

Application of combined multiplex-PCR and RT-LAMP to detect Dengue Fever, from clinically infected patients in Surabaya, Indonesia

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Abstract

Dengue virus is an important *Flavivirus* causing Dengue fever, a substantial public health cases in Indonesia. This article reported the application of multiplex PCR and RT-LAMP to detect Dengue virus from suspected infected-patients hospitalized in Dr. Soetomo hospital, Surabaya. In this study both RT-PCR and RT-LAMP could provide a definitive results for less than 2 h of work. Therefore, this experiment showed that the method could be applied for Dengue virus circulating from field samples and be used as routine laboratory testing or further epidemiology study.

Keywords: Dengue, detection, RT-PCR, RT-LAMP, Indonesia

INTRODUCTION

Dengue virus is an important *Flavivirus* causing Dengue fever, a substantial public health cases in most all tropical and subtropical countries (Banu et al., 2014; Dupont-Rouzeyrol et al., 2014; Guzman et al., 2010). Dengue infections are estimated to reach 390 million per year with different level of infection severity (Bhatt et al., 2013). Indonesia is one of tropical regions with severe infection rate of Dengue annually. In general, many factors influence infection rate of Dengue in different country. One of the most important factor is climate which supports vector life, the *Aedes* mosquitos (Colon-Gonzalez et al., 2013; Naish et al., 2014). Apparently, the effect of rainy season is count to be prominent in Indonesia. Index of rain level is correlated with Dengue incidences in areas known having highly rain level such as Jakarta and West Kalimantan (Anonym b, 2009). The natural geography location of Indonesia with its climate condition make Dengue becoming difficult to be eradicated and becoming a challenge. In the year of 2009, 77 % Indonesias municipalities that are distributed in 97 % of total provinces are endemic for Dengue fever. The incidence rate of Dengue case is 60-70 per 100,000

citizen (Anonym b, 2009). In comparison to other Asia regions such as Phillipine, Singapore, India, Myanmar and Thailand, Indonesias cases are classified into high infection rate (Anonym a, 2009).

Severe case of Dengue exhibits significant bleeding, altered level of conciousness, gastrointestinal problem and acute organ impairment (Anonym a, 2009). Dengue infection lead to case fatality rate reaching 0.89 % in Indonesia (Depkes RI, 2009, WHO, 2012). Although some vaccines of Dengue are on trial (Cunningham and Hayney, 2014; Mullard, 2014; Villar et al., 2014), no effective antiviral agents exist to treat infected patients so far and therefore the treatments to reduce disease fatality are remaining supportive. The critical factor to reduce Dengue severity and patient mortality is appropriate treatments which are largely depends on succesfull detection system in the early disease onset. The routine laboratory diagnosis such as haemagglutination inhibition and ELISA have developed earlier. These methods can not detect the serotype of dengue infecting virus and highly cross reactive with other flaviviruses. Whilst, the virus isolation and identification methods consume time

as well as providing low successful rate due to handling difficulties (Johnson et al., 2000). New development of NS1 antigen-based method gave possibility of false positive reaction in detecting clinical patients (Chung et al., 2014; Colombo et al., 2013).

The development of molecular biology-based approaches such as polymerase chain reaction (PCR) yield promising results on its specificity, sensitivity, time and cost efficiency. It is able to differentiate dengue virus serotypes in the plasma of serum patients. The developments of the system are varying starting from nested-PCR, multiplex-PCR and TaqMan real-time PCR with different target of amplification in the virus genome (Lanciotti et al., 1992; Seah et al., 1995; Waggoner et al., 2013) and becoming essential test for Dengue detection system. The development of loop-mediated isothermal amplification (LAMP) provides possibility of easier and more simple technical system (Notomi et al., 2000; Parida et al., 2005). It can proceed amplification of the sample with a single temperature instrument or waterbath without thermal cycle which fits for laboratories in developing countries.

This article reports the application of multiplex PCR and RT-LAMP to detect Dengue virus from suspected infected-patients hospitalized in Dr. Soetomo hospital, Surabaya. The successful application of RT-LAMP confirmed by multiplex-PCR and followed by DNA sequencing gave us the evidence that the system used can be applied in the clinical sample for common Dengue virus serotype circulating in Indonesia and be used as routine laboratory testing.

MATERIALS AND METHODS

Patient samples

The blood sampling from all patients in this experiment were taken with permission based on accepted ethical clearance no. 19/ Panke. KKE/ II/ 2011, issued by ethical committee RSUD Dr. Soetomo, Surabaya, Indonesia. Suspected infected-patients were diagnosed based on the clinical signs and haematological changes as guided by WHO (Anonym a., 2009). Peripheral blood samples were collected in a 9 mL-heparin vacutainer (Greiner Bio-One). To keep RNAs integrity, the blood were mixed with *RNA-later* (Ambion) at the volume ratio of 1:3 and keep on 4°C until use in no more than 3 days.

