DETERMINATION OF DENGUE VIRUS SEROTYPES IN THAILAND USING PCR BASED METHOD

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Abstract. A reverse transcriptase-polymerase chain reaction (RT-PCR) and a single-tube multiplex PCR assay was modified for typing of dengue virus in different geographical areas of Thailand during 2000-2001. A set of primers (D1 and D2) was used to generate the RT- PCR product of 511 bp in size which subsequently underwent a single-tube multiplex PCR amplification using the highly specific primers for each of the dengue virus serotypes (D1, TS1, TS2, TS3 and DEN4). The PCR products of 482, 119, 290 and 392 bp in size were generated for dengue virus serotypes 1, 2, 3, and 4, respectively. Each set of specific primers showed no amplification of non-specific and non-target PCR products from human genomic DNA. The method was applied for investigation of 637 human blood samples in Thailand during 2000-2001 and found that 71, 43, 28, and 43 patients were classified as having a single infection with serotypes 1, 2, 3, and 4, respectively. Multiple infections with two or more dengue virus serotypes were also detected.

INTRODUCTION

Dengue viruses cause one of the most important diseases in tropical and subtropical areas of the world, especially in Southeast Asia. Dengue infection is responsible for more than 100 million cases of dengue and more than 250,000 cases of hemorrhagic fever (Halstead,1988; Pinheiro and Corber,1997). They (family Flaviviridea, genus *Flavivirus*) occur as four antigenically distinct serotypes 1, 2, 3, and 4. Infection with any of them generally leads to a mild self-limiting illness (dengue fever). A more severe form of the disease, involving vascular and hemorrhagic fever-dengue shock syndrome (DHF-DSS) is responsible for a high mortality rate, primarily among children. These viruses are transmitted between humans primarily by the *Aedes aegypti* and *Ae. albopictus* mosquitos and are endemic in most areas in which the vectors occur. At present, the diagnosis of DHF is based on a viral culture that is time-consuming and costly. The immunological diagnosis is uncertain and inappropriate for serotyping due to extensive cross-reactivity of antibodies among dengue viruses. The PCR was recently developed for the diagnosis of each serotypes of dengue virus (Mason *et al*, 1987; Deubel *et al*, 1990; Eldadah *et al*, 1991; Henchal *et al*, 1991; Harris *et al*, 1993; Yenchitsomanus *et al*, 1996). However, it needed a single PCR amplification for each serotype which was time-consuming and labor-intensive when a large number of samples were investigated. A clear need exists for an assay that can be performed rapidly and that is sufficiently sensitive and specific to be clinically and epidemiologically useful. Here, we have modified a simple, sensitive, and rapid method for the detection, identification and typing of dengue viruses by using a combination of a RT-PCR and a single-tube multiplex PCR that could be implemented for epidemiological studies and detection of the viruses in carriers.

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MATERIALS AND METHODS

Clinical samples

Two milliters of blood from patients with clinical symptoms of dengue were collected in a commercial EDTA anticoagulant tube. The blood sample was centrifuged at 3000 rpm for 5 minutes at 4ºC to separate the red blood cells from

the plasma. The plasma was transferred to a new tube and re-centrifuged at 8000 rpm for another 10 minutes at 4ºC. Most of the plasma was removed and the approximately 100-200 µl left was further stored at -20ºC until testing within 1-2 weeks and at -70ºC for a long period of storage.

