Comparison of the FDA-Approved CDC DENV-1-4 Real-Time Reverse Transcription-PCR with a Laboratory-Developed Assay for Dengue Virus Detection and Serotyping

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Dengue virus (DENV) is the agent of the most common vector-borne disease worldwide. Using 199 clinical samples collected from Nicaragua and Sri Lanka, a laboratory-developed DENV multiplex real-time reverse transcription-PCR (rRT-PCR) proved more clinically sensitive than the FDA-approved CDC assay for DENV serotypes 1 to 4 when measured against a composite reference standard, with sensitivities of 97.4% versus 87.1%, respectively.

Infection with one or more of four related serotypes of dengue virus (designated DENV-1 to -4) results in a range of clinical manifestations, spanning asymptomatic infection, dengue fever (DF), and severe dengue (1). The signs and symptoms of dengue overlap significantly with those of other systemic febrile illnesses in the tropics, and diagnosis therefore rests on specific laboratory tests (2).

The CDC DENV-1-4 real-time PCR is the first RT-PCR assay approved for DENV detection (3). Experiments performed during FDA approval showed that this assay compared favorably to envelope (E) gene sequencing and anti-DENV IgM seroconversion when samples collected within the first 5 days of illness onset were tested (3). However, a comparison with another real-time RT-PCR (rRT-PCR) assay was not reported.

In this study, we compared a laboratory-developed DENV multiplex rRT-PCR with the CDC DENV-1-4 assay using 199 clinical samples collected from suspected dengue cases between day 2 and 9 of illness.

The DENV multiplex rRT-PCR was performed as previously described (4) with the following modifications. (i) A redesigned FAM-labeled molecular beacon probe (CGCGATCTTCAGCATA TTGAAAGACGGTCGGATCGCG) was used. (ii) Cycling conditions were the following: 52°C for 15 min (RT step); 94°C for 2 min; 45 cycles of 94°C for 15 s, 55°C for 40 s, and 68°C for 20 s. (iii) DENV-2 and DENV-4 primers were used at final concentrations of 350 nM and 400 nM, respectively. The analytical performance of this assay was equivalent to that of the original DENV multiplex rRT-PCR, though cross-reactions in the green channel (DENV-1) were eliminated (data not shown) (4).

The CDC DENV-1-4 real-time RT-PCR kit was obtained from the CDC Dengue Branch. The assay was performed in multiplex on the Rotor-Gene Q instrument as described in the package insert.

A total of 199 precollected and deidentified clinical samples from Nicaragua ($n = 160$) and Sri Lanka ($n = 39$) were tested using both the DENV multiplex rRT-PCR and the CDC DENV-1-4 assay. These clinical samples were described previously (5). Briefly, the Nicaraguan samples were collected between 23 September 2008 and 23 December 2011 as part of the Nicaraguan

Pediatric Dengue Cohort Study as well as a hospital-based study to assess risk factors for severe dengue. Samples were collected at presentation from 141 patients. Ten patients from the hospital-based study had serial samples drawn on day 5 of illness (the day of presentation), day 6 ($n = 9$), and day 7 ($n = 10$). Samples were also collected from 39 children on presentation to the Lady Ridgeway Hospital (Colombo, Sri Lanka) with an acute febrile illness, clinically suspected to be dengue. These samples were collected between 18 March and 28 May 2012.

Results obtained using the DENV multiplex and CDC DENV-1-4 assays were compared with each other and with a composite reference standard (Table 1). This standard incorporated the results from the present study with the results of previous testing using a heminested RT-PCR (6) and a laboratory-developed pan-DENV rRT-PCR (5). Samples that tested positive by two or more assays were considered positive. Those that tested positive by only one RT-PCR or tested negative by all assays were considered negative. For statistical analysis, two-tailed Fisher's exact tests, unpaired t tests, and kappa statistics were performed using GraphPad software (GraphPad, La Jolla, CA).

Compared to the composite reference, the DENV multiplex rRT-PCR was more clinically sensitive than the CDC DENV-1-4 assay, detecting 151/155 (97.4%) samples compared to 135/155 (87.1%), respectively ($P = 0.001$) (Table 1). Assay specificity was not significantly different (40/44 [90.9%] versus 43/44 [97.7%], respectively; $P = 0.36$). The DENV multiplex and CDC DENV-1-4 assays displayed good overall agreement (Table 1) (kappa, 0.63); however, discordant results were obtained for 29 samples (Table 2). The sensitivity of the DENV multiplex rRT-PCR did not differ significantly from the sensitivity of the original version (151/

TABLE 1 Comparison of the DENV multiplex rRT-PCR and CDC DENV-1-4 assay with a composite reference standard and direct comparison of the two tests[a]

Test	Result	No. of samples with result in comparator test		
		Positive	Negative	Total
		Composite reference		
DENV multiplex	Positive	151	4	155
	Negative	4	40	44
	Total	155	44	199
		Composite reference		
CDC DENV-1-4	Positive	135	1	136
	Negative	20	43	63
	Total	155	44	199
		DENV multiplex		
CDC DENV-1-4	Positive	131	5	136
	Negative	24	39	63
	Total	155	44	199

[a] The composite reference standard required a sample to test positive by two or more RT-PCR tests.

