Indonesian Journal of Biotechnology, December, 2006 Vol. 11, No. 2, pp. 928-932

Rapid Detection and Molecular Typing of Dengue Virus by Using Multiplex-Nested-RT-PCR

Nastiti Wijayanti*[1], Tri Wibawa[1,2], Hera Nirwati[2], Aris Haryanto[1,3], and Sutaryo[1]

[1]Research Center for Biotechnology, Gadjah Mada University, Yogyakarta 55281, Indonesia.
[2]Microbiology Department, Faculty of Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia.
[3]Biochemistry Department, Faculty of Veterinary Medicine, Gadjah Mada University 55281, Indonesia.

Abstract

The dengue viruses (genus Flavivirus) are mosquito borne and cause dengue fever in most tropical areas of the world. We have evaluated the combination of one-step RT-PCR and multiplex nested PCR assays for detecting dengue viruses from clinical samples. Twelve patients were screened for the dengue virus, using a pair of primers that conserve for several Flavivirus. The results showed that in 12 suspect patients, 100% were positive for Flavivirus and there are some genotypic variation among them, that indicated by several RT-PCR products higher than 511 bp, the expected product for RT-PCR. Further assay was performed to clarify the presence and serotypes of dengue virus using multiplex nested PCR. Serotyping results indicated that 83,3% of samples can be confirmed for dengue virus. Among the dengue virus positive 16,7 % are dengue-2, 16.7 % are dengue-3, and the most common 50% are dengue-4, whereas dengue-1 were not found among the patients. The combination of RT-PCR and multiplex nested PCR assay can be used for rapid analysis dengue samples in early phase which is potentially useful for clinical, epidemiology and also evolutionary studies.

Key words: Flavivirus, dengue virus, serotype, RT-PCR, multiplex nested PCR

Introduction

Dengue fever is caused by four distinct serotypes (dengue-1 to dengue-4) of dengue virus (genus Flavivirus, family Flaviviridae), which are transmitted to humans by the domestic mosquitoes *Aedes aegypti* and *Aedes albopictus* (Harris *et al*., 1998). The four serotypes are closely related serologically; however, they are antigenically and genetically distinctive (Gubler, 1998, Kao *et al*., 2005). Dengue fever is one of the great emerging diseases. It is estimated that around half of the world population is at risk with an annual incidence of 100 million cases of dengue fever and over 45,000 cases of dengue hemorrhagis fever (DHF) (Monath, 1994).

Infection with one of the four dengue serotypes will provide lifelong immunity to the infecting serotype, but there is no cross-protective immunity against infection from other serotypes. Reinfection with a second serotype has been associated with a more severe form of disease and is a significant risk factor for DHF (Henchal and Putnak, 1990). DHF and dengue shock syndrome (DSS), which are the two main severe clinical manifestations of dengue, continue to be major causes of human morbidity and mortality in tropical areas (Sinniah *et al*, 1995; Gubler, 1998). Kumaria and Chakravarti (2005) reported that mortality can rise to as high as 20% among

*Corresponding author: Nastiti Wijayanti, Research Center for Biotechnology, Gadjah Mada University, Yogyakarta 55281, Indonesia. Ph: 0274-564305, Fax: 0274-520842, E-mail: nastitiw@yahoo.com

complicated cases if the diagnosis is missed in the acute phase, therefore rapid diagnostic test in the early phase of illness is crucial.

The immunoglobulin M (IgM) enzyme-linked immunosorbent assays (ELISA) are the most widely used technique for serological diagnosis, but they do not identify the dengue virus serotype responsible for the current infection (De Paula, 2004). The IgM is generally produced within 5 days of disease onset with subsequent production of IgG (Lindegren *et al.*, 2005). Serological assays also showed cross-reactivity with epitopes among flaviviruses (Harris *et al*, 1998; Johnson *et al*, 2005). Nowadays various types of sensitive, specific and rapid diagnostic tools are available for dengue viruses detection, each having its own advantages and disadvantages and can be effective depend on the laboratory conditions. Samuel and Tyagi (2006) showed that the best method for diagnostic is considered the one that is rapid, specific and inexpensive.