RNA extraction

Virus strains were kindly provided by the Armed Forces Research Institute of Medical Science (AFRIMS) as follows: serotype 1: dengue virus (dengue-1; strain Hawaii); serotype 2: dengue virus (dengue-2; strain 16681); serotype 3: dengue virus (dengue-3; strain H-87); and serotype 4: dengue virus (dengue-4; strain 703-4). A 250 µl sample of human plasma or supernatant fluid from virus-infected cells was mixed with 750 µl TRIzol® reagent (Gibco BRL, Island, NY, USA). The solution was agitated for 30 seconds and incubated at room temperature for 30 minutes. Then, 300 µl of chloroform was added to the tube and followed by agitation for 30 seconds. The solution was centrifuged at 1200 rpm for 15 minutes. The aqueous phase was transferred to a clean tube, precipitated with 500 µl of isopropyl alcohol, incubated at -20ºC for 12 hours, and centrifuged at 12000 rpm for 15 minutes. The resulting pellet was then suspended at 75% cold ethanol, and centrifuged at 7500 rpm for 5 minutes. The pellet was air-dried at room temperature, resuspended in 25 µl of double distilled DEPC-H_2O and stored at -70ºC.

Selection and synthesis of oligonucleotide primers

Dengue viruses consensus primer D1 and D2 were designed from published sequences with a sequence analysis computer program. The criteria for designing the specific serotyping primers (TS1, TS2, TS3, and DEN4) were based on their maximum homology to four serotypes, high melting temperature and non-homology to other regions of the dengue virus genomes. All oligonucleotides were synthesized by using the Applied Biosystems Oligosynthesizer that were commercially available from Bio Service Unit, (BSU), as shown in Table 1.

First step: RT-PCR amplification of dengue virus RNA

Target virus RNA was converted to a cDNA amplification by the use of reverse transcriptase (RT) prior to PCR amplification using a pair of primers designed from the upstream dengue virus consensus primer (D1) and the downstream consensus primer (D2) homologous to the cDNA of the four serotypes. The PCR product obtained from RT-PCR amplification using D1 and D2 as primers was subsequently used as the template for PCR detection of the specific serotypes. All relevant RT-PCR conditions ($MgCl_2$, primers, RT, *Taq* polymerase, number of cycles and annealing temperature) were initially optimized by the use of quantitative purified dengue virus RNA to achieve a maximum level of sensitivity. The target RNA was amplified in 25 µl volumes containing the following components: 50 mM KCl, 10 mM Tris (pH 8.5) 2.4 mM $MgSO_4$, 0.01% gelatin, 400 µM each of the four deoxynucleotide triphosphate, 5 mM dithiothreitol, 1 µM each of primer D1 and D2, and 0.1 Units of RT-*Taq* polymerase and total of 2.5 to 5 µl of extracted RNA was as a template in a 25 µl reaction volume. The

Table 1
Oligonucleotide primers and their sequences used in RT-PCR and multiplex PCR for serotyping of dengue virus (a) and the sizes of PCR products (b).

a)

Name	Nucleotide sequence (5′ to 3′)
D1	TCAATATGCTGAAACGCGCGAGAAACCG
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC
TS1	CGTCTCAGTGATCCGGGGG
TS2	CGCCACAAGGGCCATGAACAG
TS3	TAACATCATCATGAGACAGAGC
DEN4	TGTTGTCTTAAACAAGAGAGGTC

b)

Serotypes	Primers	Size of PCR product (bp)
Fisrt step: RT-PCR		
All serptypes	D1 and D2	511
Second step: PCR		
Serotype 1	D1 and TS1	482
Serotype 2	D1 and TS2	119
Serotype 3	D1 and TS3	290
Serotype 4	D1 and DEN4	392

reactions were performed in a thermocycler (MJ research model PTC 200) and incubated for 1 hour at 42ºC followed by pre-denaturation at 94ºC for 2 minutes before proceeding to 35 cycles of denaturation (94ºC, 30 seconds), primer annealing (55ºC, 60 seconds), and extension (72ºC 120 seconds).

