Research Report

DIFFERENCES OF UNIVERSAL AND MULTIPLEX PRIMER FOR DETECTION OF DENGUE VIRUS FROM PATIENTS SUSPECTED DENGUE HEMORRHAGIC FEVER (DHF) IN SURABAYA

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ABSTRACT

Dengue Hemorrhagic Fever (DHF) is a global health problem in tropical and subtropical regions, as well as endemic in110 countries around the world. Indonesia is one of the largest countries in the region of endemic dengue. In Indonesia, dengue virus infection has become a contagious disease that was very important and was reported in 1968. Many molecular epidemiological approaches have been developed to look for factor that has been assumed as the cause of the increase of prevalence dengue virus infection in the world. The aim of this study is for the detection and determination of serotype of dengue virus in Surabaya. The method used was the technique of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction (PCR) with specific primers for dengue virus. Samples suspected DHF patients were obtained from various health center and hospital in Surabaya. Results of this research detected negative result for dengue virus in all samples of patients suspected DHF. Negative results caused by dengue virus titers in serum samples of patients who had been dropped due to long storage time and taken after the third day of fever in early period.

Key words: *Dengue Hemorrhagic Fever, PCR, RT-PCR, Dengue Virus*

ABSTRAK

Demam Berdarah Dengue (DBD) merupakan permasalahan kesehatan global di daerah tropis dan subtropis, serta penyakit endemik di 110 negara di dunia. Indonesia adalah salah satu negara terbesar di wilayah endemik DBD. Di Indonesia, infeksi virus dengue telah menjadi penyakit menular yang sangat penting dan dilaporkan pada tahun 1968. Banyak pendekatan epidemiologi molekuler telah dikembangkan untuk mencari faktor-faktor yang telah dianggap sebagai penyebab meningkatnya infeksi virus dengue prevalensi di dunia. Penelitian ini bertujuan untuk deteksi dan penentuan serotipe virus dengue di Kota Surabaya. Metode yang digunakan adalah teknik Reverse Transcription - Polymerase Chain Reaction (RT-PCR) dan Polymerase Chain Reaction (PCR) dengan primer spesifik untuk virus dengue. Sampel pasien diduga DHF didapatkan dari berbagai puskesmas dan rumah sakit di Kota Surabaya. Hasil penelitian terdeteksi negatif virus dengue pada semua sampel pasien yang diduga DHF. Hasil negatif yang disebabkan oleh titer virus dengue dalam sampel serum dari pasien yang telah turun karena waktu penyimpanan yang lama dan diambil setelah hari ketiga demam pada periode awal.

Kata kunci: *Dengue Hemorrhagic Fever, PCR, RT-PCR, Surabaya, Virus Dengue*

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is a global health problem in tropical and subtropical regions, also endemic diseases in 110 countries around the world.[1,2,3,4] Indonesia is one of the largest country in dengue endemic regions, with a population reaching 251 million people.[5] DHF is caused by the dengue virus and transmitted through

mosquito vector *Aedes aegypti* and *Aedes albopictus*.[6] In 2010, Indonesia became the first ranked country in ASEAN by the highest number of cases in DHF and hyperendemic predicate.[7]

The dengue virus is the Flaviviridae family members and genus flavivirus. Consists of four dengue virus serotypes (DENV-1, DENV-2, DENV-3 and DENV-4).[8,9,10] Dengue virus serotype DENV-1 was first discovered in Hawaii in 1944 and DENV-2 in Papua New Guinea in the same year. Dengue virus serotype DENV-3 and DENV-4 was found at Philippines in 1956.[11] Dengue virus is transmitted to humans by the mosquito *Aedes aegypti* and *Aedes albopticus*.[12] In Indonesia, dengue virus infection has become a contagious disease that was very important and was reported in 1968.[13,14] Many molecular epidemiological approach have been developed to look for factors that has been assumed as the cause of the increase of prevalence dengue virus infection in the world. That ranges of strains in serotypes classified in different groups can genetically sequencing revealed the dengue virus. Differences of nucleotide caused biological diversity in nature and their antigenicity.[15,16]

The aim of this study is for detection and determination of serotype of dengue virus in Surabaya. Dengue virus serotypes that have been known can be compared with previous studies, so the movement of dengue virus serotypes could be discovered.