155; 97.4%) or the pan-DENV rRT-PCR (149/155; 96.1%; $P = 0.75$) (data not shown). All assays were more sensitive than the heminested RT-PCR (119/155; 76.8%).

Serotype results agreed for 130/131 (99.2%) samples with detectable DENV (52 DENV-1, 11 DENV-2, and 67 DENV-3 samples). The single discrepant result was a Sri Lankan sample with detectable DENV that was serotyped as DENV-3 by the DENV multiplex rRT-PCR and DENV-1 by the CDC DENV-1-4 assay. For the discrepant sample, the crossing threshold (C_T) in the DENV multiplex assay was earlier than in the CDC DENV-1-4 assay (29.51 and 39.95, respectively). The cloned amplicon from the DENV multiplex assay yielded a sequence that matched DENV-3. However, the discrepancy was not fully resolved, as DENV was not detected by the heminested RT-PCR, and cloning the low-abundance amplicon from the CDC DENV-1-4 assay was unsuccessful.

When sample results were categorized by serotype, the DENV multiplex rRT-PCR proved more sensitive for the detection of DENV-1 (66/66, 100%) than the CDC DENV-1-4 assay (52/66; 78.8%; $P < 0.0001$), compared to the composite reference. The 14 DENV-1 samples with discordant results included samples from both Nicaragua ($n = 5$) and Sri Lanka ($n = 9$). The sensitivities of the DENV multiplex and CDC DENV-1-4 assays did not differ for the detection of DENV-2 (13/13 [100%] versus 11/13 [84.6%], respectively) or DENV-3 (72/76 [94.7%] versus 71/76 [93.4%], respectively).

TABLE 2 Results for 29 clinical samples with discordant results in the DENV multiplex and CDC DENV-1-4 assays

Assay	n	Mean C_T (SD)[a]	Serotype (no. of samples)
DENV multiplex	24	37.8 (3.3)	DENV-1 (16), DENV-2 (2), DENV-3 (7)[b]
CDC DENV-1-4	5	37.6 (3.0)	DENV-3 (5)

[a] C_T, crossing threshold.
[b] A single DENV-1/DENV-3 coinfection was detected.

TABLE 3 Clinical samples positive for DENV by the composite reference stratified by patient day of illness[a]

Day of illness	No. of positive samples		
	Composite reference	Multiplex rRT-PCR	CDC DENV-1-4
<5	61	61/61 (100)	57/61 (93.4)
≥5	82	80/82 (97.6)	67/82 (81.7)
Total	143	141/143 (98.6)	124/143 (79.0)

[a] Samples without day-of-illness data were removed from the analysis ($n = 42$). Results for the DENV multiplex rRT-PCR and the CDC assay are expressed as number positive/total (percent).

Results for 157 samples were further stratified by the day of illness of sample collection (Table 3). For 42 samples, this information was not available. When sample collection occurred within 5 days of illness onset, the sensitivities of the DENV multiplex (61/61; 100%) and CDC DENV-1-4 assays (57/61; 93.4%) did not differ significantly (compared to the composite reference standard; $P = 0.12$). However, when samples were collected on or after day 5 of illness, the DENV multiplex rRT-PCR proved more sensitive (80/82; 97.6%) than the CDC DENV-1-4 assay (67/82; 81.7%; $P = 0.001$). As expected, the C_T values for samples collected within 5 days of illness onset were lower (mean, 24.08; standard deviation, 6.46) than those for samples collected on or after day 5 of illness (mean, 34.17; standard deviation, 4.45; $P = 0.0001$). The sensitivities of the two assays were also significantly different when only the subset of patients who had detectable IgM at presentation were considered (DENV multiplex, 33/34 [97.1%]; CDC DENV-1-4, 24/34 [70.6%]; $P = 0.006$).

The improved clinical sensitivity of the DENV multiplex rRT-PCR was partly the result of improved DENV RNA detection in samples collected ≥5 days after illness onset. This finding is notable as data submitted for FDA approval included only samples collected on or after day 5 of illness, which is consistent with many previously published DENV nucleic acid amplification test (NAAT) validations (7–9). Symptomatic dengue cases do not necessarily present early in the disease course, and in a study from Martinique, patients who developed severe dengue presented later than patients with DF (median day of illness, 6 versus 2; $P < 0.001$) (10). Typical findings in dengue, such as thrombocytopenia and leukopenia, may not develop until day 5 of illness or later, which may further delay testing (11). RT-PCR assays, evaluated in our laboratory, perform similarly with samples collected within 5 days of illness onset (5). Rather, it is the ability to detect and serotype DENV later in the course of disease that separates different molecular assays and may meaningfully improve the state of DENV diagnosis and management (4, 12–15).

FDA approval has been highlighted as a particular strength of the CDC DENV-1-4 assay (16). This process does not guarantee optimal assay performance, however, and a number of examples in the literature identify FDA-cleared molecular virology assays that ultimately required redesign (17, 18). Further laboratory evaluation is therefore critical to recognize the potential limitations of cleared tests and guide future modifications. Indeed, the DENV multiplex rRT-PCR reported in this study was modified from the original version to simplify interpretation.

In conclusion, the DENV multiplex rRT-PCR demonstrated higher clinical sensitivity than the CDC DENV-1-4 assay. This finding resulted from improved detection of DENV-1, particu-

larly in samples collected from patients presenting ≥5 days after illness onset or with detectable anti-DENV IgM.

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