Dengue virus as positive control

Dengue virus as positive control are obtained from Department of Parasitology, Faculty of Medicine Gadjah Mada University, previously confirmed as reference isolate originated from Indonesia (Umniyati et al., 2008). Virus was cultured by monolayer of C6/ 36 cell line kept in 28°C, 5% CO₂. The infected monolayer was

supplemented with DMEM (Gibco) containing 2% fetal calf serum, 100 U/mL penicillin, 1X non-essential amino acids, and 100 µg/mL streptomycin.

Total RNAs isolation

The positive controls were taken both from the medium supernatant and infected cells remaining on the inoculated flasks after one week culture period. Whilst, the blood from patients collected (see 2.1) were served as samples. Total RNA isolation was performed using the RNeasy kit (Qiagen) according to manufacturer's protocol. For total RNA isolation, 600 µl of 70 % ethanol were added to the suspension, resuspended and transferred to the column tube. The samples were then centrifuged (10,000xg, 1 min) and flow-throughs were discarded. Then the samples were washed once with 500 µl buffer RW1 (10,000xg, 1 min) and twice with 500 µl buffer RPE (10,000xg, 1 min). Total RNA was eluted by adding 50 µl of DEPC-treated water to the column followed by centrifugation (10,000xg, 1 min). Total RNAs were stored at -20°C until further use.

One step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT PCR was performed using Superscript Platinum Taq RT-PCR (Life Technologies) according to manufacturer's instructions. The reaction contained 12.5 µl RT-mix, 0.5 µl RT-Taq enzyme, 1 µl MgSO₄, 5 µl RNA, 1 µl of 10 pmol forward primer and 1 µl of each 10 pmol reverse primer in the total 25 µl reaction volume. The primers (Lanciotti et al., 1992) were purchased from 1st BASE (Singapore) as follows: universal forward 5'TCAATATGCTGAAACGCGCGAGAAACCG3', DEN-1 reverse 5'CGTCTC AGTGATCCGGGG3', DEN-2 reverse 5'CGCCACAAGGGCCATGAACAG3', DEN-3 reverse 5'TAACATCATCATGAGACAGAGC3' and DEN-4 reverse 5'TGTTGTCTTAAA CAAGAGAGGTC3'. The reaction in the thermal cycler (Eppendorf) consist of: incubation at 42°C for 60 min for reverse transcription followed by 30 cycles of denaturation 94°C for 30 sec, annealing 54°C for 1 min, elongation 68°C for 1 min and 1 cycle of final extension 68°C for 10 min. The amplified product were loaded onto 1.5% agarose gel supplemented with SYBR Safe (Life Technologies) and visualized in UV light.

DNA sequencing

All the amplicons obtained from RT-PCR controls and samples (n=30) were sequenced (1st BASE, Singapore) and analyzed by BLASTN (<http://www.ncbi.nlm.nih.gov/blast>) to confirm sequence similarity with other Dengue viruses isolates submitted in genbank databases.

Table 1. RT-LAMP primers

Serotype	Primer	Sequence 5'→3'
DEN-1	F3	GAGGCTGCAAACCATGGAA
	B3	CAGCAGGATCTCTGGTCTCT
	FIP	GCTGCGTTGTGTCTTGGGAGGTTTTCTGTACGCATGGGGTAGC
	BIP	CCCAACACCAGGGGAAGCTGTTTTTTGTTGTTGTGCGGGGG
	FLP	CTCCTCTAACCACTAGTC
	BLP	GGTGGTAAGGACTAGAGG
DEN-2	F3	TGGAAGCTGTACGCATGG
	B3	GTGCCTGGAATGATGCTG
	FIP	TTGGGCCCCCATTTGTTGCTGTTTTAGTGGAAGTACGGTTAGAGG
	BIP	GGTTAGAGGAGACCCCCCAATTTGGAGACAGCAGGATCTCTGG
	FLP	GATCTGTAAGGGAGGGG
	BLP	GCATATTGACGCTGGGA
DEN-3	F3	GCCACCTTAAGCCACAGTA
	B3	GTTGTGTCATGGGAGGG
	FIP	TGGCTTTTGGGCTGACTTCTTTTTGAAGAAGCTGTGCAGCCTG
	BIP	CTGTAGCTCCGTCGTGGGATTTTCTAGTCTGCTACACCGTGC
	FLP	CCTTGGACGGGGCT
	BLP	GGAGGCTGCAAACCGTG
DEN-4	F3	CTATTGAAGTCAGGCCAC
	B3	ACCTCTAGTCCTTCCACC
	FIP	TGGGAATTATAACGCCTCCCGTTTTTCCACGGCTTGAGCAAACC
	BIP	GGTTAGAGGAGACCCCTCCCTTTTAGCTTCCTCCTGGCTTCG
	FLP	GGCGGAGCTACAGGCAG
	BLP	TCACCAACAAAACGCAG

