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Highly Sensitive Detection of Dengue Virus Nucleic Acid in Samples from Clinically Ill Patients[▽]

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Dengue virus (DENV) is a major cause of febrile illness and hemorrhagic fever in tropical and subtropical regions. Typically, patients presenting with acute dengue disease are viremic but may not have yet developed detectable titers of antibody. Therefore, early diagnosis depends mostly on detection of viral components, such as the RNA. To define the potential use of transcription-mediated amplification (TMA) DENV RNA as a diagnostic tool, we first compared its analytic sensitivity using a routine real-time reverse transcription (RT)-PCR and found that TMA is approximately 10 to 100 times more sensitive. In addition, we tested acute-phase serum samples (<5 days post-symptom onset) submitted as part of laboratory-based surveillance in Puerto Rico and determined that among patients with serologically confirmed dengue infection, TMA detected DENV RNA in almost 80% of serum specimens that were negative by the RT-PCR test used for diagnosis and in all specimens with positive RT-PCR results. We conclude that TMA is a highly sensitive method which can detect DENV RNA in approximately 89% of clinical, acute-phase serum specimens.

Dengue virus (DENV) is currently the most disseminated mosquito-borne virus. DENV groups with *West Nile virus* (WNV), *Yellow fever virus*, *Japanese encephalitis virus*, and approximately 70 other viruses in the *Flaviviridae* family. Concomitant with population growth and increased international travel and trade in the last two decades, DENV and its principal vector, *Aedes aegypti*, have expanded their geographical distribution throughout the tropical and subtropical areas in South East Asia and the Americas (1, 7, 19, 24, 25, 30). The World Health Organization estimates that 50 million to 100 million cases of dengue-related disease occurred in 2004, including cases of dengue fever, 500,000 cases of dengue hemorrhagic fever (DHF), and 20,000 consequent deaths (36). This wide spectrum of clinical disease is produced by the four DENV serotypes (DENV-1 to -4). Infection with one serotype provides lifelong immunity to the infecting serotype only. Residents of dengue-endemic countries are susceptible to sequential infections by different DENV serotypes, and secondary infections may correlate with higher levels of viremia, placing them at greater risk for developing DHF (10, 28, 34). Viral characteristics may also determine disease severity (6, 16).

Early diagnosis of dengue has been challenging. Patients most commonly present with undifferentiated fever and unspecific symptoms within 5 to 7 days postinfection, after which the viremia is cleared with the rise of antibodies (5, 8, 14, 21, 34, 35). Serological testing of paired acute- and convalescent-phase specimens has been the foundation of dengue diagnostics; but generally, this approach confirms dengue cases only after patients recover. Until recently, diagnosis with single, acute-phase serum specimens depended on laborious tissue culture or mosquito inoculation practices for virus detection. Reverse transcription (RT)-PCR approaches are being increasingly used for dengue diagnosis with acute-phase serum samples (9, 15). The sensitivity of RT-PCR assays in serologically confirmed cases ranges from 40 to 80% and may decrease as the interval from symptom onset to specimen collection increases (23, 29). The sensitivity of molecular diagnostics may be affected by such factors as the temperature of the sample during transport and storage, RNA extraction procedures, or the method of detection of PCR products.

All four DENV serotypes are endemic in Puerto Rico. In the last 12 years, an average of approximately 5,500 suspected cases have been reported to a laboratory-based, island-wide surveillance system during nonoutbreak years. During occasional epidemics, the number of suspected cases has ranged from 6,000 to more than 20,000 (26, 27). A serum specimen from approximately 70 to 80% of suspected cases is submitted within the first 5 days of symptoms. Since 2005, the CDC Dengue Branch in Puerto Rico has tested acute-phase serum specimens (≤5 days post-symptom onset) using an automated, one-step, multiplexed RT-PCR assay (4). Convalescent-phase sera are often not submitted in passive surveillance, and with the current sensitivity of this RT-PCR, only 45% of total suspected cases are laboratory confirmed on the island.

Transcription-mediated amplification (TMA) assays are currently employed to screen blood donations for human immunodeficiency virus type 1 (HIV-1), hepatitis B virus, hepatitis C virus, and WNV nucleic acids (31, 32). All blood donations in the United States and Canada are screened for WNV with one of two licensed nucleic acid amplification tests (17, 22, 33). A DENV TMA assay has recently been developed which has analytical sensitivity (approximately 15 copies/ml; 95% limit of

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detection) comparable to that of the WNV TMA-based assay (18). The use of this unlicensed DENV TMA assay for blood donation screening has established in two research studies the presence of DENV RNA in blood donor samples from Honduras, Brazil (18), and Puerto Rico (20).

