marrow suppression and depressed platelet and leukocyte synthesis and resulted in thrombocytopenia and leukopenia [7], [17].

## Conclusion

Two or more serotypes of dengue were found to infect a patient in West Sumatra. DENV-2 serotype was found predominantly in West Sumatra (n = 36, 39.56%) in patients with single infection. The molecular detection of dengue RNA by RT-PCR is a sensitive, rapid, and simple method. The RT-PCR method can detect the multiple dengue infection in clinical samples. The level of DENV viremia in early infection might be predictive for the development of severe disease later that can be beneficial for the management of patients with dengue virus infection.

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