MATERIAL AND METHODS

Samples

Serum samplesof patients suspected of dengue put in eppendorf tubes and storedina refrigerator at a temperature -80ºC in Dengue Laboratory, Institute of Tropical Disease Airlangga Univesity.

Extraction of dengue virus Ribonucleic acid(RNA)

Ribonucleic acid or RNA of dengue virus extracted from serum samples with extraction device QIAamp Viral RNA Mini Kit (QIAGEN), following a step works: put 560 μL of buffer AVL and carrier RNA in a 1.5 mL eppendorf tube, put1 40 μL of serum samples and vortex for 15 seconds, incubation at room temperature for 10 minutes and then centrifuge, added 560 μL of ethanol 96-100% then vortex for 15seconds and centrifuged, put 630μL of solution to the QIAamp mini coloumn, centrifuged at 8000 rpm for 1 minute, the remaining solution was included to QIAamp mini coloumn, centrifuged at 8000 rpm for 1 minute, put 500 μL of buffer AW1, centrifuged at 8000 rpm for 1 minute, put 500 μL of buffer AW2, centrifuged at 8000 rpm for 1min, transferred to a 1.5 mL microtube, added 60 μL of buffer AVE, incubated at room temperature for 1 minute, centrifuged at 8000 rpm for 1 minute and showed RNA.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction (PCR) for the detection and determination of dengue virus serotype

Molecular examination using RT-PCR and PCR for detecting nucleic acid of dengue virus sample and also for testing dengue virus serotypes is known as serotyping. The function of RT-PCR is to transcript the RNA into cDNA, then thecDNAwas amplified byPCR. Primers used for serotyping is D1 (forward), TS1, TS2, TS3 and TS4 (reverse).

In the process of RT-PCR was performed with 3 stages: the first stage component of reagents consists of 1 μL of primer, 5 μL of RNA, 1 μL of dNTP, and 7 μL of water; the second stage components of reagents consists of 4μLof 5x FS buffer, 1 μL of DTT, 0.5 μL of water, 0.5 μLof RNAse out, and 0.5 μLof superscript; the third stage was added 0.5 μL RNAseH. The first stage of the RT-PCR reaction carried out at a temperature of 65 °C for 5 minutes, the second stage carried out at a temperature of 55 °C for 50 minutes and 85 °C for 5 minutes, and the third stageincubated at a temperature of 37 °C for 20 minutes. In the process of PCR, component of reagents consists of 5 μLof cDNA, 2 μL of 10x PCR buffer, 2 μLof dNTP, 2 μL of primer, 0.1 μL of rTaq, and 9 μL of water. PCR reactions were performed as many as 30 to 40 cycles of PCR, the temperature was 94 °C for 4 minutes (pre-denaturation), 94 °C for 1 minute (denaturation), 50 °C for 1 minute (annealing), 72 °C for 12 minutes (extension) and 4 °C. Temperature 94 °C for 5 minutes in a PCR reaction aiming to denature double-stranded DNA, so DNA into single strands. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and staining is done with ethidium bromide. Marker used is a 100 bp ladder. On the implementation of the detection

Table 1. Primer oligonucleotides used for amplification and determination dengue virus serotypes

Primer	Sequences
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'
TS1	5'-CGTCTCAGTGATCCGGGGG-3'
TS2	5'-CGCCACAAGGGGCCATGAACAG-3'
TS3	5'-TAACATCATCATGAGACAGAGC-3'
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'

According Lanciotti *et al.* (1992) [17] and Harris *et al.* (1998) [9], dengue virus serotype determination was based on the size of DNA band formed after visualization on agarose gel electrophoresis, 482 bp for DENV-1, 119 bp for DENV-2, 290 bp for DENV-3, and 389 bp for DENV-4.

