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Improved Serotype-Specific Dengue Virus Detection in Trinidad and Tobago using a Multiplex, Real-Time RT-PCR

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Abstract

Dengue virus (DENV) transmission occurs throughout the Caribbean, though laboratory confirmation and epidemiologic surveillance is limited by the availability of serotype-specific molecular diagnostics. In this study, we show that a serotype-specific DENV multiplex, real-time RT-PCR detected DENV RNA in significantly more samples (82/182) than a reference hemi-nested RT-PCR (57/182; $p=0.01$).

Over the past 50 years, dengue virus (DENV) has re-emerged in countries of the Caribbean through a series of sequential epidemics involving all four serotypes (designated DENV-1-4) (Allicock, et al., 2012; Carrington, et al., 2005). In 2013, over 2 million dengue cases were reported to the Pan American Health Organization, though only a small minority of cases received a serotype-specific diagnosis (Pan American Health Organization, 2013). This situation is particularly apparent in Trinidad and Tobago (Allicock, et al., 2012; Carrington, et al., 2005), where transmission of all four DENV serotypes has been documented, but none of the 3,289 cases reported in 2013 had documented laboratory confirmation (Pan American Health Organization, 2013).

Recently, our group developed a single-reaction, multiplex real-time reverse transcriptase PCR (rRT-PCR) for the serotype-specific detection of DENV-1-4 (referred to as the DENV

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multiplex) (Waggoner, et al., 2013b; Waggoner, et al., 2013c). In large, extensive method comparison studies, this assay proved significantly more sensitive than a widely-used hemi-nested RT-PCR and the FDA-approved CDC DENV-1-4 Real-Time RT-PCR (Waggoner, et al., 2013a; Waggoner, et al., 2013b). Despite the use of 199 clinical samples from Nicaragua and Sri Lanka, this sample set did not include specimens positive for DENV-4. While the analytical performance of the DENV multiplex for DENV-4 has been clearly documented, the clinical performance of this assay for DENV-4 detection has not yet been demonstrated (Waggoner, et al., 2013c). In the current study we address this limitation by performing a comparison of the DENV multiplex and hemi-nested RT-PCR in Trinidad using samples from patients infected with DENV-1 and -4.

One hundred eighty-two archived, de-identified serum samples, from 155 suspected dengue cases in Trinidad, were included in the study. Samples were obtained during the symptomatic stage of infection. When available, clinical information was recorded for individual samples. Serum was stored at -80°C until use. Nucleic acid extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD) as described (Waggoner, et al., 2013c).

The DENV multiplex was performed on the Applied Biosystems (ABI) 7500 instrument (Life Technologies, Grand Island, NY) using 5 μL of extracted RNA in 25 μL reactions. Reaction set-up was performed as previously described (Waggoner, et al., 2013b). Cycling conditions were the following: 52°C for 15min; 94°C for 2min; 45 cycles of 94°C for 15sec, 55°C for 40sec, 60°C for 20sec, and 68°C for 20sec. Detection was performed at 55°C during the last 40 amplification cycles. Analysis was performed on the linear scale with auto baseline normalization. Thresholds were set for each run using the negative control and positive controls for each serotype. Exponential curves that crossed this threshold were considered positive. Separate internal control reactions, based on RNase P detection, were performed for all samples using identical set-up and cycling conditions. The RNase P primers and probe were used as described (Waggoner, et al., 2013a), and analysis was performed as above.

The hemi-nested RT-PCR was performed by one author (NS) as described (Lanciotti, et al., 1992). Samples were tested in the DENV multiplex by a second author (JW), who was blinded to these results. Samples were also tested by the Trinidad Public Health Laboratory (TPHL) using enzyme-linked immunosorbent assays (ELISAs) for DENV non-structural protein-1 (NS1; Standard Diagnostics, Republic of Korea) and anti-DENV IgM (Focus Diagnostics, Cypress, CA). Fisher's exact tests for assay comparisons were performed using GraphPad software (GraphPad; La Jolla, CA).

Available clinical information obtained from 155 patients included in the final assay comparison is shown in Table 1. The internal control reaction was positive in all samples, indicating adequate nucleic acid extraction and the absence of PCR inhibitors.

