Rapid detection and serotyping of dengue virus by multiplex RT-PCR and real-time SYBR green RT-PCR

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ABSTRACT

Introduction: Dengue fever and dengue haemorrhagic fever currently rank highly among the newly-emerging infectious diseases, and are considered to be the most important arboviral disease worldwide. The definitive diagnosis is culture analysis, but practical considerations limit its use. Also, the period for viral detection is limited. Within a day or two after fever subsides, rising levels of antibodies interfere with viral cultures. An alternative to this quandary is the use of viral RNA detection assays. In our laboratory, a reverse transcriptase polymerase chain reaction (RT-PCR) assay was developed using a set of degenerate primers.

Methods: This multiplex RT-PCR assay was evaluated with 280 samples collected during the year 2003. These groups include prototype dengue virus (serotypes 1-4), acute serum from which the dengue virus was isolated, seronegative acute samples (culture negative) but whose convalescent samples seroconverted, and sera positive for other microbial diseases. This assay was then modified into a real-time SYBR Green RT-PCR assay. Sensitivity and specificity of both assays were compared.

Results: The multiplex RT-PCR assay was able to detect 134 samples whereas SYBR Green RT-PCR assay was able to detect 178 out of 306 samples. Both assays were 100 percent specific. Further analysis of 53 samples showed that the virus could be amplified at IgM positive/negative values of up to 4.2, and up to six days after onset of fever. The viral detection rate was inversely proportional to the day of fever onset as well as IgM values.

Conclusion: The sensitivity and specificity of the conventional multiplex RT-PCR assay are 98.18 percent and 100 percent, respectively, and for the real-time SYBR Green assay, 99.09 percent and 100 percent, respectively. The melting curve analysis allows all four dengue serotypes to be discriminated based on distinct melting temperature value. The accuracy and speed of this multiplex RT-PCR assay makes it a suitable test for the diagnosis of dengue and for epidemiological surveillance.

Keywords: conventional multiplex RT-PCR, dengue virus, melt curve analysis, real-time SYBR Green RT-PCR

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INTRODUCTION

Dengue virus is a mosquito-borne flavivirus and the most prevalent arbovirus in tropical and subtropical regions of Asia, Africa, and Central and South America.[1] The dengue virus belongs to the family of *Flaviviridae*, and it consists of four closely-related but antigenically distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4). All four serotypes of the dengue virus can be distinguished by both serological and molecular methods.

The dengue virus is a single-stranded ribonucleic acid (RNA) positive-strand virus. The total genome size of the dengue virus is approximately 11kb in length[2] and the entire genome for these four serotypes of dengue virus has been sequenced. The genome is composed of three structural protein genes, encoding the nucleocapsid or core protein (C), a membrane-associated protein (M), an envelope protein (E), and seven nonstructural (NS) protein genes.[3] Both ends of the open reading frame are flanked by a untranslated region (UTR). The genome organisation is 5'UTR-C-prM (M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'UTR.

Dengue fever (DF)/ dengue haemorrhagic fever (DHF) is a major public health problem in tropical and subtropical countries.[4,5] It has been estimated that approximately 100 million dengue cases occur annually worldwide.[5–7] Infection with dengue virus will produce a wide spectrum of clinical manifestations ranging from completely asymptomatic, or non-specific febrile illness,

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to DF and the severe, and potentially fatal, DHF/dengue shock syndrome.[5,8] Important risk factors for DHF include the strain and serotype of the infecting virus, as well as the age, immune status, and genetic predisposition of the patient.[4]

The current gold standard for dengue virus detection and identification involves viral isolation in cell cultures or *Toxorhynchites* species mosquito larvae, followed by direct detection using immunofluorescence (IF). However, this requires cell culture facilities or mosquito colonies, which are difficult to maintain in laboratories.[9] Viral isolation usually takes longer than seven days to complete. Two different methods have been demonstrated to be useful in dengue diagnostics, and this includes serological diagnosis of antibodies based on capture immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA), and genomic RNA detection by molecular technique such as reverse transcriptase polymerase chain reaction (RT-PCR).[10,11] Recently, we had developed a multiplex RT-PCR assay as a rapid detection system for dengue diagnostics, and later adapted it to a real-time RT-PCR system by using SYBR Green I dye.

