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A Dry-format Field-deployable Quantitative Reverse Transcriptase-polymerase Chain Reaction Assay for Diagnosis of Dengue Infections

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Abstract. We have systematically evaluated a dry-format, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assay developed by Tetracore Inc. for the Cepheid SmartCycler platform to facilitate rapid diagnosis of dengue virus infections. A panel of related flaviviruses was used to evaluate the clinical specificity of the assay, and it was found to be specific to dengue. Eighty-one clinical samples previously confirmed dengue positive by virus isolation, along with 25 dengue negative control specimens were used to validate this new diagnostic assay. Using these clinical samples, the assay exhibited 98.77% sensitivity and 100% specificity. Over 85% of the clinical specimen exhibited viral loads ranging from 10^3 to 10^7 plaque-forming units per milliliter (PFU/mL). In addition, this dry-format assay is stable at ambient temperatures and requires minimal technical expertise to perform in a small thermocycler platform. These characteristics make it a promising candidate for diagnosis of dengue in mobile laboratories in the field.

INTRODUCTION

Dengue fever is one of the fastest growing global health problems today. Dengue is caused by a single-stranded, positive sense enveloped RNA virus that belongs to the family *Flaviviridae*.[1,2] The virus is transmitted by *Aedes aegypti* and *Aedes abopictus* mosquitoes and is endemic in most tropical and sub-tropical areas where these vectors are found. Nearly 50 million dengue cases are reported each year, and ~2.5 billion people are at risk for infection in sub-tropical climes worldwide.[3] The human immune system recognizes four antigenically distinct serotypes of dengue (i.e., DEN 1–4),[4,5] and a primary infection with any one of them usually results in a sub-clinical or self-limited febrile disease. Symptoms include headaches, pain in various parts of the body, myalgia, arthralgia, leukopenia, and rashes.[2,6] Primary exposure typically results in life-long immunity to that serotype. In some cases, fever from this exposure can progress to dengue hemorrhagic fever (DHF), where the patient experiences considerable fluid loss caused by internal hemorrhage.[3,7] A secondary infection with a different serotype has been shown to substantially increase the risk of DHF, and ~5–10% of secondary cases proceed to this potentially life threatening stage.[8,9] The reasons behind this increased risk for DHF during secondary exposure are not well understood. Furthermore, without proper medical attention DHF can lead to a critical condition known as dengue shock syndrome (DSS). Approximately 500,000 cases of DHF are reported each year, which lead to 25,000 deaths worldwide, mostly among children.[3] Currently, there is no vaccine to prevent dengue and no antiviral drug to cure dengue. The only available treatment option is supportive—bed rest, fluids, and symptomatic relief using analgesics.

Accurate diagnosis is critical to tackling the problems posed by dengue. Distinguishing dengue from diseases with similar initial symptoms such as malaria and influenza can help medical professionals provide appropriate care for the patient in a timely manner. In acute cases, this can involve careful monitoring for signs of hemorrhage followed by intravenous fluid replacement. Accurate identification can also enable officials to focus public health efforts toward better preventative strategies and eradication of vector populations. Traditional laboratory techniques for dengue diagnosis includes viral isolation followed by indirect immunofluorescence assay (IFA), or serologic assays such as plaque reduction neutralization test (PRNT), hemagglutination inhibition (HI), and IgM antibody capture enzyme-linked immunosorbent assay (ELISA).[10–12] However, these techniques are time consuming, labor intensive, and require trained personnel. Some of these techniques can take days or weeks to complete, limiting their value as diagnostic tools. Some assays also require the use of reagents that may be difficult to produce or may not be commercially available. By contrast, relatively new approaches such as quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) offer a promising alternative for sensitive detection of dengue viral RNA in a couple of hours with reasonably modest requirements of infrastructure and training. The objective of this study was to evaluate a dry-format dengue group–specific qRT-PCR assay developed by Tetracore Inc. (Rockville, MD) that can detect all four serotypes of the dengue virus. Several qRT-PCR assays have been developed for the detection of the dengue virus in the past.[13–17] However, this is the first evaluation of a dry-format qRT-PCR assay aiming for field diagnosis by mobile laboratories or small hospitals. We demonstrate that the detection limits of this assay are well below the virus load usually observed in a clinical setting to provide high analytical sensitivity, and we have validated the assay for sensitivity and specificity using a panel of clinical specimens.

