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Cost-Effective Real-Time Reverse Transcriptase PCR (RT-PCR) To Screen for Dengue Virus followed by Rapid Single-Tube Multiplex RT-PCR for Serotyping of the Virus▽

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Virus detection methodology provides detection of dengue virus in the early phase of the disease. PCR, targeting cDNA derived from viral RNA, has been used as a laboratory-based molecular tool for the detection of *Dengue virus*. We report the development and use of three real-time one-step reverse transcriptase PCR (RT-PCR) assays to detect dengue cases and serotype the virus involved. The first RT-PCR assay uses SYBR green I as the reporting dye for the purpose of cost-effective screening for dengue virus. The detection limit of the SYBR green I assay was 10 PFU/ml (0.01 equivalent PFU per assay) for all four dengue virus serotypes. The second RT-PCR assay is a duplex fluorogenic probe-based real-time RT-PCR for serotyping clinical samples for dengue viruses. The detection threshold of the probe-based RT-PCR format was 0.1 PFU for serotypes Dengue-1 and Dengue-2, 1 PFU for serotype Dengue-3, and 0.01 PFU for serotype Dengue-4. The third is a fourplex assay that detects any of the four serotypes in a single closed tube with comparable sensitivity. Validation of the assays with local clinical samples collected from 2004 to 2006 revealed that there was an 88% positive correlation between virus isolation and RT-PCR with regard to dengue virus detection and a 100% correlation with seroconversion in subsequent samples. The serotyping results derived from duplex and fourplex assays agree fully with each other and with that derived from immunofluorescence assays.

Dengue is a mosquito-borne disease that is prevalent in tropical and subtropical regions. *Dengue virus* is a positive-strand RNA virus that belongs to the *flavivirus* genus in the *Flaviviridae* family. Dengue virus is transmitted principally by *Aedes aegypti*, and its secondary vector is *Aedes albopictus*, both of which can be found in many urban and suburban areas (6). Dengue has four distinct serotypes (Dengue-1, -2, -3, and -4) that can be differentiated by molecular methods.

Dengue fever (DF) and Dengue hemorrhagic fever (DHF) continue to be important global public health problems (4, 9, 12, 16). In 2004, the World Health Organization reported 414,785 cases worldwide. Of these, 182,004 cases were from South-East Asia and Western Pacific regions. These regions were deemed the most seriously affected by dengue (24, 25). Singapore, located in the midst of a dengue hyperendemic region, has been at the forefront of dengue control campaigns since the 1960s when DF was first reported (5). In 2004 9,459 cases (224.2 per 100,000 population) were reported in Singapore, and in 2005 the number of dengue cases increased markedly to 14,210 cases reported.

DF presents with undifferentiated clinical symptoms, and thus it is important to have access to rapid and reliable laboratory-based detection methods specifically designed to enable the detection of dengue antigen or antibodies (10, 14). Virus isolation is often regarded as the "gold standard" in dengue diagnostics. However, the 7- to 10-day period required for the dengue viruses to grow on cell lines and this method's lack of sensitivity have taken the luster off this approach.

When virus detection fails, clinicians usually resort to serology tests for the detection of specific antibodies to dengue virus. Various serological detection methods have been described (1, 17, 19, 22). The presence of anti-dengue immunoglobulin M (IgM) would be indicative of a recent dengue virus infection. However, IgM can only be detected around 5 days from the onset of illness, rendering earlier diagnosis impossible. Moreover, anti-dengue IgM could last for several months. Unless consecutive samples were tested, this test could lead to false positives due to a recent subclinical dengue virus infection prior to the current illness under investigation. Cross-reactivity of anti-dengue IgM with other flaviviruses is also a well-known problem. A rapid detection of viral material provides a direct indication of the pathogen involved. In dengue, the 5- to 8-day viremic period experienced by a dengue patient offers a good window of opportunity for diagnosis via PCR. Early accurate diagnosis not only aids in the administration of proper clinical treatments but is also important for disease surveillance, epidemiological investigation, and implementation of a dengue control response. PCR has the added advantage of being able to provide information on circulating serotype, which is essential for virus surveillance.