METHODS

A total of 280 samples collected in the year 2003 were used to evaluate these primers (Table I). These samples include: (a) acute serum from which the dengue virus was isolated (n = 95). The isolated virus was obtained by inoculating C636 *Aedes albopictus* cell lines with serum samples and then serotyped using serotype specific monoclonal antibodies (gift from Division of Vector-borne Diseases, Centers for Disease Prevention and Control, USA); (b) seronegative acute (culture negative) samples, where the convalescent samples seroconverted (n = 15); (c) dengue specific IgM positive sera (n = 100); and (d) a panel of negative controls. The negative controls used in this study consisted of serum samples positive for other disease agents (n = 70), including herpes simplex virus (n = 6), cytomegalovirus (n = 6), varicella-zoster virus (n = 6), measles (n = 6), rubella (n = 6), mumps (n = 6), Widal-Weil Felix positives (n = 6), Japanese encephalitis virus (n = 15), West Nile virus (n = 4), *Salmonella typhi* (n = 2), *Legionella* spp. (n = 1), *Leptospira* spp. (n = 2), *Chlamydia* spp. (n = 2), and *Mycoplasma* spp. (n = 2). 140 µL of every sample was subjected to RNA extraction using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored in -70°C until use.

The following four serotypes of dengue virus nucleotide sequence were retrieved from GenBank (accession no.): DEN 1 isolate BR/01-MR (AF513110), DEN 2 strain BR64022 (AF489932), DEN 3 isolate D3/H/IMTSSA-SR1/2000/1266 (AY099336), and DEN 4 strain 814669 (AF326573). Nucleotides were aligned using ClustalX 1.83 for conserved region identification. A forward conserved primer was designed against 5' UTR region and 4 reverse primers were designed to target specific areas of the M and C proteins of the respective dengue virus 1, 2, 3, and 4. All primers were designed using similar parameters so that they would have similar melting temperatures (Tm), and GC content (GC %), thus allowing all of them to work synchronously under identical conditions. All the primers were then blasted through the National Centre for Biotechnology Information database of dengue and related flaviviruses to ensure their specificity. The sequences of the primers are shown in Table II.

The assay was optimised against viral RNA extracted

Table I. Samples used in the evaluation of the multiplex and real-time SYBR Green RT-PCR.

Category	DEN 1	DEN 2	DEN 3	DEN 4	Total
(a) Acute serum from which dengue virus was isolated	25	25	25	20	95
(b) Seronegative culture negative acute samples but convalescent samples seroconverted	–	–	–	–	15
(c) IgM positive sera	–	–	–	–	100
(d) Other disease agents (negative control)	–	–	–	–	70

Table II. Primer sequence, position in the dengue genome and size of RT-PCR product and generated combination of primers.

Virus serotype	Primer	Primer sequence	Primer position	Size of amplicon & primer combination
	Dcon	5'– AGT TGTTAGTCTACGTGGACCGACA	1–25	
DEN 1	D1	5'– CCCCGTAACACTTTGATCGCTCCATT	317–342	342 bp (Dcon and D1)
DEN 2	D2	5'– CGCCACAAGGGCCATGAACAG	231–251	251 bp (Dcon and D2)
DEN 3	D3	5'– GCACATGTTGATTCCAGAGGCTGTC	514–538	538 bp (Dcon and D3)
DEN 4	D4	5'– GTTTCCAATCCCATTCCTGAATGTGGTGT	726–754	754 bp (Dcon and D4)

Dcon: dengue conserved region.

from virus stocks provided by the University Malaya Medical Centre virology unit. The four strains of dengue viruses that were used as positive control were DENV-1 (Hawaii), DENV-2 (New Guinea C), DENV-3 (H87) and DENV-4 (H241). The viral stock was propagated in C6/36 cells, and the viral RNA was extracted from 140 µL of the cell culture supernatants and eluted in 60 µL diethyl pyrocarbonate (DEPC)-treated water.

