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Early Detection and Serotyping of Dengue Viruses by Using Reverse Transcription Polymerase Chain Reaction (RT-PCR) 2 Primers

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Abstract

Recently several methods for confirming dengue virus infection have been developed such as virus isolation, detection of virus antigen, and nucleic acid detection by using RT-PCR method. It has been reported that rapid detection method to confirm dengue virus infection by using Multiplex RT-PCR had been successfully developed. It was reported more effective than the other methods with 100% sensitivity and specivicity at the early phase (day 1-3) of infection. This study was aimed to develop rapid detection and serotyping methods for dengue virus using RT-PCR 2 primers (Dcon and preM). The whole blood samples were collected from suspected dengue fever patients that had been confirmed with NS1 detection kit from during Februari-August 2009. The PCR products showed that in 12 samples, 100% were positive with different pattern among the serotypes especially for DEN1 and DEN2, but not for DEN3 and DEN4. This method was also able to confirm the double infection DEN2-DEN3, but not for the other ones because of the unspecific pattern. It is indicated that the RT-PCR 2 primers was a promising method for early detection and serotyping dengue virus infection.

Keywords : Dengue Virus, DHF, early detection, serotyping, RT-PCR 2 primers.

Introduction

Dengue virus infection is one of the most common health problems in the tropical and subtropical countries (Pinheiro and Corber, 1997; Gubler, 1998; Lindegren *et al*., 2005). Dengue virus included into the member of the family Flaviviridae, and genus Flavivirus. Which comprised of four serotypes (DEN1, DEN2, DEN3 and DEN4) (Trent *et al*, 1990; Kuno *et al*. 1998; Harris *et al*., 1998; Yong *et al*., 2007). DEN1 was firstly found in Hawai in 1944, DEN2 in Papua New Guinea in the same year. DEN3 and DEN4 were found in Philipines in 1956 (Ananthanarayan, 2000). Dengue virus is transmitted between human primarily by *Aedes aegypti* and *Aedes albopticus* mosquitoes which are endemic in the most areas except the 1000 meters above sea level areas (Kristina *et al*., 2004).

In Indonesia, dengue virus infection has been a very important infectious disease and firstly reported in 1968 but the virologist confirmation was informed in the next two years. Since that period until recently, several large epidemics of dengue virus infection have been reported (Soedarmo, 1995; Hadinegoro *et al*., 2006). The largest epidemics of dengue virus infection was occurred in 2007 with the Incidence Rate (IR) more than 300 per 100.000 peoples. The increasing of morbidity and mortality rate of dengue virus infection were associated with delayed management and missed diagnosis. Dengue virus infection is often diagnosed as other disease, such as influenza or typhoid fever (Malavige *et al*., 2004). The effective

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surveillance and efficient control depend on rapid and accurate laboratory diagnosis (Dinas Kesehatan RI, 2009).

Diagnostic methods of dengue virus infection have been developed with their advantage and disadvantage. Serological assay for IgM and IgG by using ELISA is one of the most well known technique, but can not differenciated the four dengue virus serotypes (Innis *et al.*, 1989; DePaula, 2004). IgM response is usualy detected after the 5 day of illness, and followed by IgG response (Lindegren *et al*., 2005). The assay of IgM and IgG is usefull to confirm the primer secary infection. It is very important for patient management in spite of epidemiology, because the condition will be worst in the secary infection.

The diagnostic method which is able to confirm the dengue virus in the early phase of illness has been developed, ie NS1 antigen detection. NS1 is one of nonstructural protein of the dengue virus, NS1 antigen detection is usefull to confirm dengue virus infection in the acute phase. Several researches reported that the sensitivity of NS1 antigen detection is better than virus isolation or IgM and IgG antigen. NS1 antigen detection method has 100% specivicity compared to the gold standard of virus isolation (Samuel and Tyagi, 2006).

Recently, several methods are developed based on *Reverse Transcriptase-Polymerase Chain Reaction* (RT-PCR) by amplifying segment of dengue virus genome. This approach has several advantages, such as early detection of the viral genome in the first to fifth days infection that is a window period for IgM and IgG detections and the ability to detect serotype which is lack in the antigen NS1 detection. Previously, Wijayanti *et al*. (2006) has been conducted similar research in Yogyakarta. This research has used Multiplex-Nested RT-PCR as a dengue virus detection method by amplifying preM genes.

Multiplex RT-PCR method disadvantages is employed many primers to amplify the dengue virus target genes. Simpler and cheaper methods for early detection and serotyping method of dengue virus should be developed. We designed reverse primer which is correspond to the *preM* which is able to detect and serotyped by one step RT-PCR using two primers.