Shu et al. (18) reported oa rapid and simple real-time reverse transcriptase PCR (RT-PCR) using SYBR green I as the reporter dye for the detection of dengue RNA. However, the need for a pair of generic primers and four pairs of serotype-specific primers limited the number of samples that could be run simultaneously for the determination of serotypes. Five

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positions in the PCR thermal cycler would have to be allocated to each sample.

Other researchers have used fluorogenic probes to differentiate the four serotypes in real-time assays (3, 11, 13, 15, 23). Although some assays required major manipulation of the sample since each of the four serotypes required an individual assay for serotyping, Johnson's assay involved a sensitive single reaction with multiple primer sets that directly performed serotyping without prior screening. The former approach will drive up the costs of reagents and limit the number of samples to be tested in a single PCR equipment. The latter method is suited for serotype surveillance but may not be cost-effective for routine diagnosis since only a low percentage of the samples are usually positive for dengue.

We report the development of three different assays and a cost-effective approach for diagnosis and virus surveillance. This method was effectively used during the dengue outbreak in Singapore in 2005. A cost-effective RT-PCR assay based on SYBR green I has been developed for general screening of samples from suspected cases. This pan-dengue system only reports the quantitative status of the sample as either positive or negative for dengue virus. By augmenting the same pairs of primers with serotype-specific fluorescent probes, we were able to perform duplex assays or fourplex assays to directly determine the serotype of the virus in clinical samples. The choice of duplex or fourplex is dependent on the PCR equipment used (i.e., the availability of filters and lasers). Although the former test offers cost-effective and quick screening, particularly important for dengue surveillance in regions where the disease is endemic, the latter duplex and fourplex assays will reduce the serotyping cost per sample and minimize the manipulation of both reagents and samples. In addition, more samples can be processed in one PCR run than in a four-tube assay for each sample, leading to higher throughput. These assays allowed rapid screening and serotyping of dengue viruses in clinical samples.

The RT-PCR (probe) results were compared to virus isolation and IFA for its sensitivity and specificity.

MATERIALS AND METHODS

Clinical samples. Two sets of clinical serum samples collected from 2004 to 2006 were used in the present study. Set A of plain blood was collected through a research project and consisted of three consecutive collections from each suspected dengue patient. The first collection was within 72 h of fever onset, the second collection took place 3 days after first collection, and third collection occurred 21 days after fever onset. Collected from a total of 110 suspected dengue patients, the samples were characterized and used to validate the RT-PCR against seroconversion. Comparison with virus isolation and immunofluorescence assay (IFA) were also made. Set B was single collection of plain blood from 149 suspected dengue cases. This set of samples was sent from hospitals and primary health care clinics and was used for validation of the RT-PCR against virus isolation accompanied by IFA and for assessing the performance of the newly developed RT-PCR in a diagnostic setting.

RNA extraction. All viral RNAs were extracted from the first serum portion or virus culture supernatant by using a QIAGEN QIAamp viral RNA minikit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

Design of oligonucleotide primers and FRET probes. The generic pan-dengue primers used, which targeted the 3′ noncoding region of dengue viruses, were pan-dengue forward (5′-TTGAGTAAACYRTGCTGCCTGTAGCTC-3′) and pan-dengue reverse (5′-GAGACAGCAGGATCTCTGGTCTYTC-3′). The forward primer was designed by G. J. Chang (Division of Vector-Borne Diseases, NCID, Fort Collins, CO) and L. J. Chien (Center for Disease Control of Taiwan) and was shared during the Asia Pacific Economic Congress workshop on the molecular epidemiology of dengue viruses held in Taipei in December 2002. The reverse primer was designed to be compatible with the four pairs of fluorescence resonance energy transfer (FRET) probes that would be positioned internally in the amplified fragments. The sequences of the four pairs of probes used in this assay are provided in Table 1. Figure 1 shows the binding sites of the primers and probes used.