One step Reverse Transcription-Loop-Mediated Isothermal Amplification Assay (RT-LAMP)

The primer sets for RT-LAMP reaction performed in this experiments were based on Parida et al. (2005) with slightly modifications. Primers (1st BASE, Singapore) were consist of 6 pairs for each Dengue serotype showed in Table 1. The reaction compositions were as follows: 50 pmol of FIP and BIP primers, 10 pmol F3 and B3 primers, 25 pmol of FLP and BLP, 2.5 µl amplification buffer (New England Biolabs), 10 mM MgSO₄, 2 mM dNTPs, 10 U *Bst* polymerase (New England Biolabs), 0.5 U ThermoScript™ reverse transcriptase (Life Technologies) and 5 µl isolated RNAs in 25 µl total reaction volume. Tubes were placed in the waterbath at 64°C for 1 h. The amplifications were checked by loading onto 1% agarose gel and direct visualization under UV light/ transilluminator.

RESULTS AND DISCUSSION

The correct diagnosis of Dengue Fever becomes a key role to choose the appropriate treatments for patients and therefore able to reduce the disease fatality and mortality rate (Aurpibul et al., 2014; Simmons et al., 2012; Tomashek et al., 2014; Whitehorn et al., 2014). Detection system based on the virus genome will give accurate

result in the early fever period due to high circulating virus in the blood (Anonym a, 2009; Drosten et al., 2002).

Dengue virus serotypes cultured in the C6/ 36 cell line served as control for initial reaction test. The cytopatic effect of virus infections one week after inoculation did not show clear differences on each serotype (Figure 1.A).

RT-PCR detection showed different amplicons produced of DEN-1 482 bp, DEN-2 119 bp, DEN-3 290 bp and DEN-4 389 bp (Figure 1. B1). RT-LAMP reaction also able to be performed resulting loop-amplicon product as shown on Figure 1. B2. Highly DNA abundance amplified by RT-LAMP allows the positive amplification can be seen by naked eye on the tubes without agarose gel electrophoresis (Figure 2. B3).

The optimized reactions were then applied to patients sample as shown in Figure 2. Sequencing of multiplex PCR product showed highly similarity of Dengue viruses from field samples as follows: DEN-1 genbank no. EU 848545 (99.8%), DEN-2 genbank no. JF 327392 (99.9%), DEN-3 genbank no. AY 858047 (98.3%), DEN-4 genbank no. GQ 868594 (99.45%). The highest incidency was DEN-3 (40%) followed by DEN-2 (33.3%), DEN-4 (20%) and DEN-1 (6.7%). Overall reactions for RT-LAMP were finished 1 h to produce positive results as observed in Figure 2. B. Sample producing smear band on RT-PCR (lane 11, Figure 2.A) could be amplified by RT-LAMP succesfully as observed by thick continuous-loop band on