MATERIALS AND METHODS

Materials. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) reagents, primers, and probes were obtained from Applied Biosystems Inc. The reaction mixes containing all the reagents except template RNA were dried down in SmartCycler reaction tubes by Tetracore Inc. Dengue viruses used (DEN-1: WP74; DEN-2: 16803; DEN-3: CH53489; DEN-4: 341750) for the evaluation of the qRT-PCR assay where tissue culture-derived laboratory virus

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stocks propagated in Vero monkey kidney cells at the Naval Medical Research Center (NMRC). Known titers in plaque-forming units per milliliter (PFU/mL) of virus stocks were diluted using phosphate buffered saline (PBS), tissue culture medium (minimal essential medium with 10% fetal bovine serum [FBS]), 1% glutamine, 1.5% sodium bicarbonate, and 1% penicillin-streptomycin), or normal human serum as indicated for the experiments. The cell line DNA extracts that were used for cross-reactivity testing, were used at a concentration equivalent to 1,000 cells per reaction. The RNA from related flaviviruses were isolated from viral stocks that were diluted to 10^6 copies/mL. All extractions were done as noted for the dengue samples. De-identified clinical serum samples of dengue viremic patients were obtained using field study human protocols from endemic regions. The Medical Research Center Detachment (NMRCD) in Lima, Peru and the Naval Medical Research Unit-2 (NAMRU-2) in Jakarta, Indonesia collected these samples, tested them for dengue, and shipped them to NMRC for qRT-PCR-based testing. Serum samples from dengue-negative individuals were obtained from a dengue vaccine trial at the NMRC and a blood bank at the Walter Reed Army Medical Center, both from non-endemic areas of the United States. All of these specimens were obtained with Institutional Review Board (IRB) approval.

RNA extraction and qRT-PCR assay protocol. The RNA extraction was performed with a QIAGEN mini-kit using 140 μL of the clinical serum sample or diluted virus stocks, according to manufacturer's protocols (QIAGEN Inc., Valencia, CA). First, qRT-PCR assay tubes containing dried reagents were re-hydrated by adding 22.5 μL of rehydration buffer according to manufacturer's protocol (Tetracore Inc.). The 2.5 μL of the extracted RNA was then added to each reaction tube. The tubes were snapped shut and briefly centrifuged using a bench-top microfuge specifically designed for SmartCycler reaction tubes. The rehydrated mastermix and RNA extract were then mixed by pinching the bulb of the reaction tube 8–10 times. The tubes were then run on the SmartCycler system using the following cycling conditions: a 20 minute reverse transcription (RT) step at 60°C, a 15 second denaturation step at 95°C, followed by 45 cycles of 95°C for 2 seconds and 60°C for 30 seconds. The primer and probe sequences that were used to manufacture the dry-format dengue assay are: GGA CTA GAG GTT AKA GGA G (Forward), GCG TTC TGT GCC TGG AAT GAT (Reverse), and CCC AGC GTC AAT A (Probe). The results were visualized in real-time on the computer monitor. A graphical display of the FAM (6-carboxyfluorescein) dye fluorescence is a direct measurement of qRT-PCR amplification. The *X*-axis represents the qRT-PCR threshold cycle number (Ct value). The *Y*-axis represents the relative fluorescence of the signal. The threshold for the FAM fluorescent dye was manually set at 70 fluorescent units. The ideal shape of qRT-PCR amplification for a positive sample is a sigmoid curve with two inflection points. On the basis of our preliminary study, a sample was considered positive if the signal crossed the set threshold level before cycle 40 (Ct cutoff value based on manufacturer's specifications) and had at least one inflection point. A run was considered valid only if a positive control (RNA extracted from 10^3 PFU/mL of stock virus) tested positive, and a negative control (Tris-EDTA buffer) remained negative until the assay endpoint of cycle 45.