TABLE 1. Sequences of FRET probes and primers used in the dengue virus real-time RT-PCR assays

Probe or primer	Sequence (5′–3′)
Probes	
Den1-FL	CAGGATACAGCTTCCCCTGGTGTTG-fluorescein
Den1-LC	LC640-GCCCCGCTGCTGCGTTATGT-phosphate
Den2-FL	CTTACAAATCGCAGCAACAATGGG-fluorescein
Den2-LC	LC705-GCCCAAGGTGAGATGAAGCTGTAGTC-phosphate
Den3-FL	GCCCGAGCACTGAGGGAAGCT-fluorescein
Den3-LC	LC640-ACCTCCTTGCAAAGGACTAGAGGTTATAGG-phosphate
Den4-FL	ATCACTGACAAAACGCAGCAAAAG-fluorescein
Den4-LC	LC705-GGCCCGAAGCCAGGAGGAAG-phosphate
Primers	
Pan-dengue forward	TTGAGTAAACYRTGCTGCCTGTAGCTC
Pan-dengue reverse	GAGACAGCAGGATCTCTGGTCTYTC
D1 cloning F	TCCAAGGACGTAAAATGAAGT
D1 cloning R	TCTGTGCCTGGAATGATGC
D2 cloning F	AAAAACTATGCTACCTGTGAG
D2 cloning R	CATTTTCTGGCGTTCTGTG
D3 cloning F	GCCACCTTAAGCCACAGTA
D3 cloning R	TGATTCAACAGCACCATTCC
D4 cloning F	CAACAACAAACACCAAAGGCTATT
D4 cloning R	AATCCATCTTGCGGCGCTCTGTG

Detection by SYBR green I-based real-time RT-PCR. All RT-PCRs were performed with 1 μl of RNA template in 10-μl reactions. The RNAs of Dengue-1 (S144), Dengue-2 (ST), Dengue-3 (SGH), and Dengue-4 (S006) were included as external controls in every RT-PCR run. These positive controls were derived from virus cultures isolated from clinical samples obtained from dengue patients in Singapore. All clinical RNA samples were first screened by SYBR green I-based real-time RT-PCR. One-step SYBR green I-based RT-PCR was carried out on the LightCycler system (LC 1.2; Roche Diagnostics, Penzberg, Germany). Samples were assayed with an optimal concentration (0.4 μM) of each primer in a 1× final concentration of LightCycler RNA master SYBR green I and 3 mM manganese acetate (Roche Diagnostics). The RT-PCR conditions for the one-step SYBR green I RT-PCR consist of a 10-min reverse transcription step at 60°C and then 1 min of *Taq* polymerase activation at 95°C, followed by 35 cycles of PCR at 95°C without holding time (denaturation), 60°C for 3 s (annealing), and 72°C for 10 s (extension). The fluorescence emitted was captured at the end of the extension step of each cycle at 530 nm.

Amplification graphs were checked for the cross-point (CP) value of the PCR product. The CP value represented the cycle by which the fluorescence of a sample increased to a level higher than the background fluorescence in the amplification cycle. Melting-curve analysis was performed after PCR amplification to verify that the correct product was amplified by examining its specific melting temperature (T_m). Reactions with a high CP value or ambiguous melting-curve results were analyzed by gel electrophoresis on a 2% agarose gel to confirm presence of product of the correct size.

Serotyping by FRET probes. Distinction between the serotypes was achieved by augmenting the pair of generic primers with serotype-specific FRET probes. In the duplex assay, probes targeting Dengue-1 and Dengue-3 were tagged with LC-Red 640 fluorescent dye, while probes targeting Dengue-2 and Dengue-4 were labeled with LC-Red 705 fluorescent dye. A duplex PCR was carried out on each sample by introducing the sample into two separate capillary tubes. One tube contained serotype-specific probes for Dengue-1 and Dengue-2, while the other tube contained serotype-specific probes for Dengue-3 and Dengue-4. A Roche LightCycler RNA master hybridization probe kit (Roche Diagnostics) was used, and each assay consisted of 1× LightCycler RNA master hybridization probe mix, 3.25 mM manganese acetate, a 0.4 μM concentration of forward primer, a 0.4 μM concentration of reverse primer, and a 0.3 μM concentration of each probe.