Given the potential of TMA to detect DENV in serum with high sensitivity, we assessed its performance with serum specimens submitted to the laboratory-based surveillance system in Puerto Rico. Our results indicate that TMA detects DENV RNA in approximately 89% of acute-phase serum specimens and therefore may provide a useful diagnostic test for acute DENV infections.

MATERIALS AND METHODS

Dengue TMA assay. The qualitative DENV RNA assay (18, 20) uses the same technology as the FDA-licensed Procleix WNV assay (2, 11, 17, 33). Briefly, 500 μl of specimen was added to a detergent solution (target capture reagent), and viral RNA was isolated using magnetism-based target capture. An internal control RNA was added to each sample via the target capture reagent to determine individual reaction validity. The captured RNA was washed twice to remove extraneous specimen components and potential amplification inhibitors from the reaction tube. Target amplification occurred via TMA, which is a transcription-based nucleic amplification method. Detection was achieved by hybridization of chemiluminescence-labeled nucleic acid probes. As with the Procleix WNV assay, each reaction was carried out in a single tube from specimen processing to detection, and the entire amount of RNA isolated from the 500-μl specimen was used in the TMA reaction. The assay oligonucleotides target sequences of DENV that are conserved among all four serotypes (12). Testing was performed using the semiautomated Procleix system, in which one operator can complete up to 200 tests in 5 to 6 h (17). The assay can also be carried out on the fully automated Tigris system, which can complete up to 100 tests in 14 h (18, 20). Due to limited sample volumes, all specimens in the study reported here were manually pipetted and tested on the semiautomated platform. Assay results were reported in relative light units, which were used to derive signal-to-cutoff (S/CO) values. Cutoff values for the DENV TMA internal control (IC) and analyte signals were calculated with the same formulae used for the Procleix WNV assay (17). A serum sample was considered reactive if the analyte S/CO was $\geq$1.0, nonreactive if the analyte S/CO was $<$1.0 and the IC signal was above the IC cutoff, and invalid if the analyte S/CO value was $<$1.0 and the IC signal was below the IC cutoff. Comparable sensitivity of the TMA assay in detecting RNA from the four DENV serotypes had previously been established by running the assay on serially diluted stocks of DENV-1, -2, -3, and -4 RNA (18).

DENV multiplex real-time RT-PCR assay. A volume of 280 μl of each sample was processed for RNA extraction using MagAttract virus minikits (Qiagen). Ten microliters of the 50 μl eluted RNA was mixed with 25 pmol of each primer, 2.5 pmol of each serotype-specific probe, and the iScript One Step RT-PCR kit for probe mix (Bio-Rad) to make a 50-μl-final-volume reaction mixture. The previously published NS5 TaqMan real time RT-PCR protocol was strictly followed (4). Briefly, a set of two flavivirus primers was used to amplify a gene segment located near the 3′ region of NS5, flanked by conserved regions among all four dengue serotypes: mFU1 and CFD2. DENV serotypes were detected using the serotype-specific TaqMan probes FAM-D1p-BHQ1, Texas Red-D2p-BHQ2, Cy5-D3p-BHQ3, and HEX-D4p-BHQ1, for which sequences have been reported previously (4). Amplification and real-time fluorescence detection consisted of a 30-min reverse transcription period at 50°C, one initial 15-min incubation at 95°C, followed by 30 s at 50°C and 1 min at 72°C; and 45 continuous fluorescent data collection cycles at 95°C for 15 s and at 48°C for 3 min. To determine the analytical sensitivity of this assay, eight replicas of serially diluted DENV-1, -2, -3, and -4 in vitro RNA transcripts were tested according to previously published protocols (3). Briefly, pCR-II-TOPO plasmids (Invitrogen) containing the PCR amplicons generated with the above-mentioned primers served as templates for in vitro RNA transcription using a DuraScribe T7 transcription kit (Epicentre). This kit introduces two nucleotide analogs that generate RNase-resistant RNA transcripts, resilient to repetitive handling but less sensitive to PCR amplification. The serial dilutions were used to set a standard curve and calculate a linear regression where we determined that one genome copy is equivalent to one RNA transcript. Sensitivity was then established at 1×10^4 to 2×10^4 genome copy equivalents (GCE) per reaction (DENV-1 and -4) for a 100% detection limit with a correlation coefficient of 0.99.

