Development and Evaluation of Fluorogenic TaqMan Reverse Transcriptase PCR Assays for Detection of Dengue Virus Types 1 to 4

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The fluorogenic TaqMan reverse transcriptase PCR (RT-PCR) assay was developed for detecting each of the dengue virus (DV) types 1 to 4. DV genome was detected in all the 35 serum samples from confirmed dengue cases by the TaqMan RT-PCR, although it was not detected in 13 and 21% by conventional type-specific and cross-reactive RT-PCR, respectively.

Dengue virus (DV) infections occur in most tropical and subtropical areas of the world (10). The number of travelers to areas where dengue is endemic has been increasing. Consequently, the number of imported dengue cases has also been increasing. A sensitive and specific assay to detect and discriminate a wide range of isolates of DV types 1 to 4 is needed. A fluorogenic probe-based assay has advantages over the conventional reverse transcriptase PCR (RT-PCR) (1, 3, 7, 8, 15). In the present study, we developed an improved serotype-specific fluorogenic (TaqMan) RT-PCR assay for detecting dengue virus genome. We then examined the newly developed TaqMan RT-PCR for sensitivity and specificity in comparison with conventional RT-PCR, by using serum samples from Japanese dengue patients.

DV types 1, 3, and 4, isolated from Japanese dengue fever patients, were propagated in C6/36 cells. DV type 2, Trinidad strain TR1751, was propagated in suckling mouse brain. West Nile virus, New York and Eg101 strains, Kunjin virus, K47382 and OR393 strains, Japanese encephalitis virus, Gar01 strain, and yellow fever virus, 17D strain, were propagated in Vero cells and used in flavivirus cross-reactivity studies.

One serum sample each was collected from 35 Japanese dengue patients. These patients visited countries where DV was endemic, including India, Sri Lanka, Bangladesh, Thailand, Cambodia, Laos, Singapore, Malaysia, East Timor, Indonesia, the Philippines, Tahiti, and Samoa during the years of 1994 to 2003. Some patients visited more than one country. Of 35 patients, 18 patients were infected with DV type 1, 8 with DV type 2, 6 with DV type 3, and 3 with DV type 4. DV infection was confirmed by immunoglobulin M capture enzyme-linked immunosorbent assay, conventional RT-PCR, or plaque titration assays, as reported by Yamada et al. (21, 22) and Nawa et al. (13).

All the envelope (E) gene nucleotide sequences available in GenBank were retrieved and aligned using CLUSTAL X (version 1.8) (17) to prepare optimized primers and probes for the TaqMan RT-PCR. We used the E gene because it had been best analyzed among dengue genes (2, 4, 5, 6, 9, 14, 16, 18, 19).

The strains retrieved from GenBank are shown in Table 1. Primer and probe sequences were selected using the Primer Express software (PE Applied Biosystems, Foster City, Calif.).

Viral RNA was isolated using a QIAamp viral RNA kit (QIAGEN, Valencia, Calif.). The conventional RT-PCR was performed with a TITAN One-Tube RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, Ind.), according to the method of Yamada et al. (21). The cross-reactive and type-specific primers for DV types 1 to 4 were previously reported and evaluated by Morita et al. (11, 12) and Yamada et al. (21) (Table 1). In the TaqMan assay, 5 μl of RNA isolated from a serum sample was mixed with 100 pmol of each primer and 15 pmol of a probe in a 25-μl reaction volume, using a TaqMan RT-PCR Ready-Mix kit (PE Applied Biosystems). The samples were subjected to amplification in an ABI Prism 7000 Sequence Detection System instrument (PE Applied Biosystems). The TaqMan RT-PCR assay consisted of a 30-min RT step at 48°C and 45 cycles of the PCR step (95°C for 15 s and 57°C for 60 s).

PCR products were directly detected by monitoring the increase in fluorescence of a dye-labeled oligonucleotide probe with an ABI Prism 7000 sequence detector. Assay specificity was evaluated by testing serotype-specific probe and primer sets against virus panels that included DV types 1 to 4, West Nile virus (New York strain, 1.0×10^6 PFU/tube; Eg101 strain, 5.4×10^4 PFU/tube), Kunjin virus (K47382 strain, 1.8×10^7 PFU/tube; OR393 strain, 1.6×10^8 PFU/tube), Japanese encephalitis virus (7.6×10^5 PFU/tube), and yellow fever virus (6.0×10^3 PFU/tube). The detection threshold of each TaqMan assay was determined using 10-fold serial dilutions of RNA extracted from stock DV. The threshold was defined as 0.5. This corresponds to the level at which specific viral RNA was consistently detected and at which no nonspecific viral RNA was detected. The threshold cycle value (Ct) was defined as the number of amplification cycles at the threshold. Ct remained within a standard curve having a minimum correlation coefficient of 0.98.