Second step: multiplex PCR amplification with serotype-specific primers

A PCR amplification reaction was performed for differentiation of dengue virus serotypes. The reaction was conducted after the first step reaction of RT-PCR that used D1 and D2 as primers. One µl of diluted material (1:100 ratio of first RT-PCR solution to sterile distilled water) was added to the PCR mixture that contained all the components described for the amplification reaction using D1 and the dengue virus type specific primers (TS1, TS2, TS3, and DEN4). The PCR mixtures were conducted for 35 cycles of denaturation (94ºC for 30 seconds), primer annealing (55ºC for 60 seconds), and extension (72ºC for 120 seconds) in the Thermal Cycler (MJ research model PTC 200). A 15 µl portion of the reaction product was electrophoresed on a 1.5% gel agarose in 0.5 x TBE buffer (89 mM Tris borate, 2.5 mM EDTA, pH 8.3). The size of the resulting DNA band was characterized for each dengue virus type with a 100 bp ladder as a size standard (Gibco BRL).

RESULTS AND DISCUSSION

Upon RT-PCR amplification of dengue virus RNA using D1 and D2, a 511bp PCR fragment was generated for all types of dengue virus. The 511 bp RT-PCR product was used as a cDNA template and subsequently amplified in the second step of a single tube multiplex PCR containing a set of serotype-specific primers (D1, TS1, TS2, TS3, and DEN4). The primers used in this multiplex PCR showed highly specific amplification of each type of virus cDNA without the presence of non-specific amplification to each other and non-target PCR products such as human DNA (Fig 1). The sensitivity of the primers was tested and revealed that they could detect the virus cDNA in amounts as little as 1pg-10fg (Fig 2). The data implied that the technique was suit-

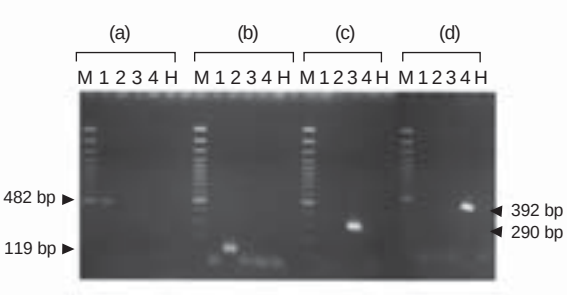

Fig 1– Specificity testing of PCR primers for typing of dengue viruses. Four sets of specific primers were (a) D1 and TS1, (b) D1 and TS2, (c) D1 and TS3, and (d) D1 and DEN4. Lanes 1-4 represent dengue virus serotypes 1, 2, 3 and 4, respectively. Lane M represents the 100 bp ladder marker. Lane H represents human DNA.

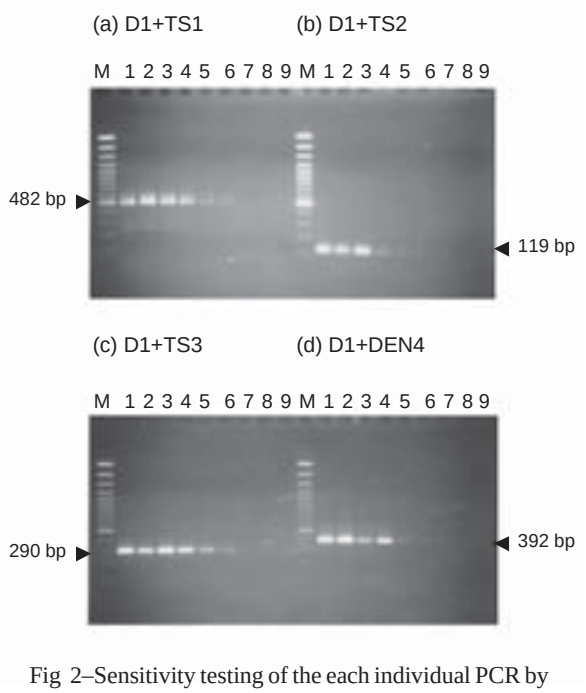

Fig 2–Sensitivity testing of the each individual PCR by using each pair of primers. Four sets of specific primers were (a) D1 and TS1, (b) D1 and TS2, (c) D1 and TS3, and (d) D1 and DEN4. Single PCR was manipulated using four serotypes of dengue virus cDNA at concentration of 200 µg (lane 1), 100 µg (lane 2), 1 µg (lane 3), 10 ng (lane 4) 0.1 ng (lane 5), 1 pg (lane 6), 10 fg (lane 7), 1 fg (lane 8) and 0.1 fg (lane 9). The PCR condition using for each reaction was based on the optimized condition for those of multiplex PCR as described in Materials and Methods. Lane M represents the molecular weight size markers containing the fragments of 100-1,500 bp.

