DEVELOPMENT OF SINGLE-TUBE MUTIPLEX RT-PCR FOR DENGUE VIRUS TYPING

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Abstract. This study evaluated a single-tube multiplex RT-PCR with a primer focusing on nonstructural protein region 5 (NS5) and a highly conserved region for dengue virus serotyping. The method was compared with conventional PCR. This new method had a sensitivity of 96.7% and a specificity of 96.7% for disease detection. The new method also proved suitable for use in the field as it reduces time and decreases risk of contamination.

INTRODUCTION

Dengue is an important public health problem in tropical and subtropical regions. Dengue infection is distributed worldwide due to international travel (Wilder-Smith and Gubler, 2008).

Dengue virus (DV) is a single-stranded RNA virus in the genus *Flavivirus*, family Flaviviridae. Secondary infection with a different strain of one of the four strains of dengue virus (DV1-4) can result in more severe disease (Coffey *et al*, 2009). Clinical signs and symptoms vary among those with dengue fever, dengue hemorrhagic fever and dengue shock syndrome. Unfortunately, there is no vaccine and the treatment is only supportive, thus proper management of infection is important.

To facilitate a prompt diagnosis, several methods have been developed employing antibody or antigen detection methods (Guzman and Kouri, 2004). The antibody detection method uses paired sera with result in a delay in laboratory diagnosis. Antigen isolation methods, such as virus isolation by cell culture, are time consuming. Another method for viral detection, antigen detection, can be achieved during the early stage of infection; this includes Reverse Transcriptase and Nested Polymerase Chain Reaction (RT/ Nested PCR) for typing dengue infection (Lanciotti *et al*, 1992). This method is prone to contamination during the RT step and Nested PCR diagnosis. The purpose of this study was to develop a single-tube multiplex RT-PCR with a consensus primer of the nonstructural protein region 5 (NS5), a highly conserved region, and to evaluate the dengue serotype which can be used for disease surveillance and epidemiological investigations.

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MATERIAL AND METHODS

Blood samples

Blood samples positive for dengue were obtained from the microbiology and immunology laboratory at the Faculty of Tropical

Table 1
Sequence of single-tube multiplex RT-PCR primers.

Sero-types	Sequence (5'-3')		Product size
	Sense primer	Antisense primer	
DV1	CAGGAAGAAGAAGCGTCTCAGG	AGGTTGTCCAAGGCATTCTGG	109 bp
DV2	CAATGAGATGGTGTTGCTGCAG	GTCTCTTTCTGTATCCAATTTGACCC	139 bp
DV3	CCAGGCATTTTCCAGACAACAAC	GCGCTATTCCACTGACATAGCC	169 bp
DV4	GACTGAAGCCAAGTCTGCCC	ACGTTCTTGAGTGTCGCCATG	199 bp

Medicine, Mahidol University. The use of unlinked specimens was approved by the Ethics Committee of the Faculty of Tropical Medicine, MUTM 2006-050.

Viruses/virus propagation

Japanese encephalitis (JE) virus (Beijing strain) and all 4 types of dengue viruses were obtained from the Faculty of Tropical Medicine Dengue Diagnostic Center (TDC), Mahidol University. DV1 strain Hawaii, DV2 strain 16681, DV3 strain H87, DV4 strain H241 were prepared by the laboratory using viral cultivation in a mammalian cell line (LLC-MK2) using the plaque assay process.

With the plaque titration assay, LLC-MK2 cells were used for double overlay plaque assay, modifying the protocol originally described by Malewicz and Jenkin (1979). Briefly, LLC-MK2 cells were maintained in GM-DMEM. Ten-fold dilutions of each sample were diluted in 5% RPMI medium. LLC-MK2 cells were washed once with Hank's balance salt solution and then inoculated with approximately 250 µl of virus suspension per well in a 6 well-plate with constant rocking at room temperature. This process was followed by adding the first overlay. After initial overlay solidification, cells were inverted and incubated at 37ºC in a CO_2 incubator for 7 days. A second overlay containing 1% neutral red was then added and incubated at 37ºC in a CO_2 incubator. The plaques appeared and increased in size by 24 hours of incubation.

The laboratory strains of *Plasmodium falciparum* were obtained from Professor Srivicha Krudsood at the Critical Care Research Unit, Department of Tropical Medicine, Faculty of Tropical Medicine, Mahidol University.

Primers and RNA extraction

Consensus primers were designed for the nonstructural protein region (NS5), which is a highly conserved region. The product sizes were DV1 109 bp, DV2 139 bp, DV3 169 bp, DV4 199 bp. The primer sequences are shown in Table 1. Viral RNA extraction were performed by a viral RNA mini kit (QIAamp, QIAGEN GmbH, Hilden, Germany) following their protocol.

RT-PCR

For the single-tube multiplex RT-PCR, the one step RT PCR kit (QIAGEN, GmbH, Hilden, Germany) was used with Taq polymerase (QIAGEN, GmbH, Hilden, Germany) which was then mixed with designed primers and extracted RNA. The PCR step started with RT at 50ºC for 30 minutes then preheated with Taq-PCR at 94ºC for 15 minutes followed by 35 cycles of denaturing at 94ºC for 30 seconds each, then annealing at 60ºC for 1 minute, and finally extension at 72ºC for 2 minutes.