Table 2. Primer oligonucleotides used for detection of flavivirus group

Primer	Sequences
cFD2	5'-GTGTCCCAGCCGGCGGTGTCATCAGC-3'
MAMD	5'-AACATGATGGGRAARAGRGARAA-3'
FS778	5'-AARGGHAGYMCDGCHATHTGGT-3'

reaction and serotyping, always included a positive comparison (positive control dengue virus).

Additionally, in this studyuseda specific primer for flavi virus, which cFD2, MAMD, and FS778. The process of RT-PCR with specific primers for flavi virus was done in 3 stages: the first stage component of reagent sconsists of 1 µL of cFD2 primer, 5 µL of RNA, 1 µL of dNTP, and 7 µL ofwater; the second stage components of reagent sconsists of 4 µL of 5 xFS buffer, 1 µL of DTT, 0.5 µL of water, 0.5 µL of RNAse out, and 0.5 µL of superscript; the third stage was added 0.5 µL of RNAseH. The first stage of the RT-PCR reaction carried out at a temperature of 65°C for 5 minutes, the second stage carried out ata temperature of 55°C for 50 minutes and 85°C for 5 minutes, and the third stage incubated at a temperature of 37°C for 20 minutes.

In the process of PCR, component of reagents consists of 5 µL of cDNA, 2 µL of 10x PCR buffer, 2 µL of dNTP, 2 µL of primer (1 µL of cFD2 and 1 µLof MAMD), 0.1 µL rTaq, and 9 µL of water. PCR reactions were performed by 25 cycles of PCR, the temperature was 94°C for 4 minutes (pre-denaturation), 94 °C for 1 minute (denaturation), 54°C for 1 minute (annealing), 72°C for 1 minute (extension), and 72°C for 10 minutes (extension). Next process was heminested-PCR, component of reagents consists of 5 µL of DNA,2 µL PCR buffer 10x, 2 µL of dNTP, 2 µL primer (1 µL of cFD2 and 1 µL of FS778), 0.1 µLof rTaq, and 9 µL of water.

Heminested-PCR was performed by 25 cycles of PCR, 94°C for 2 minutes (pre-denaturation), 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), 60°C for 4 minutes (extension), and 60°C for 10 minutes (extension). Heminested-PCR products were analyzed by electrophoresis method on a 1.5% agarose gel and staining is done with ethidium bromide. Marker used is a 100 bp ladder.

RESULT AND DISCUSSION

Reverse transcription-polymerase chain reaction or RT-PCR and polymerase chain reaction or PCR for detection of dengue virus performed on serum samples of patients suspected of DHF were taken from Medokan Ayu Health Center, Manukan Kulon Health Center, Pacar Keling Health Center, Tenggilis Health Center, Krembangan Selatan Health Center,and Soerya Hospital Child and Maternity. This method, obtained negative results for all
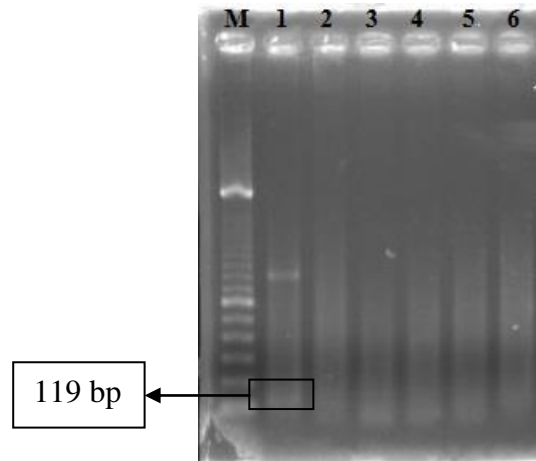
Figure 1. Results of electrophoresis of PCR samples obtained from Soerya Hospital Child and Maternity with primer D1, TS1, TS2, TS3, TS4. M: Marker; 1: Positive Control DENV-2; 2-6: Samples.
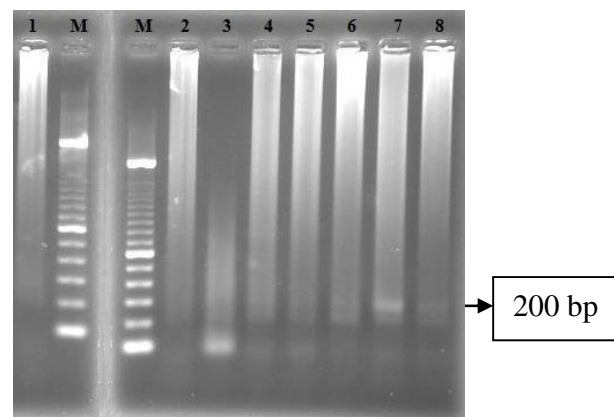
Figure 2. Results of electrophoresis of PCR samples with primer cFD2, MAMD, and FS778. M: Marker; 1: Medokan Ayu Health Center; 2: Manukan Health Center; 3: Pacar Keling Health Center; 4: Tenggilis Health Center; 5: Krembangan Selatan Health Center; 6: Manukan Health Center; 7: Positive Control; 8: Positive Control.