Reverse transcription was carried out at 61°C for 15 min, followed by *Taq*

FIG. 1. Alignment of the 3′ untranslated region of dengue viruses and binding sites for RT-PCR primers and probes. Except for Dengue-2, each serotype is represented by a Singaporean and two foreign isolates. Primer 1, pan-dengue forward primer; 2, pan-dengue reverse primer–reverse complementary; 3, D1 fluorescein-labeled probe–reverse complementary; primer 4, D1 LC Red-labeled probe–reverse complementary; primer 5, D2 fluorescein-labeled probe; 6, D2 LC Red-labeled probe; 7, D3 fluorescein-labeled probe; 8, D3 LC Red-labeled probe; 9, D4 fluorescein-labeled probe; and 10, D4 LC Red-labeled probe.

polymerase activation at 95°C for 1 min. A 45-cycle PCR amplification was performed with denaturation at 95°C (without holding time), annealing at 60°C for 15 s, and extension at 72°C for 10 s. The fluorescence emitted from the assay was captured during the annealing phase of each cycle at 640 and 705 nm. The results were analyzed with LightCycler software version 3.5.

A fourplex single-tube format RT-PCR assay was developed by using a more advanced version of the LightCycler (LC 2.0; Roche Diagnostics). The addition of two filters allowed the reading of fluorescence at two extra wavelengths, i.e., 610 and 670 nm, compared to the older version LC 1.2. In the fourplex assay, probes targeting Dengue-1 were tagged with LC-Red 610 fluorescent dye, and those targeting Dengue-2 were tagged with LC-Red 670 fluorescent dye. Fluorescent dyes for Dengue-3 and Dengue-4 remained the same as for the duplex assay. A fourplex RT-PCR was carried out in a single tube on each sample containing all of the four fluorescent dyes for each respective dengue serotype. Each assay consisted of 1× LightCycler RNA master hybridization probe mix (Roche Diagnostics), 3.25 mM manganese acetate, a 0.3 μM concentration of forward primer, and a 0.3 μM concentration of reverse primer. The concentration of the probes was optimized to eliminate cross talks. The probe concentrations used in each reaction tube were 0.3 μM for Dengue-1, 0.15 μM for Dengue-2, 0.12 μM for Dengue-3, and 0.2 μM for Dengue-4.

Reverse transcription was carried out at 61°C for 20 min, followed by *Taq* polymerase activation at 95°C for 1 min. A 45-cycle PCR amplification was performed with denaturation at 95°C for 1 s, annealing at 59°C for 15 s, and extension at 72°C for 10 s. The fluorescence emitted from the assay was captured during the annealing phase of each cycle at 610, 640, 670, and 705 nm, respectively. The results were analyzed by using LightCycler software version 4.05.

Preparation of DNA constructs for sensitivity tests. Table 1 shows the primers used for amplification of the target fragment of each dengue virus serotype. The amplified fragment of each serotype was cloned into the pCR-Blunt II TOPO vector by using the Zero Blunt TOPO PCR cloning kit (Invitrogen) according to the manufacturer's instructions. A plasmid construct for each serotype was prepared by using the QIAprep spin miniprep kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Virus isolation and IFA. A serum portion was utilized for virus isolation in the *Aedes albopictus* mosquito cell line C6/36 cell line (ATCC CRL-1660), followed by serotyping using IFA. Briefly, cell cultures were incubated for 10 days at 33°C after a 1-h adsorption period at 37°C. Cells were harvested after 10 days for staining in IFA. Cells were reacted with either dengue virus group-specific or dengue virus serotype-specific monoclonal antibodies derived from the clarified supernatant from hybridoma cultures (ATCC HB-46, HB-47, HB-48, and HB-49). The fluorescein isothiocyanate-conjugated goat anti-mouse antibody was used as the detector. A maximum of three passages was done for each sample if it was negative in the first or second passages.