Since the existing diagnostic methods for suspected clinical cases are complicated by the short duration of viremia, reverse transcription (RT)-PCR- and real-time PCR-based methods have recently been developed to detect dengue viral RNA during the viremic phase (Henchal and Putnak 1990; Callahan *et al.*, 2001). RT-PCR is definitely the most satisfactory test that can be used on these infections, since it has been shown to be able to detect dengue viruses up to the 10th day after the onset of the symptoms (De Paula, 2004).

The aim of this work was to optimize methods for confirmation of dengue virus infection and for detection as well as serotyping of dengue virus by PCR-based test.

Materials and Methods

Materials

Whole blood samples from 12 patients collected during November 2006. Samples was collected from suspected children, within their early fever periods (day 1-3). Suspected patient were assigned based on the clinical symptoms of dengue fever, by an experience pediatrician.

RNA extractions

RNA was extracted from the whole blood. Extraction of viral RNA was done manually using High Pure Viral Nucleic Acid Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. The viral RNA was stored at -20°C prior to a complementary DNA (cDNA) synthesis.

Reverse transcription-PCR and Nested PCR

Reverse transcription was conducted at 60°C for 60 min and 94°C for 2 min, followed by 35 amplification cycles of 94°C for 30 sec, 55°C for 1 min and 68°C for 2 min, with a final extension at 68°C for 5 min using thermocycler (Biorad 10487). Five micro liters of extracted RNA was used as a template in a 25 µl reaction volume. RT-PCR was done using Superscript™ III One-Step RT-PCR system with Platinum (Invitrogen), which is a one-step RT-PCR kit, it was possible to convert the target viral RNA to a complementary DNA (cDNA) and to perform the DNA amplification in a single tube. The amplification products (10 µl) were analyzed by electrophoresis on a 1.5% agarose gel and stained in 1x TBE buffer containing 0.5 µg / ml ethidium bromide for ultraviolet visualization.

The multiplex nested PCR was performed with 5 µl of diluted (1:10) material from initial amplification reaction (RT-PCR) and used as a template for the second PCR in a 25 µl reaction volume. Nested PCR was done at 94°C for 2 min,

followed by 20 amplification cycles of 94ºC for 30 sec, 62ºC for 1 min and 72ºC for 2 min, with a final extension at 72ºC for 5 min. This assay was done using PCR Core Kit (Roche Diagnostic GmbH, Mannheim, Germany). Ten micro liters of the 25 µl reaction mixtures was electrophoresed on 1.5% agarose gel in 1x TBE buffer containing 0.5 µg/ml with ethidium bromide for ultraviolet visualization.

One set of primer corresponding to the C/prM region of the virus, designed by Lanciotti *et al.* (1992), the D1 (nt 134-161) and D2 (nt 616-644) primers, were used in the RT-PCR assay. D1 primer and 4 serotype-specific primers (TS1, TS2, TS3 and DEN4) as described by Harris *et al.* (1998) were used in the multiplex nested PCR assay (More detail see Tabel 1).

Table 1. Nucleotide sequence of primers

Primer	Nucleotides sequences
D1	5' TCA ATA TGC TGA AAC GCG CGA GAA ACC 3'
D2	5' TTG CAC CAA CAG TCA ATG TCT TCA GCT TC 3'
TS1	5' CGT CTC AGT GAT CCG GGG G 3'
TS1	5' CGC CAC AAG GGC CAT GAA CAG 3'
TS3	5' TAA CAT CAT CAT GAG ACA GAG C 3'
DEN4	5' TGT TGT CTT AAA CAA GAG AGG TC 3'

Results and Discussion

This study was trialed the cost-effective diagnostic test to detect dengue virus infection cases and to determine serotype of dengue viruses. To improve the sensitivity of dengue serotyping, we have performed a nested PCR using specific primers.

Viral RNA was isolated from the whole blood samples of the suspect dengue fever patients which were collected during the early phase. Since in this phase IgM antibodies specific to the dengue viral may not be detectable (Henchal and Putnak, 1990), our approach is an alternative for detection of dengue virus infection in early period.