Virus isolation and immunofluorescence assay. The positive and negative clinical samples were diluted 1:10 in tissue culture medium and inoculated into the *Aedes albopictus* mosquito cell line C6/36 cell monolayers. After a 1 hour adsorption of the inoculum onto the cells at 28°C, cell cultures were incubated for 14 days at 28°C. Cells were harvested after 14 days for identification of virus by immunofluorescence assay (IFA) as previously described.[6] The serotype–specific mouse monoclonal antibodies used were 15F3 for DEN-1, 3H5 for DEN-2, 5D4 for DEN-3, and 1H10 for DEN-4. Fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse antibody was used as the detector and the results were observed under a fluorescent microscope.

RESULTS

Limit of detection using dengue virus stocks. To determine the ability of the dengue group assay to detect four dengue serotypes, we made serial dilutions of each virus stock in PBS. The RNA was extracted from 140 μL of the diluted virus using a QIAGEN RNA extraction kit. From an extraction yield of 60 μL, a template volume of 2.5 μL of RNA was tested in each reaction. The lowest level of viral RNA detected by each assay was adjusted from PFU per milliliter to PFU per volume tested by taking into account the original viral titer, the volume used for the RNA extraction procedure (140 μL), and the volume of extracted RNA used as an analyte for testing (2.5-μL sample). Thus, if the stock virus dilution was 1,000 PFU/mL an equivalent of 5.8 PFU (1,000 PFU/1,000 μL × 140 μL × 2.5 μL/60 μL) likely makes it into the qRT-PCR reaction tube, assuming 100% recovery. In PBS, the assay was able to detect as little as 10 PFU/mL of each of the four serotypes (Table 1). This corresponded to a limit of detection of less than 0.05 PFU of dengue virus per reaction tube.

The assay was repeated with the known quantities of virus spiked in tissue culture medium and normal human serum. We spiked tissue culture medium with dengue virus to simulate the environment in which the virus is grown in Vero cells. Similarly, dengue virus stocks were spiked into normal human serum, which was tested negative for dengue virus, to simulate a clinical specimen. The RNA was extracted from these dilutions, and the dengue qRT-PCR assay was performed (Figure 1). The assay performance in tissue culture medium and normal human serum was comparable with a slope of

TABLE 1

Limits of detection in phosphate buffered saline (PBS) and normal human serum; the Ct values of DEN1–4 at different dilutions are provided*

	10^{-1}	10	10^1	10^2	10^3
PBS					
DEN-1	–	–	37.20	30.71	28.36
DEN-2	–	–	34.54	30.95	27.12
DEN-3	–	–	33.82	29.53	25.98
DEN-4	–	–	37.60	30.72	27.08
NHS					
DEN-1	–	–	–	–	28.47
DEN-2	–	–	37.92	32.77	27.85
DEN-3	–	–	–	29.58	27.21
DEN-4	–	–	37.64	30.27	27.43

* Reactions that did not produce a positive result in 40 cycles were considered negative, and are marked with a minus (–).