The extracted samples were then subjected to a one-step multiplex RT-PCR by using AccuPower RT-PCR PreMix (Bioneer, Seoul, Korea). This premix contains optimal concentrations of all the components necessary for cDNA synthesis and amplification in a single 0.2 ml tube. Each premix tube contains a stabiliser and is preserved in a lyophilised form. Thus the premix can be used easily by simply distributing the 15 µL of diluted primer mix into each tube, followed by adding the 5 µL of RNA template (approximately 1.0 µg). The primer mix was prepared by mixing 400 nM of forward conserved primer (Dcon) and 200 nM of each reversed primer (D1, D2, D3 and D4) with appropriate volume of DEPC-treated distilled water. The lyophilised pellet in the tube was dissolved by vortexing, followed by a brief spin. The thermal cycling profile of this assay consists of a 30-min RT step which is performed at 50°C, 15 min of *Taq* polymerase activation at 95°C, followed by 40 cycles of PCR at 95°C denaturation for 30 s, 60°C of annealing for 30 s and 72°C extension for 1 min. RT-PCR was performed in a Mastercycler gradient machine (Eppendorf, Hamburg, Germany) and it takes about 2½ hours.

The PCR results were then analysed by gel electrophoresis. 5 µL of the 20 µL PCR product were loaded into a 1.5% (W/V) agarose gel (Cambrex, Rockland, ME, USA) in 0.5× Tris-Borate-EDTA buffer with a 100-bp ladder as molecular weight marker. The expected size of the amplicons were 342 bp (D1), 251 bp (D2), 538 bp (D3) and 754 bp (D4) (Fig. 1).

The same set of samples were also subjected to the one-step SYBR Green I real-time RT-PCR. This assay was performed in a iCycler Real Time PCR machine (BioRad, Hercules, California, USA) using a one-step QuantiTect SYBR Green kit (Qiagen, Hilden, Germany). After optimisation, the RNA from 357 pre-collected sera were assayed in a 25 µL reaction containing 5 µL of the sample RNA, optimal concentration of primer (75 nM each), and 3 mM of $MgCl_2$ as final concentration. The thermal cycling profile of this assay consisted of a 30-min RT step which was performed at 50°C, 15 min of *Taq* polymerase activation at 95°C, followed by 45 cycles of PCR at 95°C denaturation for 30 s, 60°C of annealing for 30 s and 72°C extension for 1 min (Fig. 2).

Following amplification, the melting curves were analysed. This is to verify the specificity of the PCR product by looking at its Tm. Melting curve analysis consisted of a denaturation step at 95°C for 1 min, lowered to 55°C for 30 s, and followed by 80 cycles of incubation in which the temperature is increased to 95°C at a rate of 0.5°C/10 s/cycle. The Tm of each specific PCR product was analysed using iCycler™ iQ optical system software, version 3.0a (BioRad, Hercules, California, USA). The Tm for each sample was used to identify the dengue serotype and the samples sharing the same Tm were interpreted as belonging to the same serotype. The results of the one-step SYBR Green RT-PCR assay were also compared to the results obtained by conventional multiplex RT-PCR assay.

The limit of sensitivity of the assay was carried out with known quantitative RNA standards prepared using the method of Yong et al.[21] Briefly in-vitro transcribed dengue RNA was serially diluted ten-fold and RT-PCR was performed as described above.

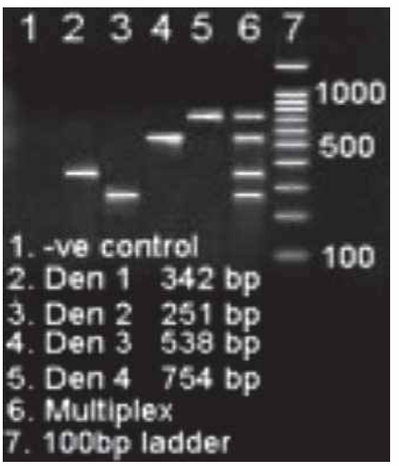

Fig. 1 Gel electrophoresis analysis of the products from RT-PCR. The RT-PCR product shown in lane 6 was generated from a mixture of all four serotypes of dengue viral RNA in equal amounts, which served as a positive control to the assay.