Materials and Methods

Samples

This study was carried out among 14 dengue virus infection patients that had been confirmed by NS1 antigen detection kit during Februari-August 2009. The whole blood was collected from the patients within their early fever periode on day one to three. Twelve sampels were single infection and two sampels were double infection of dengue virus that was confirmed by multiplex one-step RT-PCR using serotype-specific primers for dengue virus designed by Yong *et al.* (2007).

RNA extraction

RNA was extracted from the whole blood. The extraction was manually done using *High Pure Viral Nucliec Acid* kit (Roche, Mannheim, Germany) according to the recommended procedure of the manufacturer.

Primer design

Dcon primer which was designed by Yong *et al.* (2007) was used as forward primer, and *preM* primer as a reverse primer. The *preM* primer was designed after aligned several *preM* sequences of dengue virus isolates. The *prM* primer was correspond to the. conserve sequence in the *preM* of those dengue virus isolates.

Table 1. Nucleotide sequences of primer pairs which were designed to amplify preM gene of Dengue Virus.

No	Primer	Sequence	Product (bp)
1.	Dcon	5'-AGTTGTTAGTCTAC GTGGACCGACA-3'	700
2.	preM	5'-GCCACTGATCGTTT CTCTCGTC-3'	

Detection and serotyping of Dengue Virus by using RT-PCR 2 Primers.

Detection and serotyping of dengue virus was carried out in on tube reaction by amplifying *preM* gene using 2 primers (Dcon and preM). RT-PCR 2 primers was performed with 5 µL extracted RNA in a 25 µL reaction volume. RT (reverse transcription) reaction was run at 55°C for 30 min. The preM amplification was run at 94°C for 2 min, followed by amplification 35 cycles of 94°C for 45 sec, 57°C for 1 min, and 68°C for 1 min, with a final extension at 68°C for 7 min.

The product were analyzed by electrophoresis on a 1% agarose gel and stained with Gold View™ Nucleic Acid Stain in 1x TBE buffer. 100 bp ladder (Microzone) was used as a marker. Gel was exposed on the UV transiluminator.

Results and Discussion

Detection and Serotyping of Dengue Virus from Clinical Samples

Detection and serotyping of the the clinical samples by using multiplex one step RT-PCR was done to confirm presence and serotype of the dengue virus. The Multiplex RT-PCR products showed that there were four different serotypes present in our patients (Figure 1). We used 12 samples which were positive for single serotype dengue virus, which were consist of three samples for each serotypes (DEN1, DEN2, DEN3 and DEN4) and two samples which were double infected with two different serotypes (one sampel with DEN2 and DEN3 and one sampel with DEN2 and DEN4)

Molecular techniques are particularly useful for the detection and typing of dengue virus and several RT-PCR protocols have been described. Identification of the four serotypes can be achieved by nested amplification of a primary product generated with universal dengue virus primers (Lanciotti *et al*., 1992; Haris *et al*., 1998), hybridization of a universal RT-PCR product with type-specific probes (Deubel *et al*., 1990; Henchal and Putnak, 1990), and simultaneous amplification with a single 5′ universal primer and four type-specific 3′ primers (Yong *et al*., 2007). However, the nested PCR amplification increases the risk of cross-contamination, especially during the routine analysis. Therefore, it is necessary to simplify the RT-PCR procedure and minimized the number of primers required for detection and serotyping of dengue viruses.

Detection and serotyping of Dengue Virus by using RT-PCR 2 primers.

The *preM* genes was successfully amplified by RT-PCR 2 primers method. The expected size of RT-PCR products using primer Dcon and preM was 700 bp. RT-PCR amplification using 2 primer was able to detect dengue virus in all clinical samples of 14 patients enrolled in this study. The results showed that beside the expected product, there were other RT-PCR bands appeared. There were three patterns of the PCR products on the electrophoresis gel agarose (Figure 2A). These patterns are consistent with serotypes, especially for DEN1 and DEN2. However, amplified

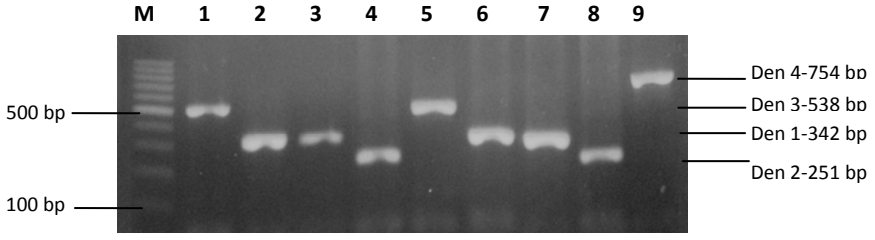

Figure 1. Representative results of serotyping dengue virus in the clinical samples obtained from DKI Jakarta dan DI Yogyakarta by using *One Step Multiplex* RT-PCR with primers which were designed by Yong *et al.* (2007). Lanes 2 ,3, 6 and 7 are DEN1; Lanes 4 and 8 are DEN2; Lanes 1 and 5 are DEN3; Lane 9 is DEN4; and M is 100 bp marker.