Results of the comparison of the DENV multiplex and hemi-nested RT-PCR are shown in Table 2. The DENV multiplex detected DENV RNA in significantly more samples than the hemi-nested RT-PCR ($p=0.01$). Concordant serotype results were obtained for 50/52

(96.2%) samples that tested positive in both assays. Two samples with discordant serotype calls had results consistent with DENV-4 in the hemi-nested RT-PCR and DENV-1 and DENV-2, respectively, in the DENV multiplex. Additional specimen was not available for further testing to resolve these serotype calls, and these samples were removed from subsequent comparisons based on the infecting serotype. DENV-1 RNA was detected in significantly more samples using the DENV multiplex [47/50 (94%) samples with DENV-1 detected by one or both assays] compared to the hemi-nested RT-PCR [27/50 (54%); p 0.0001]. DENV-4 RNA was also detected in more samples using the DENV multiplex [33/35 (94.3%) vs. 28/35, (80.0%)], but this did not reach statistical significance (p 0.15). Thirty samples that tested positive in only one RT-PCR had results from testing at TPHL for NS1 and/or anti-DENV IgM, and 27/30 (90.0%) had positive results for one or both analytes, including 22/25 (88%) samples positive only in the DENV multiplex.

Although the hemi-nested RT-PCR was originally described in 1992, it remains in wide use for reference molecular testing throughout the world. The DENV multiplex was previously shown to be more clinically sensitive than a modified version of the hemi-nested assay for patients with DENV-1, -2, and -3 (Chien, et al., 2006; Waggoner, et al., 2013a). However, concern was raised that it had not been sufficiently evaluated in patients with DENV-4 (Munoz-Jordan & Santiago, 2014; Waggoner & Pinsky, 2014). In this report, we confirm our earlier findings and extend those results by demonstrating improved DENV detection in patients with DENV-1 and -4 infections. The high percentage of patients with discrepant RT-PCR results that had detectable NS1 and anti-DENV IgM support our conclusion that the DENV multiplex is more clinically sensitive than the hemi-nested RT-PCR. These immunoassays may provide useful diagnostic information, particularly when combined with molecular detection, though, unlike the DENV multiplex, these tests are unable to provide the serotype information required for optimal epidemiologic surveillance. Importantly, this study also provides the first description of the DENV multiplex implemented in a DENV-endemic country on the ABI 7500 instrument. This platform gained widespread use during the 2009 influenza A (H1N1) pandemic (WHO/CDC, 2009), and it remains one of the most common real-time PCR instruments in the DENV-endemic world. In conclusion, the DENV multiplex demonstrates greater clinical sensitivity in patients infected with all four DENV serotypes compared to the hemi-nested RT-PCR and should be considered by reference and public health laboratories for confirmatory testing and epidemiologic monitoring.

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Table 1

Associated clinical data available for 155 study patients included in the final comparison.

	N (%)	Median	Range
Patients - Total	155 (100)		
Day of Illness	45 (29.0)	5	2–19
Age	56 (36.1)	28	5–73
Clinical History ^a	88 (56.8)		
<i>Fever</i>	56 (63.6)		
<i>Body Pain</i>	43 (48.9)		
<i>Headache</i>	37 (42.0)		
<i>Gastrointestinal Symptoms</i> ^b	36 (40.9)		
<i>Respiratory Symptoms</i> ^c	17 (19.3)		

^a Percentages for clinical history items were calculated using the number of patients with any clinical history.

^b Includes abdominal pain, nausea, vomiting, diarrhea, loss of appetite

^c Includes cough and rhinorrhea

Table 2

Comparison of the DENV multiplex and hemi-nested RT-PCR using 182 clinical samples collected from 155 patients in Trinidad and Tobago. Comparisons are shown for all samples (A), samples positive for DENV-1 (B), and samples positive for DENV-4 (C).

A.				
		Hemi-Nested RT-PCR		
		Positive	Negative	Total
DENV Multiplex	Positive	52	30	82
	Negative	5	95	100
	Total	57	125	182
B.				
		Hemi-Nested RT-PCR		
		Positive	Negative	Total
DENV Multiplex	Positive	24	23	47
	Negative	3		3
	Total	27	23	50
C.				
		Hemi-Nested RT-PCR		
		Positive	Negative	Total
DENV Multiplex	Positive	26	7	33
	Negative	2		2
	Total	28	7	35