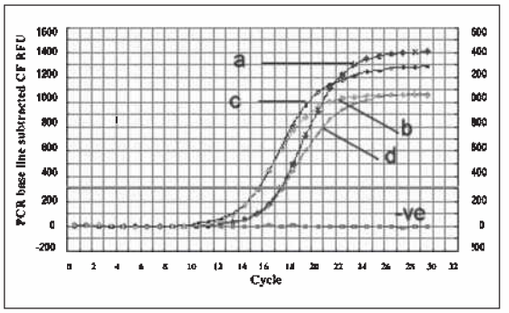

Fig. 2 Amplification curve of dengue virus one-step real-time RT-PCR. a: DEN 1; b: DEN 2; c: DEN 3; d: DEN 4. Figure shows that all positive controls can be amplified between Ct value 16 to 18.

Table III. Number of positive samples detected by multiplex RT-PCR compared to real-time SYBR Green RT-PCR.

Category	Multiplex RT-PCR					Real-time SYBR Green RT-PCR					Total samples
	DEN 1	DEN 2	DEN 3	DEN 4	Total +ve	DEN 1	DEN 2	DEN 3	DEN 4	Total +ve	
Acute serum from which dengue virus was isolated	25	25	25	20	95	25	25	25	20	95	95
Seronegative culture, negative acute convalescent seroconverted samples	10	0	2	1	13	11	0	2	1	14	15
IgM positive sera	14	5	4	3	26	43	8	9	9	69	100
Negative control	0	0	0	0	0	0	0	0	0	0	0
Total					134					178	280

Table IV. Calculation of clinical sensitivity and specificity of the assays.

	Multiplex RT-PCR		Real time SYBR Green RT-PCR	
	True positive	True positive	True negative	True negative
Positive test	108	109	0	0
Negative test	2	1	70	70
Total	110	110	70	70
Sensitivity (%)	98.18		99.09	
Specificity (%)	100		100	

RESULTS

The performance of both conventional multiplex RT-PCR and real-time SYBR Green RT-PCR assays were evaluated using 280 pre-collected samples, consisting of 210 samples which were positive for dengue infection (Table I), and 70 samples that were negative controls. The results and the comparison of detection rates between both assays are summarised in Table III. Generally, out of 210 samples, the multiplex RT-PCR assay was able to detect 134 samples, whereas SYBR Green RT-PCR assay was able to detect 178 samples. Real-time SYBR Green RT-PCR assay was seen to be more sensitive than the conventional multiplex RT-PCR. The clinical sensitivity was assessed with samples from groups (a) and (b), and are shown in Table IV. Of 110 samples tested, the RT-PCR assay detected 108, while the SYBR Green RT-PCR assay detected 109 samples (Table III). The specificity of the assay was evaluated using 70 negative control samples, and the assay showed that none of them were detected by the assay, leading to a conclusion that the both conventional multiplex RT-PCR and SYBR Green RT-PCR assays were 100% specific. The calculations for the clinical sensitivity and specificity of the assays are shown in Table IV. The two assays were then compared, with regard to their sensitivity, using 100 dengue IgM positive samples. It was observed that the RT-PCR assay amplified dengue viral RNA from 26 samples, while the SYBR Green RT-PCR assay detected dengue viral RNA in 69 samples (Table III).

The detection limit of the SYBR Green RT-PCR assay

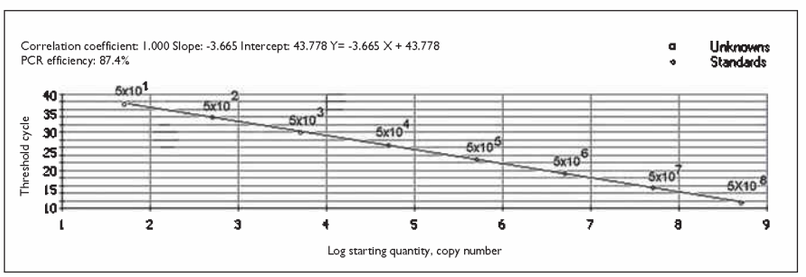

Fig. 3 Standard curve generated with in-vitro transcribed dengue virus RNA. A total of 3 ×10^8 molecule/μL RNA standard were ten-fold serial diluted and 5 μL of the diluted RNA were added to the reaction tube. The standard curve shows correlation of coefficient of 9.98 and detection limit of 50 molecule/μL.