product pattern for DEN3 and DEN4 were undistinguishable. RT-PCR 2 primer method was not able to confirm double infection case due to the unspecific DNA band on electrophoresis gel (Figure 2B).

In general, PCR-based technique can be more rapid, sensitive, and specific than other methods especially when they are made accessible by a low-cost methodology that involves simplification and effective procedures for minimizing DNA contamination (Chan *et al.* 1994). The RT-PCR 2 primers described herein was adapted from a previously reported one step multiplex RT-PCR protocols (Yong *et al.*, 2007). The reverse primer (preM) was designed to improve the efficiency of the amplification. The RT-PCR 2 primers redesigned primer was expected to become an alternative method for detection and serotyping dengue virus directly from the clinical sample. The ability of RT-PCR 2 primer method to detect DEN1 and DEN2 specifically is very usefull for dengue virus survailance in where DEN1or DEN2 is the most commonly found serotype (Haris *et al.*, 1998).

Our result indicated that the RT-PCR 2 primers is less laborious compare to the one step multiplex RT-PCR which has been accepted to detect and serotyped dengue virus from clinical samples. The fact that pattern of RT-PCR amplification product were associated with dengue virus serotype is a new approach which may develop and further scrutinize for future approach of detection and serotyping of dengue virus.

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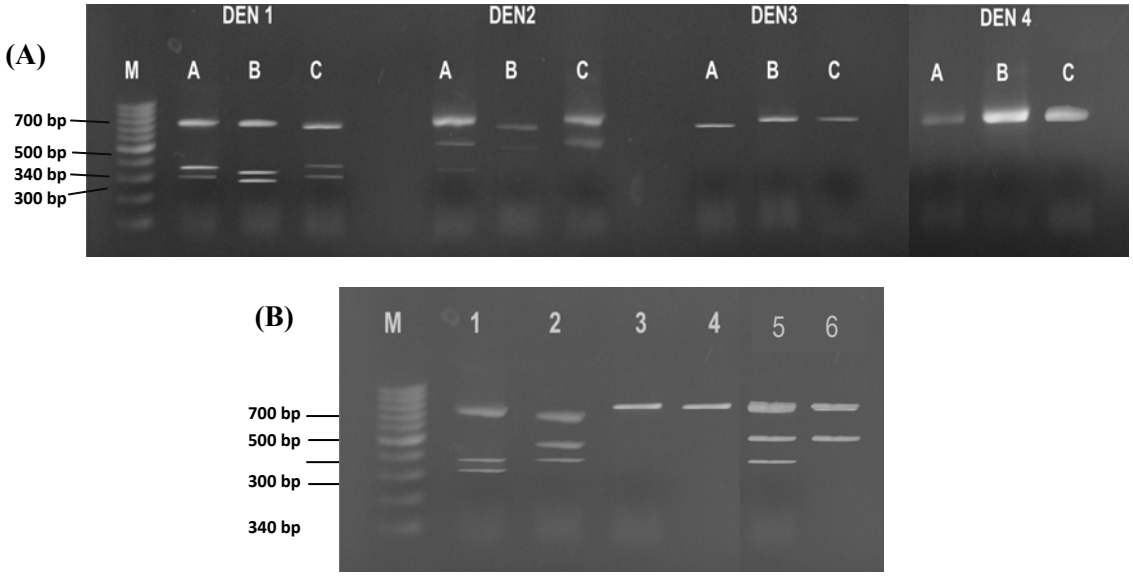

Figure 2. (A) RT-PCR 2 primers (Dcon and preM) amplification products were run on agarose gel. Each serotype was represented by 3 samples. The pattern of amplified products were consistent with DEN1 and DEN2, but not for DEN3 and DEN4 which were undistinguishable; (B) **RT-**PCR 2 primers amplified product of double infection samples. Lane 1: DEN1; lane 2: DEN2; lane 3: DEN3; lane 4: DEN4; lane 5: double infection DEN2 dan DEN3; lane 6: double infection DEN2 dan DEN4; M: 100 bp Marker.

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