Table 2
Showing the number of investigated patients from different geographical area in Thailand during year 2000-2001 using RT-PCR and a single tube multiplex PCR technique.

Area	Number of samples	Serotypes					Mix serotypes (Number)
		1	2	3	4	Neg	
Central Thailand							
Bangkok	152	64	-	-	-	88	-
Samut Prakan	38	-	1	-	1	36	-
Nakhon Sawan	24	2	2	7	6	7	-
Pathum Thani	38	-	7	-	3	28	-
Ratchaburi	36	-	3	5	8	13	Types 2+3 (1) Types 2+4 (1) Types 3+4 (4) Types 2+3+4 (1)
Nakhon Pathom	43	3	3	4	2	30	Types 2+3 (1)
Samut Songkhram	13	-	-	2	6	5	-
Northeast Thailand							
Ubon Ratchathani	80	-	4	1	4	70	Types 3+4 (1)
Udon Thani	11	-	-	-	1	4	Types 3+4 (6)
North Thailand							
Chiang Rai	30	-	3	-	5	15	Types 3+4 (7)
Phitsanulok	48	-	9	1	-	38	-
South Thailand							
Ranong	39	-	5	-	1	32	Types 3+4 (1)
Nakhon Si Thammarat	74	2	6	7	4	52	Types 1+2 (1) Types 1+3 (1) Types 1+2+4 (1)
Yala	11	-	-	1	2	2	Types 3+4 (6)
Total	637	71	43	28	43	420	32

able for early detection of the virus, as in the case of carriers. The RT-PCR and a single-tube multiplex PCR were used to analyze the plasma specimens obtained from 637 patients from different geographical areas in Thailand. Since the virus was unstable at the room temperature, the plasma was stored at -20ºC or lower. We found that the assay of the virus in plasma samples, either immediately or a few days after collection, was suitable. It was necessary to store plasma samples for longer periods of time for the re-PCR amplification in the second step or storage. This was done at -80ºC with liquid nitrogen.

Results shown in Table 2, revealed that dengue virus serotype 1 was abundant in Bangkok metropolitan whereas serotypes 2, 3, and 4 were dominant in the suburban areas. The percentage of single infection was approximately 29% (185 out of 637).

DISCUSSION

The data from the present study support the previous reports that all four types of dengue viruses were circulated in both the urban and suburban areas of Thailand (Nisalak *et al*, 1988; Nimmannitya *et al*, 1997).

Multiple infections were detected in many areas with a percentage value of 5.0% (32 out of 637). Among the multiple infections, the concomitance of serotypes 3 and 4 (25 out of 32) were found in Chiang Rai (7/32), Yala (6/32), UdonThani (6/32), Ratchaburi (4/32), Ubon Ratchathani (1/32), and Ranong (1/32). The re-

sults indicated the simultaneous endemicity of multiple serotypes occurred and led to hyperendemicity. Multiple serotype infections with dengue viruses may have resulted from the multiple feedings of female mosquitos before completing a gonotrophic cycle (Gould *et al*, 1970; Yasuno and Tonn, 1970). The classic experiment by Siler and colleaques (1926) demonstrated that female mosquitos, once infected, remained infective throughout the adult stage even after repeated bites of humans. Another possibility of multiple serotype infections with dengue viruses is related to the movement and/or migration of infected people. For example, migration of susceptible laborers from the northeast to the south in Thailand was strongly suspected to be the cause of the increased dengue incidence in the southern provinces (Sucharit *et al*, 1993).

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