First, the sensitivities of the TaqMan RT-PCR assay for DV types 1 to 4 was evaluated. Reference samples of DV types 1 to 4, which were previously quantified, were diluted 10-fold and used for sensitivity assays. Sensitivities were 1.2 PFU/tube for DV type 1 (Ct = 40.7), 0.1 PFU/tube for type 2 (Ct = 39.2), 1.0

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TABLE 1. Oligonucleotide primers and probes used in the conventional RT-PCR and TaqMan RT-PCR

Assay	Serotype	Identification	Sequence (5′–3′)	Size (bp)	Gene	No. of strains[c]
Conventional RT-PCR with cross-reactive primers		Dus (forward)	TCAATATGCTGAAACGCGCGAGAAACCG	511	C-PrM.	
		Duc (reverse)	TTGCACCAACAGTCAATGTCTTCAGGTTC			
Conventional RT-PCR with type-specific primers	1	D1s (forward)	GGACTGCGTATGGAGTTTTG	490	E-NS1	
		D1c (reverse)	ATGGGTTGTGGCCTAATCAT			
	2	D2S (forward)	GTTCCTCTGCAAACACTCCA	230	E	
		D2c (reverse)	GTGTTATTTTGATTTCCTTG			
	3	D3s (forward)	GTGCTTACACAGCCCTATTT	320	E-NS1	
		D3c (reverse)	TCCATTCTCCCCAAGCGCCTG			
	4	D4s (forward)	CCATTATGGCTGTGTTGTTT	398	NS2a-NS2b	
		D4c (reverse)	CTTCATCCTGCTTCACTTCT			
TaqMan RT-PCR with type-specific primers and probes	1	D1MGBEn469s (forward)	GAACATGGRACAAYTGCAACYAT	67	E	76[d]
		D1MGBEn493p[a] (probe)	ACACCTCAAGCTCC			
		D1MGBEn536r (reverse)	CCGTAGTCDGTCAGCTGTATTTCA			
	2	D2MGBEn493s (forward)	ACACCACAGAGTTCCATCACAGA	68	E	203[e]
		D2MGBEn545p[a] (probe)	CGATGGARTGCTCTC			
		D2MGBEn568r (reverse)	CATCTCATTGAAGTCNAGGCC			
	3	D3MGBEn1s (forward)	ATGAGATGYGTGGGAGTRGGAAAC	70	E	59[f]
		D3MGBEn27p[a] (probe)	AGATTTTGTGGAAGGYCT			
		D3MGBEn71r (reverse)	CACCACDTCAACCCACGTAGCT			
	4	D4TEn711s (forward)	GGTGACRTTYAARGTHCCTCAT	75	E	30[g]
		D4TEn734p[b] (probe)	CCAAGAGACAGGATGTGACAGTGCTRGGATC			
		D4TEn786c (reverse)	WGARTGCATRGCTCCYTCCTG			

[a] Labeled at the 5′ end with the FAM reporter dye and at the 3′ end with the quencher dye TAMRA.

[b] MGB probes were labeled at the 5′ end with the FAM reporter dye and were not labeled at the 3′ end with the quencher dye TAMRA.

[c] Retrieved from GenBank to design primers and probes.

[d] AF425639, AF514876, AF514878, AF514883, AF514885, AJ574760, AY206457, AB111064-78, AF226685, AF298807-8, AF311956-58, AF425609-38, AF513110, AJ438941, AY145121-23, AY153755, D00501-05, M23027, M87512, S64849, U88535-37, and X76219.

[e] A91810, AF004019-20, AF01041, AF022434-41, AF038402-3, AF093674, AF100459-69, AF19661, AF163096, AF195032-44, AF19678-88, AF204177-78, AF231715-20, AF264053-4, AF276619, AF295694-709, AF359579, AF363069-92, AF398106-14, AF410345-79, AF469175-6, AF489932, AF509530, AY037116, AY044442, AY079923-24, D00345-6, D10514, AB111448-54, L04561, L10040-55, M15075, M19197, M20558, M24444-51, M29095, M84727-28, U87411-12, X15214, X15433-34, X54319, and X65240.