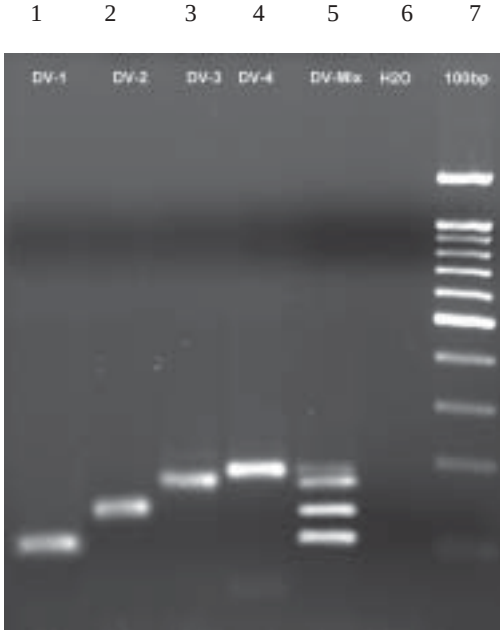

Fig 1–Gel electrophoresis of dengue virus run by a single-tube multiplex RT-PCR, DV1 was 109 bp, DV2 was 139 bp, DV3 was 169 bp, DV4 was 199 bp, H_2O was the negative control.

The gold standard used for comparison in this study used a one step RT-PCR kit (Qiagen®) and nested PCR for dengue serotyping using Taq polymerase (Qiagen®).

Electrophoresis analysis

A 10 µl volume of each reaction was run in 1.5% agarose gel containing ethidium bromide, then electrophored. The gel was exposed to a UV transilluminator and photographed by SynGene gel documentation equipment.

RESULTS

The single-tube multiplex RT-PCR

The single-tube multiplex RT-PCR for dengue positive samples was carried out using 1.5% agarose gel separately for each of the four serotypes. A mix of the 4 dengue serotypes was used as a positive control, H_2O was used as a negative control. The results are shown in Fig 1.

Sensitivity

In this study we tested the single-tube multiplex RT-PCR with each serotype (DV1 to DV4) and DV mixed samples, 30 times each. The results showed perfect detection of DV2 and DV3 but missed 1 out of 30 for serotypes DV1 and DV4 (96.7% detection) and missed 3 out of 30 with the mixed DV serotypes (90.0% detection). The single-tube multiplex RT-PCR results were compared with the gold standard (RT/Nested PCR, Lanciotti) for 30 samples: 28 known dengue positive cases (n=7 for each subtype) and 2 negative samples (n=2). As shown in Table 2, the single-tube multiplex RT-PCR was highly accurate with one false negative for each group.

To measure the lowest titration of virus the single-tube multiplex RT-PCR method could detect, the four DV serotypes were divided into three groups based on the number of virus plaque formation unit (pfu) (10^0 to 10^{-3}). The single-tube multiplex RT-PCR method detected DV1 at 10^{-1}, and DV2, DV3, and DV4 at 10^{-2} (data not shown).

Specificity

Cross-reactivity among flaviviruses was tested with Japanese encephalitis virus (JE; Beijing strain); false positive rates were determined. The specificity was 96.7%. A specificity of 100% was found with *Plasmodium falciparum* malaria infection detection (Table 3).

DISCUSSION

The viral detection using RT-PCR is a good method for early detection of dengue infection and serotyping, however, the method is time consuming and technically prone to contamination. To reduce the

Table 2
Dengue virus detection by single-tube multiplex RT-PCR and RT/Nested PCR (Lanciotti, the gold standard).

Specimen type	Positive results using single-tube multiplex RT-PCR		Known samples		
	n	Positive	*n*	Single-tube multiplex RT-PCR	RT/ Nested PCR
DV1	30	29 (96.7)	7	6	5
DV2	30	30 (100)	7	7	6
DV3	30	30 (100)	7	7	6
DV4	30	29 (96.7)	7	7	7
DV mixed	30	27 (90.0)	-	-	-
Negative control	-	-	2	2	2

Table 3
Specificity test.

Pathogen	Number tested	Number positive	Specificity (%)
JE virus(Beijing strain)	30	1	96.7
Malaria(*Plasmodium falciparum*)	30	0	100

chances of contamination, we developed a single-tube multiplex PCR with the primer detecting NS5, a highly conserved region. This method was compared with the Lanciotti *et al* (1992) as the gold standard. We tested this method with each of the dengue virus serotypes (DV1-DV4) and cross checked it with JE virus and malaria parasite. This method detected DV1 at a concentration of 10^{-1} and DV2, 3, and 4 at concentration of 10^{-2} and a sensitivity and specificity of 96.7%.

The NS5 coding region is a target site for dengue and others viruses in the flavivirus group. Diagnosis using this highly conserved region results in a test with a high sensitivity; in Brazil, Baleotti *et al* (2003) analyzed flavivirus phylogeny based on the NS5 region, and in Ecuador, Regato *et al* (2008) isolated dengue virus with the NS5 region. Ayers *et al* (2006) developed a single-tube multiplex PCR for the flavivirus group with the potential to detect each serotype of dengue at low titers of DV1 and DV2 at $3x10^{-1}$, DV3 at $6x10^{-2}$, DV4 at 2.6. Gomes *et al* (2007) developed a single-tube nested PCR using immobilized internal primers (RT-STNPCR) for dengue serotype detection with a sensitivity of 75.9% and a specificity of 100%. Saxena *et al* (2008) also developed a rapid dengue serotype diagnosis using a one step single-tube RT-PCR (M-RTPCR) which was user friendly, rapid and cost-effective.

Our study, the one step dengue serotype diagnosis using single-tube multiplex RT-PCR was effective in reduction of time and risk of contamination. Our method was easy to interpret with good sensitivity and speci-

ficity for the detection of dengue virus serotypes 1-4. This rapid detection method for early stage of dengue infection should be useful in the field and for epidemiological investigations. We plan to use this technique to develop detection methods for other vector-borne and zoonotic diseases.

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