In this study viral RNA was reverse transcripted to the cDNA by one-step RT-PCR. We used a combination of RT-PCR as described previously by Lanciotti *et al.* (1992) and multiplex PCR as described by Harris *et al.* (1998) in a nested PCR form. One set of primer, the D1 and D2 primers, was used in the RT-PCR assay for rapidly identifying Flaviviruses (Dengue, St. Louis encephalitis, and West Nile virus). D1 primer and 4 serotype-specific primers (TS1, TS2, TS3 and DEN4) used in the multiplex nested PCR assay for serotyping dengue virus.

RT-PCR products showed that the properly size DNA was 511 bp by amplification using consensus primer D1 and D2. Results of this RT-PCR assay showed that in 12 patients, 100% were positive for dengue virus infection (Figure 1). In addition, the RT-PCR assay also amplified other virus-related to flaviviruses with the same size of amplification product (West Nile and St. Louis encephalitis), however, Lanciotti *et al.* (1992) reported that they did not react with the dengue virus type-specific oligonucleotide primers in the nested PCR.

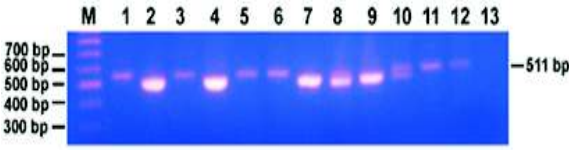

Figure 1. Detection of dengue virus by using RT-PCR assay. Lane M, DNA Marker; lane 1-12, samples from patients; lane 13, negative control (water). Expected product sizes is 511 bp. Results are representative and reproducible from three different RT-PCR.

Figure 1 showed RT-PCR assay of several samples resulted higher amplification product than 511 bp (lane 1, 3, 5, 6, 10, 11, and 12). It indicated that there is variation of nucleotide sequence among dengue viruses, which infected patients in Yogyakarta. This result is in line as reported by Johnson *et al.* (2005) that dengue viruses, a positive-strand RNA virus have a high potential for mutation, resulting in nucleotide differences between genotypes

and also within a serotype.

The expected sizes of the multiplex nested PCR amplification products using the specific oligonucleotide primers TS1, TS2, TS3 and DEN4 are 482 bp (dengue-1), 119 bp (dengue-2), 290 bp (dengue-3), and 389 bp (dengue-4). Figure 2 demonstrated that from twelve clinically suspected patients tested, 10 samples were found to be positive and could be successfully characterized into the serotypes. The serotypes detection showed that 16.7% (2/12) equally were positive for both serotype dengue-2 (lane 4 and 5) and dengue-3 (lane 7 and 12), followed by dengue-4 with 50% (6/12) (lane 1, 4, 8-11). Two samples were not detected. In our case, no samples belonging to dengue 1 serotype were detected and there are no double infection was detected simultaneously in the same patient.

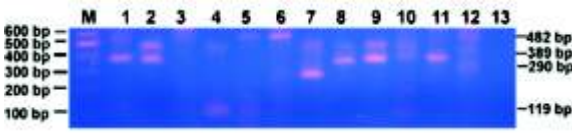

Figure 2. Serotyping of dengue virus by using multiplex nested PCR assay. Lane M, DNA Marker; lane 1-12, samples from patients; lane 13, negative control (water). Expected product sizes are 482 bp (dengue-1) 119 bp (dengue 2), 290 bp (dengue 3), and 389 bp (dengue 4). Results are representative and reproducible from three different multiplex nested PCR.

In this regard, one-step RT-PCR and multiplex (fourplex) nested PCR have been evaluated and optimized for detection of dengue viral RNA in clinical samples. In conclusion, 100 % samples were successfully detected for Flavivirus, and dengue virus infection was confirmed in 83.3% by serotyping assay. Thus, the combination of RT-PCR and multiplex nested PCR assay can be used for rapid analysis dengue samples in early phase which is potentially useful for clinical, epidemiology and also evolutionary studies. Futher studies should be done for bigger sample sizes.

Acknowledgement

This research was partly supported by Research Center for Biotechnology, Gadjah Mada University, Yogyakarta, Indonesia. We thank to Arsyah, Sujono, Tony Ruwaedi and Ipa Eka Wati for technical assistant.

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