Serology. Serology testing for anti-dengue IgM and IgG antibodies were performed by using a dengue capture IgM enzyme-linked immunosorbent assay (ELISA), a dengue capture IgG ELISA, and a dengue indirect IgG ELISA (Pan-Bio Diagnostics) according to the manufacturer's instructions.

RESULTS

Specificity of pan-dengue primers and serotype-specific probes. The pan-dengue pair of primers was tested for specificity against various flaviviruses such as Japanese encephalitis virus, yellow fever virus (vaccine strain), Kunjin virus, West Nile virus (New York and Wenglar strains), dengue virus, and an alphavirus, Chikungunya virus. These viruses are either closely related to dengue virus or cocirculate in the region with very similar symptoms. The primer pair was found to be specific to dengue viruses.

The specificity of the serotype-specific FRET probes was confirmed with an in-house collection of isolates of well-characterized serotypes, determined by IFA, and sequencing. At least three isolates of each serotype were tested.

Sensitivity of SYBR green I-based and FRET probe-based one-step RT-PCR assays. The detection limits of the three RT-PCR assays were determined according to PFU and copies of DNA in duplicates. The former was performed with 10-fold dilutions of seed viruses that had been quantitated by a plaque-forming assay. The detection threshold of the SYBR green pan-dengue assay is 0.01 PFU for all four dengue serotypes. This high sensitivity would ensure that clinical samples with a

TABLE 2. Sensitivity of each RT-PCR assay: SYBR green pan-dengue RT-PCR, serotype-specific duplex assay, and serotype-specific fourplex assay based on PFU per reaction[a]

Virus serotype	PFU	CP value for SYBR green pan-dengue RT-PCR	CP value for serotype-specific:	
			Duplex assay	Fourplex assay
Dengue-1	1,000	12.94	15.02	12.96
	100	16.90	18.75	16.30
	10	20.43	22.66	19.70
	1	24.32	25.63	22.61
	0.1	27.46	25.94	24.98
	0.01	30.48	ND	26.16
Dengue-2	1,000	16.55	15.59	13.85
	100	20.20	18.29	16.86
	10	23.89	22.31	19.70
	1	27.18	25.61	22.59
	0.1	29.86	27.25	24.95
	0.01	30.50	ND	ND
Dengue-3	1,000	12.39	14.21	12.29
	100	15.84	17.91	16.57
	10	19.66	21.67	18.46
	1	23.35	25.35	21.41
	0.1	26.71	ND	23.76
	0.01	29.72	ND	25.11
Dengue-4	1,000	15.47	15.20	16.85
	100	18.98	17.56	19.01
	10	22.90	21.07	21.89
	1	26.47	24.82	23.76
	0.1	28.72	26.21	ND
	0.01	30.67	28.71	ND

[a] Only the respective serotype was amplified. ND, not detected.

TABLE 3. Sensitivity of each RT-PCR assay: SYBR green pan-dengue RT-PCR, serotype-specific duplex assay, and serotype-specific fourplex assay based on DNA copies per reaction

Virus serotype	PFU	CP value for SYBR green pan-dengue RT-PCR	CP value for serotype-specific:	
			Duplex assay	Fourplex assay
Dengue-1	10^6	18.58	18.10	19.26
	10^5	22.52	21.82	23.63
	10^4	25.92	25.84	26.57
	10^3	26.61	27.98	29.83
	10^2	26.62	30.91	33.29
	10^1	ND[a]	ND	34.04
Dengue-2	10^6	18.73	18.63	18.75
	10^5	22.83	22.63	22.62
	10^4	25.19	25.89	25.85
	10^3	26.20	28.25	29.00
	10^2	27.98	30.13	31.91
	10^1	ND	32.73	32.86
Dengue-3	10^6	18.53	19.88	19.26
	10^5	22.85	23.66	23.04
	10^4	24.69	25.77	26.69
	10^3	24.86	28.65	29.58
	10^2	26.38	30.19	31.15
	10^1	ND[1]	31.04	ND
Dengue-4	10^6	18.80	18.92	22.90
	10^5	22.19	21.95	26.91
	10^4	25.21	25.41	29.69
	10^3	26.11	28.15	32.69
	10^2	26.15	30.52	32.72
	10^1	ND	ND	ND