TMA/RT-PCR comparisons. To compare sensitivities of the dengue RT-PCR and TMA, tissue culture stocks of DENV-1, -2, -3, and -4 were available which had been quantified by end-point titration inoculation onto Vero cells to define PFU/ml. These stocks were diluted to 1 PFU/ml and then subjected to serial threefold dilutions using defibrinated human serum (SeraCare, Milford, MA). Real-time RT-PCR was conducted on each dilution in replicas of 8, whereas 20 replicas of each of these dilutions were tested by TMA.

Specimen collection and diagnostic testing. As part of laboratory-based surveillance, physicians submit serum specimens from all patients suspected of having dengue fever or DHF to the CDC-Dengue Branch in San Juan, Puerto Rico, for diagnostic testing. We used a testing algorithm by which sera collected within 5 days of symptom onset were considered acute and were tested by RT-PCR (TaqMan Applied Biosystems, Foster City, CA) to determine the presence of DENV nucleic acid (4). Serum samples collected at least 6 days after symptom onset were considered convalescent phase and were tested for anti-DENV immunoglobulin M (IgM) antibodies using an IgM antibody-capture enzyme-linked immunoabsorbent assay. Acute-phase serum specimens from 388 patients previously collected by this surveillance effort between 2005 and 2006 were randomly selected and deidentified according to protocols approved by Institutional Review Board at the CDC and Gen Probe. Acute-phase specimens were chosen according to the following characteristics: group A, 69 acute specimens with negative DENV RT-PCR results from patients with confirmed dengue diagnosis based on seroconversion with a later convalescent-phase specimen; group B, 199 acute specimens with negative RT-PCR results from patients with suspected dengue illness but who had an indeterminate dengue diagnosis because no convalescent-phase specimen was submitted; group C, 105 acute specimens with a positive RT-PCR result; and group D, 15 specimens with negative RT-PCR and IgM enzyme-linked immunosorbent assay tests that were paired with a convalescent-phase specimen also negative for these two tests.

RESULTS

Comparison between real-time RT-PCR and TMA sensitivities. In order to compare the sensitivities of the multiplexed RT-PCR and TMA assays, serially diluted DENV-1, -2, -3, and -4 stocks were analyzed by real-time RT-PCR in replicas of 8 and by TMA in replicas of 20. The titer of each virus stock had previously been obtained by plaque assay with Vero cells and expressed in PFU per ml. Average cutoff values were obtained for the RT-PCR reactive samples. Using quantitative RT-PCR, the amount of viral RNA in GCE per reaction was obtained. For all TMA-reactive dilutions, the average S/CO values were calculated. At 0.01 PFU/ml, all 20 replicas of DENV-1, -2, -3, and -4 were detected by TMA, whereas at 0.1 PFU/ml, all 8 replicas of DENV-2, -3, and -4 and 3 of 8 replicas of DENV-1 were detected by RT-PCR (Table 1). The results indicate that these two techniques differ in their sensitivities, with TMA being 100% sensitive at dilutions 10 to 100 times higher than the dilutions at which this RT-PCR assay showed 100% sensitivity. These differences were established by comparing 95% detection limits of the 8 RT-PCR and 20 TMA replicas.

TMA testing of dengue surveillance specimens. Serum specimens collected from patients at days postonset (DPO) ranging from 0 to 5 were analyzed by RT-PCR. Group A specimens had negative RT-PCR results, and their corresponding paired, convalescent-phase specimens had positive confirmatory serological results. Group B specimens had negative RT-PCR results and had no accompanying paired specimen. Group C specimens had a positive RT-PCR result. In group A, 55 (80%) of 69 acute-phase specimens were positive by TMA, 12 specimens were nonreactive by TMA, and 2 had invalid results (due to a low internal control signal). In group B, 122 (61%) of the 199 RT-PCR-negative acute-phase specimens were positive by TMA, 75 were nonreactive by TMA, and 2 had invalid results.

TABLE 1. Comparison between RT-PCR and TMA[a]