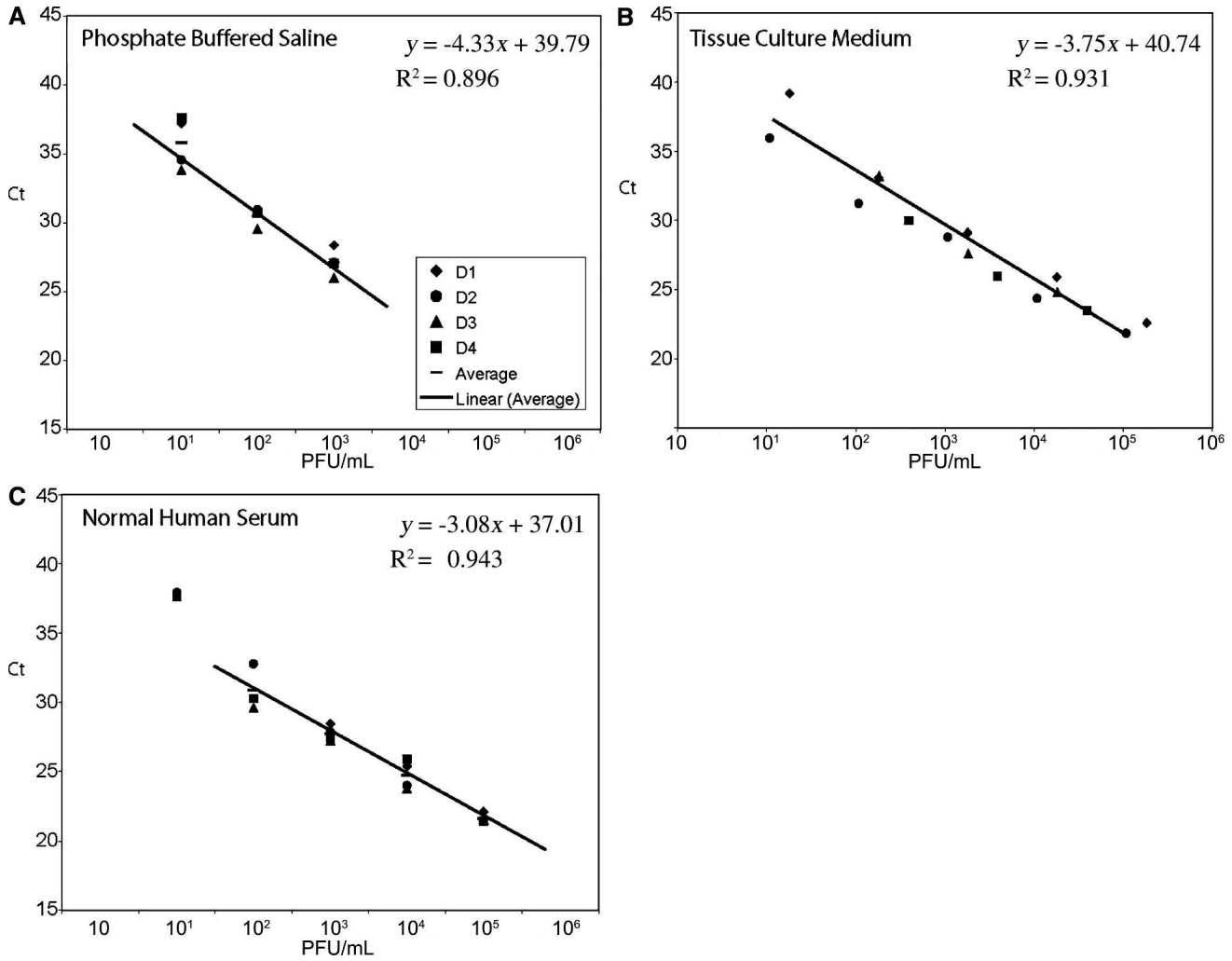

FIGURE 1. Assay performance in different media. Serial dilutions of known quantities of DEN-1: WP74; DEN-2: 16803; DEN-3: CH53489; and DEN-4: 341750 were made in **A**, phosphate buffered saline (PBS), **B**, tissue culture medium, and **C**, normal human serum. The *X*-axis represents the concentration of the live virus stock in plaque-forming units per milliliter (PFU/mL). The *Y*-axis represents the Ct value. Regression lines were also calculated where *y* represents Ct and *x* represents $\log_{10}$ (PFU/mL of virus).