[a] ND, not detected.

low virus titer would be recognized as dengue positive (Table 2). The detection threshold of the FRET probe-based duplex RT-PCR assay was 0.1 PFU for Dengue-1 and Dengue-2, 1 PFU for Dengue-3, and 0.01 PFU for Dengue-4. In the case of fourplex RT-PCR assay, the sensitivity was comparable, with the following results: 0.01 PFU for Dengue-1 and Dengue-3, 0.1 PFU for Dengue-2, and 1 PFU for Dengue-4 (Table 2).

The second method was performed with 10-fold dilutions of plasmid containing target fragment of each of the four serotypes. The detection threshold of the SYBR green pan-dengue assay was 10^2 DNA copies for all four dengue serotypes (Table 3). The detection threshold of both the FRET probe-based duplex and the fourplex RT-PCR assay was 10 to 100 DNA copies for all serotypes (Table 3). This method provides us with an idea of the sensitivity of the assays but is limited since it only provides information on the sensitivity of the PCR component of the RT-PCR and does not take the RT efficiency into consideration.

Validation of RT-PCR assays through seroconversion among samples with three consecutive collections. Validation of the SYBR green RT-PCR was performed with sample set A, with three collections at different time points from patients suspected of having dengue. RT-PCR was performed on the first collection, and the subsequent two collections enabled confirmation of RT-PCR diagnosis via anti-dengue virus IgM and IgG seroconversion assays. Among a total of 110 sets of samples (each complete with three different collections), 90 samples tested positive for SYBR green RT-PCR in the first collection, and all exhibited seroconversion (anti-dengue virus IgM or IgG) in later subsequent samplings. Seventeen individuals who did not display anti-dengue virus IgM seroconversion displayed seroconversion when tested using dengue capture IgG ELISA. None of the other 20 sets of serum samples that tested RT-PCR negative in the first collection displayed any seroconversion (Table 4). The results demonstrated the 100% sensitivity and 100% specificity of our SYBR green RT-PCR assay when performed within 3 days of illness. Table 5 shows

TABLE 4. Comparison of SYBR green RT-PCR assay with seroconversion status

Collection	Seroconversion status	No. of patients with the indicated PCR result in the first collection (<72 h from onset of fever)	
		+	−
First collection (IgM)	+	0	0
	−	90	20
Seroconversion in second or third collection (IgM or IgG)	+	90	0
	−	0	20

TABLE 5. SYBR green RT-PCR assay

Set	No. of samples with CP value of:			
	≤20.0	20.1–25.0	25.1–30.0	>30
Set A	51	30	8	1
Set B	19	18	8	7

the range of CP values for the positive samples. Among the 90 PCR-positive samples, 79 were positive for virus isolation. This translated to 87.8% correlation.

All RT-PCR (SYBR green)-positive samples could be serotyped by using the FRET probe-based duplex and fourplex RT-PCR. Among the 90 RT-PCR samples, 38 were found to be Dengue-1, 4 of them were Dengue-2, and 48 were Dengue-3. Among those that could be isolated, the serotype results determined by IFA correlate fully with the results obtained from the duplex and fourplex RT-PCRs.