Virus serotype	Virus concn (PFU/ml)	No. of samples reactive/no. tested by RT-PCR	Avg CT value by RT-PCR	No. of GCE/ml detected by RT-PCR	No. of samples reactive/no. tested by TMA	Avg S/CO by TMA
DENV-1	10	8/8	32.06	2.51E+05	20/20	30.17
	1	8/8	37.13	1.02E+04	20/20	27.66
	0.1	3/8	42.27	4.07E+02	20/20	22.70
	0.01	0/8	NA	NA	20/20	7.50
	0.001	0/8	NA	NA	4/20	2.61
	0.0001	0/8	NA	NA	0/20	0.08
	0.00001	0/8	NA	NA	0/20	0.06
	0.000001	0/8	NA	NA	0/20	0.05
	0	0/8	NA	NA	0/20	0.05
DENV-2	10	8/8	26.96	8.71E+05	20/20	28.78
	1	8/8	31.38	6.47E+04	20/20	28.05
	0.1	8/8	35.28	6.61E+03	20/20	27.51
	0.01	3/8	40.18	3.66E+02	20/20	24.96
	0.001	0/8	NA	NA	20/20	18.49
	0.0001	0/8	NA	NA	11/19	7.82
	0.00001	0/8	NA	NA	1/19	4.01
	0.000001	0/8	NA	NA	1/19	8.08
	0	0/8	NA	NA	0/20	0.11
DENV-3	10	8/8	28.43	2.57E+05	20/20	27.58
	1	8/8	32.76	2.57E+04	20/20	27.24
	0.1	8/8	36.3	3.98E+03	20/20	26.18
	0.01	5/8	41.61	2.40E+02	20/20	22.88
	0.001	0/8	NA	NA	20/20	10.09
	0.0001	0/8	NA	NA	10/20	2.78
	0.00001	0/8	NA	NA	1/20	1.77
	0.000001	0/8	NA	NA	0/20	0.13
	0	0/8	NA	NA	0/20	0.09
DENV-4	10	8/8	29.45	2.69E+05	20/20	26.96
	1	8/8	32.77	4.79E+04	20/20	26.69
	0.1	8/8	37.02	5.25E+03	20/20	24.26
	0.01	3/8	38.52	2.40E+03	20/20	20.67
	0.001	0/8	NA	NA	13/20	10.27
	0.0001	0/8	NA	NA	4/20	7.87
	0.00001	0/8	NA	NA	1/20	27.53
	0.000001	0/8	NA	NA	0/20	0.05
	0	0/8	NA	NA	0/20	0.05

[a] CT, cutoff; NA, not applicable.

In group C, 103 (98%) of the 105 RT-PCR-positive acute-phase specimens were also positive by TMA and 2 specimens had invalid results. Of the 105 specimens from group C, 6 specimens had previously been identified as DENV-1, 41 as DENV-2, 56 as DENV-3, and 2 as DENV-4. The two invalid results in group C corresponded to one DENV-2 specimen and one DENV-3 specimen. The 15 specimens from group D, which had negative IgM antibody tests and were paired with convalescent-phase specimens also having negative IgM tests, showed no TMA reactivity (Table 2).

DISCUSSION

Early detection of DENV in presumptive cases is difficult due to transient, low levels of viremia; therefore, health care decisions are often made in the absence of laboratory confirmation. Delays in identifying dengue cases also hinder our ability to promptly recognize outbreaks. Our unpublished data indicate that approximately 45% of confirmed cases identified through our surveillance system could be detected by the multiplexed RT-PCR assay used in this study. The comparison of limits of quantitation of the real-time RT-PCR versus TMA used in this study demonstrated that the TMA is 10 to 100 times more sensitive than the multiplexed RT-PCR (Table 1). TMA can detect DENV in all RT-PCR-positive serum samples and in 80% of the samples coming from cases later confirmed by seroconversion that had negative RT-PCR results. Therefore, considering the 80% sensitivity of TMA in the remaining 55% RT-PCR-negative specimens, it is reasonable to predict that approximately 89% of all acute-phase specimens received for diagnosis can be confirmed by TMA. This is particularly advantageous given that 70 to 80% of all specimens received for laboratory diagnosis are from the acute, viremic period of disease. Early and rapid detection of DENV in acutely ill patients could expedite case detection and determine clinical management for these patients. High-throughput and sensitive tests could potentially direct and expedite community vector control interventions to prevent the spread of disease.

TMA is highly sensitive and has been used for detection of DENV in healthy blood donations from three Latin American

TABLE 2. Sensitivity of TMA with surveillance specimens[a]

Group	No. of specimens tested	DPO	% R by DPO	% of reactive specimens by group (96% CI)[b]
A	3	0	67	80 (0.071, 0.89)
	4	1	25	
	7	2	83	
	14	3	93	
	18	4	82	
	23	5	87	
	12	0	65	61 (0.54, 0.75)
	24	1	25	
B	40	2	58	
	34	3	42	
	41	4	74	
	48	5	83	
	4	0	100	97 (0.98, 0.99)
	18	1	100	
C	21	2	95	
	30	3	96	
	23	4	100	
	9	5	100	
D	15	1–3	0	NA
Total	388			

[a] % R, percent reactive; NA, not applicable; CI, confidence interval.
[b] Statistically significant association between sample type and TMA reaction; continuity correction applied.

locations (18, 20). Also, in concordance with results of previous studies that showed comparable sensitivity of TMA for detection of the four DENV serotypes (18), our analysis of RT-PCR-positive specimens by TMA showed detection of all serotypes in diagnostic specimens. The reported limit of detection of our RT-PCR assay for different flaviviruses ranges between 10^3 and 10^4 GCE per reaction (3). Our quantitative results using this assay indicate comparable levels of detection for all four DENV serotypes.