–3.75 and –3.08, respectively, indicating high qRT-PCR efficiency. The regression coefficients (R^2) values were 0.931 for tissue culture and 0.942 for normal human serum, indicating that the various data-points corresponding to different serotypes and dilutions follow the regression line closely. This close correlation also suggests that the regression equations derived from these experiments may be used as standard curves to predict viral loads in dengue viremic samples by dengue qRT-PCR assays.

Validation of dengue qRT-PCR assay using clinical samples. To determine the sensitivity of the dengue qRT-PCR, the assay was performed using 81 de-identified clinical serum samples collected in Peru and Indonesia from patients exhibiting dengue clinical symptoms. These samples were tested positive when collected in Peru or Indonesia for dengue using virus isolation method followed by IFA with anti-dengue antibody. These samples were then shipped to NMRC and tested using the dengue qRT-PCR (Supplementary Table 1, available at www.ajtmh.org). Of these 81 samples, 80 tested positive using the qRT-PCR assay. Using the virus isolation data as the gold standard, sensitivity of the assay was then calculated to be 98.77% (Table 2). Another panel of serum samples was collected from volunteers with no previous exposure to dengue. This panel of 25 samples was collected during a phase-1 vaccine trial at NMRC, and from the blood bank at the Walter Reed Army Medical Center. These negative samples were screened using PRNT and ELISA to con-

TABLE 2

Comparison of quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) dengue assay (test method) with viral isolation (reference method)

	Virus isolation		
qRT-PCR assay	Positive	Negative	
Positive	80	0	
Negative	1	26	
Total	81	26	n* = 107
	% Sensitivity†	% Specificity‡	% Agreement§
	98.77 (80/81)	100 (26/26)	99.06 (106/107)

* n = total number of serum samples tested.

† Capacity to define true positives ([number of assay positive sera that were also viral isolation-positive/total number of viral isolation-positive sera] × 100%); 95% confidence interval (CI) 96.36% to 101.17%.

‡ Capacity to define true negatives ([number of assay-negative sera that were also viral isolation-negative/total number of viral isolation-negative sera] × 100%); 95% cannot be calculated.

§ Accuracy of the assay (number of sera positive by both methods + number of sera negative by both methods/total number of sera tested) × 100%; 95% CI 97.24% to 100.89%.

TABLE 3
Cross reactive panel of closely-related Flaviviruses and cell line DNA extracts

Virus	Strain or source	Dengue qRT-PCR assay
DEN-1	West Pac	Pos
DEN-2	New Guinea C	Pos
DEN-3	CH53489	Pos
DEN-4	H241	Pos
Japanese encephalitis	SA 14-14-2	Neg
Yellow fever	17D-204	Neg
West Nile	NY Crow 394-99	Neg
Murray Valley encephalitis	IITRI	Neg
Kunjin	K5374	Neg
St. Louis encephalitis	TBH-28	Neg
Cell line DNA extracts	**Origin**	
SF9	Spodoptera frugiperda (fall armyworm) pupal ovary	Neg
L929	Mouse, C3H/An, connective, fibroblast	Neg
Hela	Human, black, cervix, carcinoma, epitheliod	Neg
SL29	Chicken, embryonic fibroblast	Neg
Vero 76	Monkey, African green, kidney	Neg
C6/36	Aedes albopictus (mosquito), hatched larvae	Neg
BHK-21	Hamster, Syrian kidney	Neg

firm dengue seronegativity (Supplementary Table 1). Among all 25 normal human serum samples, none of them tested positive for dengue with the dengue qRT-PCR assay, which demonstrates 100% specificity to dengue (Table 2). To determine the microbial specificity of the assay, a cross-reactivity panel of closely related flaviviruses and genomic DNA extracts from other pathogens was used. The viral panel included Japanese encephalitis, yellow fever, West Nile, Murray Valley encephalitis, Kunjin, and St. Louis encephalitis. No cross-reactivity was observed yielding a microbial specificity of 100% based on the selected panel (Table 3). Together, a high sensitivity and specificity for dengue virus combined with low cross-reactivity to related pathogens suggests that this assay can be used effectively for clinical diagnosis of dengue infection.