[f] AF147456-60, AF317645, AF349753, AF533079, AY038605, AY135419, AY145712-30, AB111080-84, L11422-42, L11619-20, L41125, and NC_001475.

[g] AF231722-25, AF289029, AF326573, AF375822, AB111085-88, NC_002640, and U18425-42.

PFU/tube for type 3 (Ct = 38.2), and 0.1 PFU/tube for type 4 (Ct = 38.8). The primer and probe pairs were also tested for virus specificity and did not detect other DV types or other flaviviruses. These results indicate that the newly developed TaqMan RT-PCR assays for DV types 1 to 4 are highly specific.

DV genome was detected in all the 35 serum samples (100%), when tested by the TaqMan RT-PCR. When tested by conventional RT-PCR, DV genome was detected in 27 of 31 serum samples (87%) with type-specific primers and in 26 of 33 samples (79%) with dengue cross-reactive primers (Table 2). These results suggest that sensitivity of TaqMan RT-PCR is higher than those of conventional RT-PCRs ($P < 0.02$).

We next examined the relationship between virus titers and Ct values by analyzing serum samples from DV type 1-infected patients. There was an inverse correlation between virus titer and Ct (Fig. 1). Additionally, seven serum samples from non-dengue patients were tested by TaqMan RT-PCR. DV genome was not detected in any of these samples (data not shown).

We detected DV genome in all the 35 tested serum samples from dengue patients by TaqMan RT-PCR, while 13% (type specific) and 21% (cross-reactive) were determined to be negative by the conventional RT-PCR. The newly developed TaqMan RT-PCR is, therefore, a useful tool for testing acute-phase serum samples from clinically suspected dengue cases. We did not observe any cross-reactions to other dengue types

TABLE 2. Virus gene detection of serum samples from confirmed dengue patients by TaqMan RT-PCR and conventional RT-PCR

Ct range of TaqMan RT-PCR (cycles)	Detection by:		
	TaqMan RT-PCR	Conventional RT-PCR	
		Type specific	Cross-reactive
<25	10/10 (100)[a]	10/10 (100)	9/9 (100)
25–29.9	7/7 (100)	6/7 (86)	6/6 (100)
30–34.9	7/7 (100)	6/6 (100)	7/7 (100)
35–39.9	6/6 (100)	3/4 (75)	3/6 (50)
≥40	5/5 (100)	2/4 (50)	1/5 (20)
Total	35/35 (100)	27/31 (87)	26/33 (79)

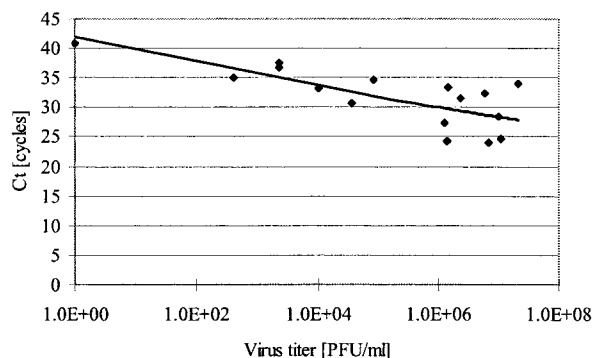[a] Positive samples/number of tested samples (%).

FIG. 1. A log-scale plot of the virus threshold cycles (Ct) versus the virus titer for dengue virus type 1. Correlation coefficient, 0.502 (6-carboxyfluorescein [FAM] filter).

or other flaviviruses. This indicates that the E gene displays a high degree of sequence variation among flaviviruses, which enables us to construct primers and probes with high specificity and sensitivity. To our knowledge, a TaqMan RT-PCR system with a high specificity and high sensitivity has not been reported using other target regions. For example, Warrilow et al. (20) reported a group assay to detect all four dengue serotypes, using the 3′ noncoding region as the target area, but the sensitivity was not high. Callahan et al. also document a group assay with two sets of primers and probes, using the NS5 and C regions as the target area (1). Although they succeeded in detecting the DV types 1 to 4 with a high sensitivity using group assays, they did not obtain high sensitivities in type-specific assays. Additionally, since their group assay requires two sets of primers and probes, its advantage over a type-specific approach is reduced. Furthermore, in contrast to our results, the sensitivity of their type-specific TaqMan assay was not consistent with plaque titration results. Unfortunately, neither we nor Callahan et al. (1) converted the sensitivity units from PFU per milliliter to RNA copies. We will create a stable internal positive control for quantization, using plasmids containing the targeted viral nucleotide sequence, and further standardize the assay in the future.

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