samples. Negative results caused by dengue virus titers in serum samples of patients who had been dropped due to long storage time and taken after the third day of feverin early period.

In Figure 1 was a serum samples obtained from Soerya Hospital Child and Maternity. The results obtained through out the sample is negative, there is no dengue virus DNA bands appearing. However, the positive control dengue virus DNA bands appear. Positive control was used the dengue virus serotype DENV-2 (119 bp).

In Figure 2 was a serum sample obtained from Medokan Ayu Health Center, Manukan Kulon Health Center, Pacar Keling Health Center, Tenggilis Health Center, and Krembangan Selatan Health Center. The negative results of the samples based on the condition that there is no DNA

band of dengue virus appearing. Negative results caused by dengue virus titers in serum samples of patients who had been dropped due to long storage time and taken after the third day of fever in early period. However, the positive control of dengue virus DNA bands appear at about 200 bp position. Primers used were primer for flavi virus, which are cFD2, MAMD, and FS778. Primer of flavi virus used to detect a group of viruses belonging to the genus *Flavivirus*.

In addition, dengue virus consists of four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4).[8,9,10] In Indonesia gained dominance serotype DENV-2, followed DENV-3 in 2003 to 2005.[18] In Indonesia, the four serotypes of dengue virus have been discovered but DENV-3 is often associated with severe dengue cases.[19,20] In Surabaya in 2005 was dominated serotype DENV-2, followed by DENV-3 and DENV-1. In Surabaya in 2008-2009 was also dominated by DENV-2.[21] According Aryati et. al. (2012),[22] in Surabaya dominated by DENV-1, followed by DENV-2, DENV-4, and DENV-3.

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 (double infection) can contribute to the severity of the infection. Then serotyping very important in the management of patients with dengue virus infection.[23,24]

Virus serotype can be demonstrated by molecular techniques such as PCRandRT-PCR. This is very important because it changes serotypes causing an indication of the threat of dengue fever in this population.[25] Unavailability of vaccines or antiviral drugs for the prevention of dengue virus infection is the cause of the development of research based surveillance system is needed in early warning (early warning) DBD. Such information can be used as apreventive measure and alert in preparation for an outbreak of dengue. Early warning is given each year prior to the extraordinary incident in dengue. Societies can play an active role in efforts to combat the vector which is an important factor for breaking the chain of transmission and prevention of dengue disease that reappeared in the future.

CONCLUSION

In this study, all samples of patients suspected of Dengue Hemorrhagic Fever (DHF) which is obtained from various health center and hospital in Surabaya detected negative and dengue virus serotype can't be known.

ACKNOWLEDGEMENTS

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