Validation of RT-PCR assays with virus isolation and IFA among routine diagnostic samples. Further validation of the RT-PCR assays and determination of their performance were performed on clinical samples that were sent for diagnostics (singly collected). A total of 149 serum samples from suspected dengue cases were screened. Of these, 52 samples (34.9%) were tested positive for dengue virus by the SYBR green-based RT-PCR. Table 5 shows the number of samples within various ranges of CP values. The CP values of most samples were less than 30 cycles, which were easily interpreted. Only 46 of the 52 PCR-positive samples (88.5%) were positive by virus isolation and IFA (Table 6, footnote *a*). All samples that were positive by virus isolation and IFA were positive in the RT-PCR. The 88.3% correlation in the two techniques determined here was comparable to that determined with samples set A. (87.8%). The six samples that were positive for PCR and negative for virus isolation/IFA were further investigated by using serology assays (Table 6). Two of the six samples had positive anti-dengue IgM and a PCR CP value of more than 31 cycles. These findings suggested that the cases were at the end of the viremic stage with low viral load, which explained the low viability rate of the virus. Three other samples had no detectable level of anti-dengue IgM. However, we detected the presence of high level of anti-dengue IgG (>37 Pan-bio units). There was insufficient sample from the last case for further serology investigation.

All RT-PCR (SYBR green)-positive samples could be serotyped by using the FRET probe-based duplex and fourplex RT-PCRs. Of the 52 dengue virus-positive samples, 45 (86.5%) were tested as Dengue-1, 2 (3.8%) were Dengue-2, 5 (9.6%) were Dengue-3, and none was Dengue-4. The serotype determined by duplex and fourplex RT-PCRs correlates 100% with each other and with that determined by the IFA. Although Dengue-4 was not detected within the test samples, it was detected in five samples in 2005, and the results were also confirmed by IFA (National Environment Agency, unpublished data).

Using serum samples spiked with various combinations of serotypes in a single sample, the fourplex assay was able to detect the presence of more than one serotype in a single

TABLE 6. Comparison of SYBR green RT-PCR assay with virus isolation[a]

Sample	Serology of samples that were PCR positive but isolation negative		
	CP value	Dengue IgM	Dengue IgG[b]
SS693Y04	>31	Pos	Neg (4.02)
SS713Y04	26.43	Neg	Pos (43.3)
SS007Y05	24.93	Neg	Pos (42.7)
SS040Y05	>31	Pos	Pos (40.63)
SS059Y05	21	Neg	Pos (37.93)
SS069Y05	24	ND[c]	ND

[a] Neg, negative; Pos, positive.
[b] Numbers in parentheses are pan-bio units: <9 = negative, 9 to 11 = equivocal, and >11 = positive.
[c] ND, not done due to insufficient serum.

sample. This demonstrated its plausible ability to detect coinfection of more than one serotype.

DISCUSSION

The RT-PCR assays developed have been shown to be more sensitive than virus isolation. The sensitivity of the method was contributed by two factors: one-step RT-PCR and real-time assay. Recently, it was reported that a one-step RT-PCR was better than a two-step RT-PCR (8). This could be explained by the fact that all of the cDNA synthesized in a one-step RT-PCR would be available for PCR amplification, and this was reflected in the high sensitivity of our RT-PCR (probe) assay. The real-time tracking of PCR products within the capillary tube as the PCR progresses ensured that every product was captured and reported compared to endpoint PCRs, where only a portion of the product was loaded for visual examination on an ethidium bromide-stained agarose gel. The added advantage of the system was the minimization of false positives due to cross-contamination.

The pan-dengue generic primers used in the present study were a modification of the ones used in previous published study (2). The original primer pair was found to cross-react with West Nile (New York strain), while the specificity of primers in the present study had been demonstrated by tests with closely related flaviviruses.