Table V. Comparison of the day of onset versus IgM value and PCR of 53 selected patients.

Category	Day of fever onset	IgM P/N* ratio	Result of PCR		
			Multiplex	SYBR Green	Tm
DEN1 isolate (serum)	1	0.723	DEN1	DEN1	82.50
DEN1 isolate (serum)	1	0.522	DEN1	DEN1	82.50
DEN1 isolate (serum)	2	1.460	DEN1	DEN1	82.50
DEN1 isolate (serum)	2	1.460	DEN1	DEN1	82.50
DEN1 isolate (serum)	2	0.884	DEN1	DEN1	82.50
DEN2 isolate (serum)	3	1.259	DEN2	DEN2	81.50
DEN2 isolate (serum)	1	0.789	DEN2	DEN2	81.50
DEN2 isolate (serum)	2	1.452	DEN2	DEN2	81.50
DEN3 isolate (serum)	3	0.858	DEN3	DEN3	82.80
DEN3 isolate (serum)	2	0.817	DEN3	DEN3	82.80
DEN3 isolate (serum)	2	0.556	DEN3	DEN3	82.80
DEN3 isolate (serum)	1	1.094	DEN3	DEN3	82.80
DEN3 isolate (serum)	3	0.685	DEN3	DEN3	82.80
DEN4 isolate (serum)	2	1.138	DEN4	DEN4	83.50
DEN4 isolate (serum)	1	0.976	DEN4	DEN4	83.50
DEN4 isolate (serum)	1	0.908	DEN4	DEN4	83.50
DEN4 isolate (serum)	3	1.342	DEN4	DEN4	83.50
Seronegative acute samples	4	1.276	DEN1	DEN1	82.50
Seronegative acute samples	3	1.505	DEN3	DEN3	82.80
Seronegative acute samples	2	0.552	DEN3	DEN3	82.80
Seronegative acute samples	1	1.276	DEN1	DEN1	82.50
IgM +ve, culture not done	3	2.207	–	DEN3	82.80
IgM +ve, culture not done	4	4.832	–	–	76.00
IgM +ve, culture not done	5	2.147	–	DEN1	82.50
IgM +ve, culture not done	4	4.382	–	DEN1	82.50
IgM +ve, culture not done	7	5.316	–	–	76.00
IgM +ve, culture not done	5	5.552	–	–	76.00
IgM +ve, culture not done	> 7	6.301	–	–	76.00
IgM +ve, culture not done	2	1.533	DEN4	DEN4	83.50
IgM +ve, culture not done	6	6.475	–	–	76.00
IgM +ve, culture not done	4	2.208	DEN1	DEN1	82.50
IgM +ve, culture not done	5	2.191	–	DEN2	81.50
IgM +ve, culture not done	8	6.372	–	–	76.00
IgM +ve, culture not done	4	4.320	–	–	76.00
IgM +ve, culture not done	6	2.088	–	DEN1	82.50
IgM +ve, culture not done	6	5.930	–	–	76.00
IgM +ve, culture not done	5	5.568	–	–	76.00
IgM +ve, culture not done	4	4.230	–	–	76.00
IgM +ve, culture not done	6	4.371	DEN2	DEN2	81.50
IgM +ve, culture not done	6	5.113	–	–	75.50
IgM +ve, culture not done	4	4.299	–	DEN1	82.50
IgM +ve, culture not done	5	4.091	–	DEN1	82.60
IgM +ve, culture not done	3	1.989	–	DEN1	82.50
IgM +ve, culture not done	6	6.052	–	–	76.00
IgM +ve, culture not done	4	4.543	–	DEN1	82.50
IgM +ve, culture not done	5	3.491	DEN1	DEN1	82.50
IgM +ve, culture not done	5	5.689	–	–	76.00
IgM +ve, culture not done	5	5.661	DEN3	DEN3	82.80
IgM +ve, culture not done	5	5.614	–	–	75.50
IgM +ve, culture not done	6	5.272	–	–	76.00
IgM +ve, culture not done	6	5.310	–	–	75.00
IgM +ve, culture not done	8	7.355	–	–	76.00
IgM +ve, culture not done	7	5.895	–	–	75.00