We used the standard curve generated by making serial dilutions of dengue virus stocks in human serum to estimate the viral titers in patients reporting dengue symptoms. We plotted the predicted viral titers (in PFU/mL) for serum samples that tested positive for dengue using viral isolation followed by IFA and the qRT-PCR assay (Figure 2A). The typical viral load in these patients reporting clinical symptoms was determined to be between 10^4 and 10^5 PFU/mL. Nearly 86% of all dengue–infected individuals had greater than 10^3 PFU/mL of dengue, and 96% had greater than 10^2 PFU/mL (Figure 2B). On the basis of the limit of detection studies using virus stocks diluted in normal human serum (Table 1), the assay was able to successfully detect dengue virus 4 out of 4 times at 10^3 PFU/mL, and 3 out of 4 times at 10^2 PFU/mL. In addition, the dengue qRT-PCR assay successfully detected dengue in all these clinical dengue cases, including those cases below the limit of detection levels. Together, these data indicate that the dengue assay is sensitive and specific for diagnosis of dengue from serum samples during clinical dengue infections.

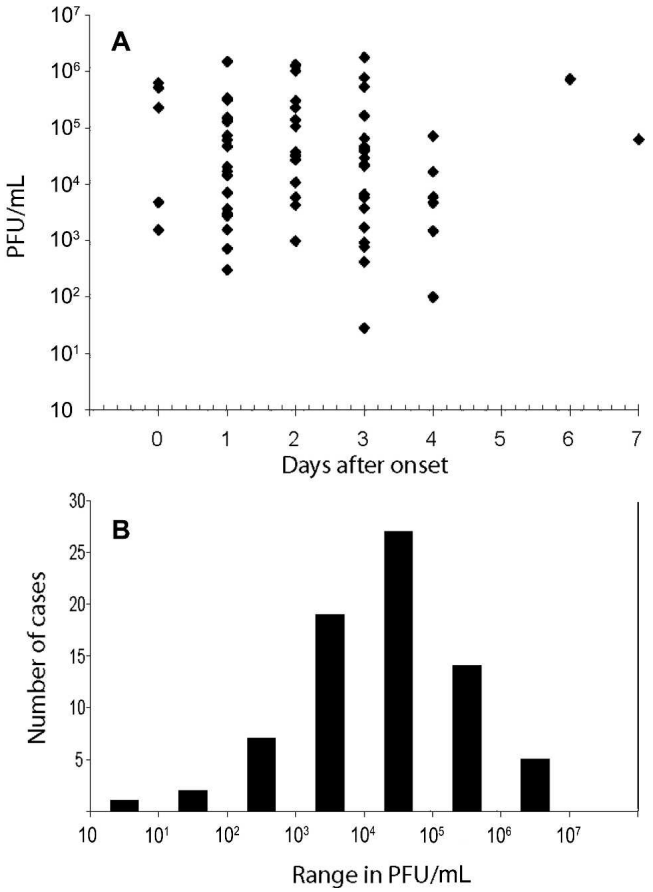

FIGURE 2. Predicted viral load in patients **A**, over days after onset of symptoms or **B**, overall. On the basis of the regression equation derived in Figure 2C from dengue virus spiked in normal human serum, viral titers were calculated for clinical dengue-positive serum samples. Figure A shows how dengue viremia varies over date after onset of symptoms. Predicted viral titer is plotted on the *Y*-axis and days after onset of symptoms are plotted on the *X*-axis. Figure B shows the typical viremia in patients reporting dengue symptoms. The number of clinical serum samples at a given range of viral titers is represented on the *Y*-axis. The viral titer range is given on the *X*-axis.