One particular point of interest is how the relative sensitivity of the pan-dengue SYBR green I-based RT-PCR and the multiplex PCR differs depending on the template used. When RNA prepared from viruses grown in cell culture or from clinical samples was used as a template, the SYBR green RT-PCR displayed equal or higher sensitivity than the probe-based multiplex assays. However, using DNA constructs as a template rendered the SYBR green assays equally or less sensitive (by 1 log unit) than the probe-based multiplex assays. This is likely due to the fact that the optimization of the assays was performed on RNA prepared from viruses grown in cell culture, and the assays may not be optimal for plasmids isolated from bacterial cell culture. Variations in salt concentration and nucleic acid interference may be some of the causative factors. Thus, the complex interplay between primer, probe, salt, and nucleic acid concentration could have resulted in the above observation. This, along with the fact that the use of DNA constructs in the assays negates the reverse transcription pro-

cess, demonstrated that sensitivity tests using DNA constructs could only provide us with an idea of the sensitivity of the assays.

Samples that were negative by virus isolation were further investigated by testing their antibody status. Our results suggested that anti-dengue antibody levels in the samples might have interfered with the growth of the virus on C6/36 cell lines. The inhibition of virus development in vitro, despite rather low CP values of 21, 24, and 26, could likely be due to the antibodies' effect on the viability of the virus or the neutralizing effect of the virus by these antibodies. On the other hand, the PCR method might be less affected by the presence of neutralizing antibodies in the samples.

As much as 88% of all PCR-positive sera yielded an isolate in cell culture. This could be due to (i) the small size of Singapore and the short transport chain required to get the samples from patient to the laboratory and (ii) early sampling of the patients due to high level of awareness for dengue and a very accessible primary health care system in dengue-endemic Singapore. The former factor ensures the integrity of the samples, and the latter could lead to samples with higher viral load and with higher viral viability.

Serotyping dengue viruses is important in the epidemiological study of the disease. Vaughn et al. (21) reported that disease severity in dengue correlates with virus titer and virus serotype. Moreover, the intrinsic incubation period differs from serotype to serotype. This may provide the clinician who is treating a patient with much-needed information on the potential complications that could arise. In addition, certain serotypes might be associated with an epidemic. A change in the predominant serotype circulating within a community may be an indicator of an imminent dengue outbreak since the population is immunologically naive against the new serotype. Thu et al. (20) made such observations in Myanmar during the 2001 outbreak when Dengue-1 became the predominant serotype circulating in the country. It was also evident in Singapore where a switch from Dengue-2 to Dengue-1 led to an outbreak in 2004 and 2005 (National Environment Agency, unpublished data).

Dengue produces a febrile disease that presents with undifferentiated symptoms. This disease can only be confirmed with antigenic or immunologic detection tests. We have reported the development of three assays that we had used during the 2005 dengue outbreak in Singapore in support of the diagnostic and surveillance efforts. Our strategy was to use the low-cost SYBR green test for screening or for diagnosis. This real-time PCR, which could be completed in 1 h, was a rapid method allowing the fast detection and confirmation of dengue viruses in clinical samples. This early diagnostic tool was useful since patients in Singapore generally seek medical attention early in the disease, within the viremic period. The use of SYBR green had reduced our material costs for diagnosis by half compared to other methods that used probes. This was particularly important since generally 30 to 50% of the samples tested are positive during an epidemic period and only less than 10% are positive during a nonepidemic season. This rapid, early, and affordable diagnostic approach can contribute to a timely response to cluster management.

The more costly RT-PCR probe assays were, and still continue to be, used only on dengue RNA-positive samples for the surveillance and epidemiological study of serotypes. The duplex and fourplex format had allowed us to increase our throughput. The former was appropriate for use in LightCycler system (LC 1.2; Roche Diagnostics), while the latter was compatible with LightCycler LC 2.0, which consisted of two additional filters that allowed fluorescence measurement at wavelengths of 610 and 670 nm. The multiplex formats were critical during the outbreak period, where high-throughput and rapid results on circulating serotypes were appreciated. The speed of the test and the high throughput capacity had allowed us to observe, almost in real time, the predominance of Dengue-1 in the Singapore 2005 outbreak and the emergence of new serotypes in parts of the country. Enhanced action could then be taken against regions with the emergence of a new serotype.

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