Generally, TMA is a highly sensitive method of nucleic acid detection. The reasons for the difference in sensitivity between TMA and the RT-PCR used in our study have not been extensively addressed. The two techniques differ in the following aspects: TMA is a transcription-mediated procedure, whereas RT-PCR is based on DNA amplification; and TMA uses 500 μl of serum as the starting material, whereas RT-PCR uses a portion of the 50 μl RNA elution obtained from 280 μl of serum sample. Therefore, TMA is processing five times more RNA than the RT-PCR used in our comparison. The use of chemoluminescence detection of TMA may provide additional sensitivity compared to the fluorescence detection used in real-time RT-PCR. The RT-PCR method used in our study may also have characteristics of its own that could account for the observed difference in sensitivity. The flavivirus genus-specific primers used in our RT-PCR assay may exhibit some degree of nucleotide mismatch with the DENV genomes, resulting in subamplification of the cDNA. In addition, the RT-PCR assay was conducted in a multiplexed configuration, which may negatively influence sensitivity compared to the single-system configuration (unpublished data). It is then reasonable to speculate that these differences may partially explain TMA's superior sensitivity over that of the multiplexed real-time RT-PCR used in this study. Other RT-PCR assays may exhibit diverse levels of sensitivity (13, 15, 23). A more robust assessment between available single and multiplexed real-time RT-PCR systems is suggested to further establish the extent to which TMA may be able to improve dengue surveillance over that of the most sensitive RT-PCR systems.

The cost effectiveness of using TMA in routine diagnostics will also be addressed. The cost of nucleic acid testing may prove to be prohibitive for many resource-limited areas in the world. However, the recent introduction of viral load monitoring for HIV-1 in several African countries and blood screening for HIV-1, hepatitis C virus, and hepatitis B virus in South Africa suggests the feasibility of performing nucleic acid testing in many areas previously thought to be outside the reach of this type of technology. The cost of the WNV TMA test per specimen currently used for routine blood screening is comparable to that of RT-PCR tests in the United States. If commercialized, the Gen-Probe DENV TMA assay would be expected to have a cost per test similar to that of other qualitative nucleic acid amplification methods.

Although in its present configuration TMA does not distinguish serotypes, its high sensitivity is particularly significant in areas like Puerto Rico, where all serotypes cocirculate and all submitted blood specimens require rapid diagnosis. While serotype-specific TMA tests may be possible in the future, the current TMA test can be efficiently used for laboratory diagnosis of acutely ill patients without the need of late, convalescent-phase specimens for confirmation. TMA is also potentially useful in dengue surveillance of large populations where specimens are tested longitudinally to evaluate epidemic trends.

Our results show that TMA detected dengue RNA in the majority of acute specimens collected between 1 and 5 days of symptoms. Previous studies using a similar TMA for the detection of blood-transfusion-related West Nile virus cases indicate that this assay could detect the virus up to 30 days after transfusion (22). The assay has also been useful in detecting DENV in asymptomatic blood donors (18, 20). We plan to analyze the sensitivity of TMA in detecting DENV in serum samples collected beyond day 5 of illness and determine the feasibility of running this test in a simple, one-specimen testing algorithm. The lack of reactivity of TMA in the confirmed dengue-negative specimens indicates that this sensitive test does not detect false-positive samples.

Secondary infections with DENV may correlate with high viremia levels, which could potentially place patients at risk of more-severe illness (34). In fact, approximately 75% of DENV-2 and 55% of DENV-3 identifications from all reported hospitalized cases in Puerto Rico correspond to patients with previous exposures to DENV (unpublished data). Of the 12 blood donations from Puerto Rico that tested positive by the dengue TMA in 2005, 8 donations presented anti-DENV IgG in the absence of IgM antibodies, indicating secondary exposures to DENV (20). Therefore, TMA, in combination with supporting serological tests, may also help us assess the primary/secondary status of DENV infections in patients presenting with symptoms.

In conclusion, TMA offers the potential for the diagnosis of DENV infection in areas where serum specimens from acutely ill patients are often not currently diagnosed as DENV positive due to the low sensitivity of current tests or a lack of paired

specimens. Use of this highly sensitive test would allow rapid clinical and epidemiological assessments that may translate into improved patient care and more timely community interventions.

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