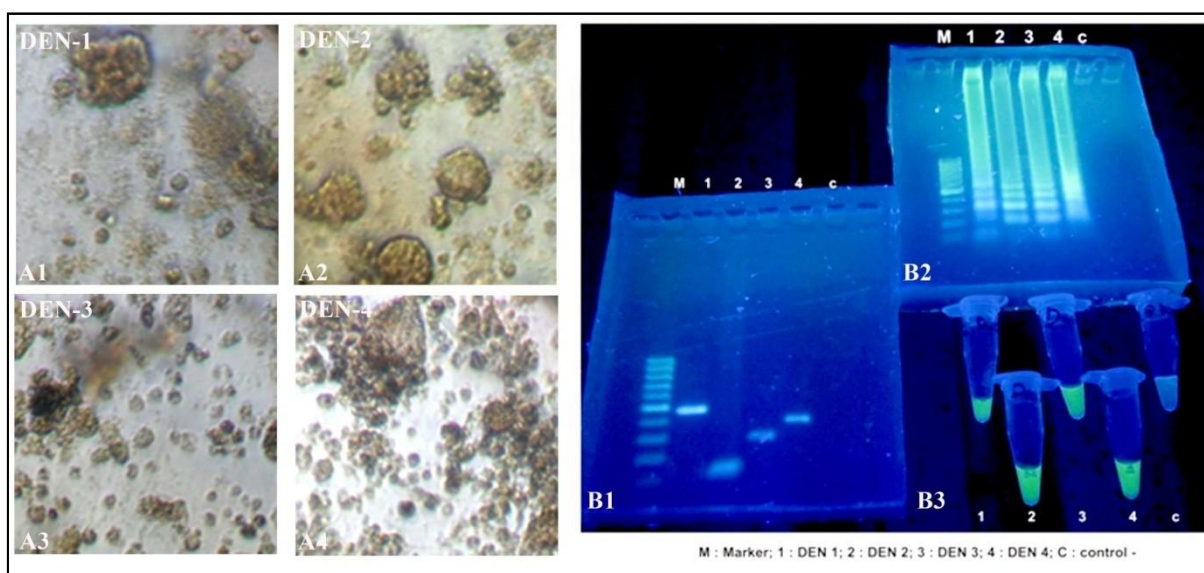


Figure 1. Cultivation on C6/36 cell line, multiplex RT-PCR and RT-LAMP of control virus.

A: Cultivation of four Dengue virus serotype; A1: DEN-1; A2: DEN-2; A3: DEN-3; A4: DEN-4. B: Multiplex RT-PCR reaction of control (B1), RT-LAMP reaction (B2) and direct visualization from RT-LAMP reaction under UV-light (B3), with the respective annotations; M: marker; 1: DEN-1; 2: DEN-2; 3: DEN-3; 4: DEN-4; c: control (H₂O).

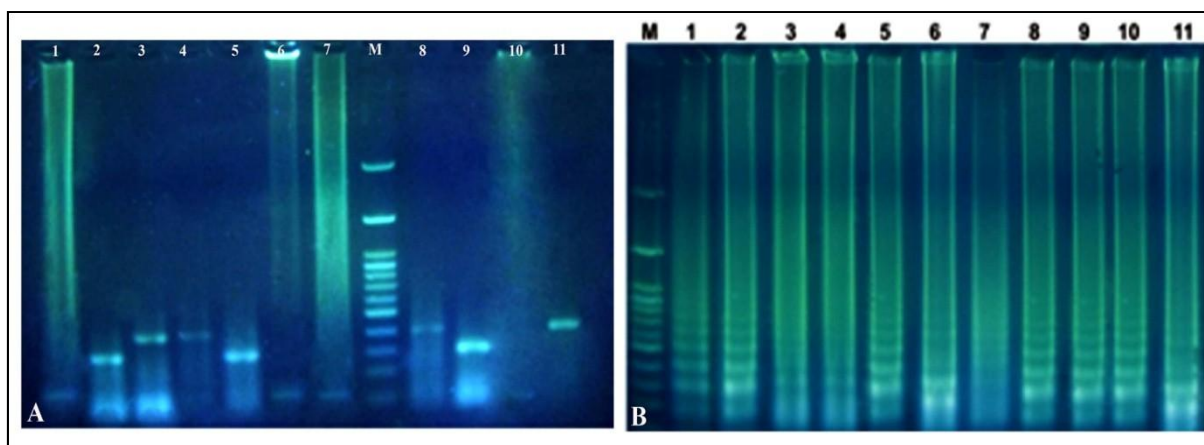


Fig. 2. Exemplary result of multiplex RT-PCR (A) and RT-LAMP (B) reactions from field samples.

agarose gel (lane 11, Figure 2.B). The results implied that RT-LAMP system used can provide fast, sensitive and simple detection for RNA samples originated from field cases.

In this study both RT-PCR and RT-LAMP could provide a definitive results for less than 2 h work. The serotypes of infecting Dengue virus were differentiated by multiplexing primers used and monitored by band migration in DNA electrophoresis. Nevertheless, the necessity of thermal cycler to perform PCR may become difficult to be provided in all periphery laboratories. Whilst RT-LAMP does not need expensive thermal cycler

to be performed. Single temperature set of reverse transcriptase and *Bst* polymerase in around 60°C made overall reaction could be established in the normal waterbath. In addition, positive reaction could be observed directly without loading onto agarose gel.

Although high genetic variability of Dengue virus are commonly occurred (Rico-Hesse, 2003, 2007), RT-PCR and RT-LAMP used here are still able to be performed without any difficult optimizations as compared to controls and confirmed by DNA sequencing. Therefore this experiment showed that the method could be applied for Dengue virus from field sample circulating in Indonesia.

The accuracy and feasibility of methods are not only intended for practical doctors but also disease investigation such as viral load quantification and epidemiology studies. The usage may also be expanded for detection on the vector carrying virus, *Aedes* mosquitos.

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