* P/N: positive/negative ratio

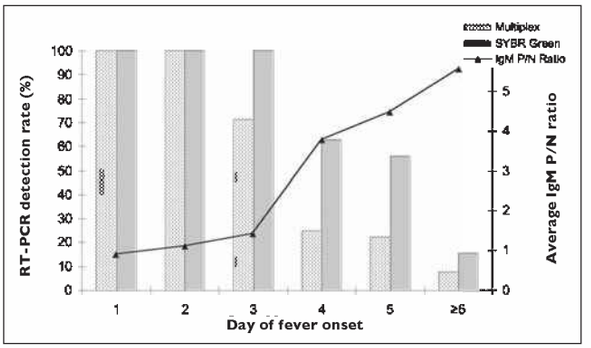

Fig. 4 Comparison between viral detection rate (%), day of fever onset and IgM P/N ratio. The percentage of viral detection was inversely proportional to the IgM titre. Generally, real-time SYBR Green RT-PCR assay has a higher detection rate over the conventional multiplex RT-PCR.

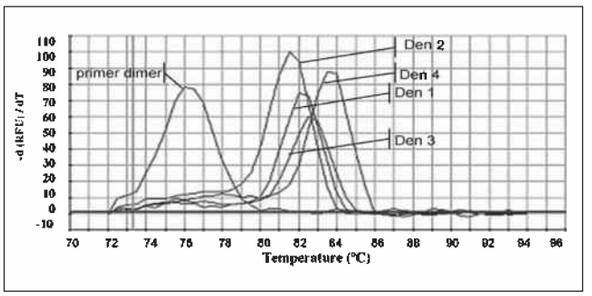

Fig. 5 Melting curves for dengue virus serotypes 1–4 based on SYBR Green RT-PCR. The mean of the melting peak of DEN 1 = 82.5°C, DEN 2 = 81.5°C, DEN 3 = 82.8°C and DEN 4= 83.5°C. The melting peak for primer dimers is at 75–77°C.

was tested by using a fixed amount of in-vitro dengue virus RNA transcripts. Using ten-fold serial dilutions of a RNA stock of 3×10^8 molecule/μL, a standard curve was plotted to show the detection limit to be 50 molecule/μL (Fig. 3). 53 out of 210 samples were selected for further analysis. These samples were selected based on the availability of data of anti-dengue IgM and onset of fever, and each patient's details are shown in Table V. IgM was detected by an in-house capture ELISA, in which the base cut-off values of positive/negative (P/N) ratio ≥ 2 was indicative of the presence of a current or recent infection. As can be seen in Table V, the dengue virus can be amplified at IgM P/N values of up to 4.2. Also IgM is detected up to six days after onset of fever. The viral detection rate was inversely proportional to the day of fever onset as well as IgM values (Fig 4).

In order to determine the value of the Tm of each serotype of dengue virus, the mean and the standard deviation of Tm were calculated for each serotype. This melting curve analysis showed that the mean Tm of DEN 1 was 82.5°C, DEN 2 was 81.5°C, DEN 3 was 82.8°C and DEN 4 was 83.5°C. As there was no amplification in the PCR reaction, the five primers in the negative control tubes tended to form primer dimers and gave Tm values at much lower temperatures, 75–77°C (Fig. 5).

DISCUSSION

The gold standard of any viral disease is the detection and/or isolation of the causative organism. The most sensitive dengue virus isolation method is the in-vivo amplification through mosquito inoculation, or mosquito-derived cell cultures, such as C6/36 cell line.[12] Serotyping of dengue virus is done by using IF staining of the infected cell with serotype-specific monoclonal antibodies.[13,14] However, viral isolation is tedious, labour intensive, and requires cell culture facilities or mosquito colonies. These are difficult to maintain in a diagnostic laboratory setting.[9] Viral isolation is time consuming, usually taking more than seven days to complete. Furthermore, when antibody levels begin to rise from Day 3 after onset of symptoms, it interferes with viral isolation, resulting in the low sensitivity of viral isolation. Therefore, serological tests provide a better diagnosis in dengue disease, as it requires less time to be completed. Both anti-dengue IgM and IgG antibodies are often found from the sera of the patients with acute infection. However, IgM levels could be low or sometimes not detected in secondary infections.[15] Also, it is rather difficult to identify the serotype of the infecting virus serologically, particularly in secondary infections, due to the "original antigenic sin" phenomenon.[6,16] At the same time, cross reaction between dengue antibodies and other flaviviral antigens may also occur, and this complicates the interpretation of a positive finding, if other flavivirus infections cannot be excluded.[17] As serological tests for IgM antibodies are only useful one week after the onset of illness, early detection cannot be done with these tests. Thus, antibody detection has less impact on patient management and disease control measures exercised by medical and public health authorities.

In contrast, identification of dengue virus by molecular methods, such as RT-PCR, is highly indicative for the acute infection of dengue virus in human serum or plasma.[18-20] PCR-based techniques are rapid, sensitive, specific and cost-effective. Several laboratories have published various RT-PCR protocols for dengue virus detection. Among these, the two-step nested RT-PCR protocol, originally reported by Lanciotti et al,[20] and later modified by Harris et al into a one-step multiplex RT-PCR for the purpose of detection and serotyping are well known.[9,16] This assay has the advantage of detecting and differentiating four dengue serotypes by determining the sizes of the amplicons using agarose gel electrophoresis.

In this study, we have developed a multiplex RT-PCR that can be used to facilitate dengue virus diagnosis. The assay reported here is adapted from that previous reports,[9,16,20] but with slight modifications in order to reduce primer-dimer formation and non-specific binding

problems. In addition, the use of AccuPower RT-PCR PreMix had greatly facilitated the routine diagnosis work. The premix consists of a strip of eight tubes with all components required for cDNA synthesis and PCR, such as M-MLV RT, ribonuclease (RNase) inhibitor, thermostable DNA polymerase, and deoxyribonucleotide triphosphates. The freeze-dried format preserves the viability of the enzymes for long periods, even beyond typical storage limits. Thus the RNA template, primer pairs (forward and reverse), and deoxyribonuclease and RNase free water, are all that is needed from the user. Since every single tube contains all the essential components for the RT-PCR premix, fewer steps are required in the protocol. This will help to reduce not only potential errors, but also time needed to prepare the assays as well as risk of cross contamination.

When the assay was adapted to a real-time RT-PCR assay by using SYBR Green 1, it increased the sensitivity of the assay. Generally, dengue viral RNA detection rate by RT-PCR is inversely proportional to the day of fever onset as the result of rise of the antibody titre. However, real-time SYBR Green RT-PCR appears to have a higher detection rate even in the presence of anti-dengue antibodies, as shown in Fig 3. Thus, this assay may be a useful diagnostic test in the early stages of DF and DHF, even until the appearance of anti-dengue IgM antibodies. We are currently assessing this assay for the detection of the virus in relation to primary and secondary infections, and the presence of IgG antibodies. In conclusion, the clinical sensitivity and specificity of the conventional multiplex RT-PCR assay are 98% and 100%, respectively, and for the real-time SYBR Green assay, 99% and 100%, respectively. The melting curve analysis results demonstrate that all four dengue serotype could be discriminated based on a distinct Tm value. The accuracy and speed of this multiplex RT-PCR assay makes it a suitable test for the diagnosis of dengue and for epidemiological surveillance. In this study, the whole process starting from RNA extraction, RT-PCR, to completing with agarose gel electrophoresis can be done within three hours. In our diagnostic laboratory, this assay has been used either as a complement to the existing technique or as a replacement.

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