DISCUSSION

In this study, we characterized the performance of a dry-format dengue qRT-PCR assay. During the course of this

study, we observed that the RNA extraction may be the most critical step in determining the sensitivity of the assay. The RNA extraction efficiency can vary significantly between different kits. One way to further increase the sensitivity of the assay is perhaps to tailor the reaction master mix to accommodate a higher volume of template RNA. For example, if 7.5–10 μL of template can be added to the reaction instead of 2.5 μL, we would have increased the sensitivity by 3- to 4-fold. Higher sensitivity can also be achieved by concentrating the RNA eluate (60 μL) by using techniques such as vacuum centrifugation, or resuspending in a smaller volume. However, on the basis of our subsequent analysis with dengue clinical samples, the assay demonstrated a sensitivity of 98.77%. This indicates that it has sufficient sensitivity to detect the majority of clinical dengue cases.

The purpose of the dengue qRT-PCR assay is to detect viral RNA from the serum of patients suspected of having dengue infection. Human serum is a complex mixture of many different kinds of compounds. Some of these compounds, such as nucleic acids co-extracted from serum or sample-associated microbes, can interfere with both RNA extraction and qRT-PCR efficiency.[18] Before testing clinical serum samples, we therefore decided to evaluate the assay using known quantities of stock virus spiked into normal human serum. The assay was also evaluated using tissue culture medium, a heterogeneous mixture in which virus is often grown *in vitro,* and PBS, which serves a buffered control environment at physiologic pH. No significant difference in assay performance or sensitivity was observed among the three different media. The primers and probes for the assay are designed to bind to the 3′ non-coding region of the dengue genome that is conserved between all four serotypes. High regression coefficients (Figure 2) obtained from our data provides experimental evidence of the low variation between serotypes, as expected by this genetic conservation. For a given starting concentration of virus, there was no significant difference in Ct value resulting from it being extracted from PBS or normal human serum ($P = 0.33$ using a two-tailed students t test statistic for 10^3 PFU/mL). However, in contrast to PBS, when diluted in human serum the assay was less predictable at concentrations less than 10^3 PFU/mL. The assay could detect dengue in all samples containing 10^3 PFU/mL of dengue virus, but only half of the samples containing 10 PFU/mL of the virus when it is spiked into human serum (Table 1).

Viral load has been correlated with the severity of clinical manifestation of dengue.[19] A correlation between viral titer in tissue culture (expressed in PFU/mL), and the limit of detection is particularly important to predict the *in vivo* biologic sensitivity of the assay. Therefore, we generated a standard curve to compare known virus stock concentrations to the Ct value of the qRT-PCR reaction. Although the assay is intended to be a diagnostic device for use in determining the presence or absence of dengue, we believe this standard curve can be used to add a quantitative dimension to the diagnosis. This is especially important for the interpretation of the qRT-PCR results. During the febrile phase, dengue viremia has been shown to range from 10^2 to 10^7 PFU/mL depending on serotype and time after onset.[14,19] Our own data is in broad agreement with these numbers, and nearly 96% of all dengue clinical samples fell within this range (Figure 2B). In the late acute phase serum, viremia may drop to below 10^{-1} PFU/mL[14], below the detection limit of this assay or other direct detection methods such as virus isolation. Immune responses to the virus are usually strong after an infection has been cleared, and therefore serologic approaches to diagnosis usually work well at this stage. For effective clinical diagnosis of dengue through all stages of infection, a combination of qRT-PCR-based and serologic diagnosis should therefore be used. In a typical clinical scenario, however, where the patient exhibits fever symptoms and needs to be diagnosed, the dengue qRT-PCR assay appears to have adequate sensitivity and specificity to diagnose effectively.

Related virus stocks demonstrated no cross-reactivity to the dengue qRT-PCR assay. Regions endemic for dengue, such as parts of Central America and Southeast Asia are often also endemic for other flaviviruses. An accurate diagnosis is especially important in these regions for the health of subjects. While minimizing cross-reactivity with other viruses, the assay also needs to detect the divergent clades of dengue that are prevalent across the world. The assay detected all four serotypes of dengue efficiently.

This dengue qRT-PCR assay is performed on the SmartCycler platform, developed by Cepheid, Inc. (Sunnyvale, CA). This machine has a smaller footprint than comparable PCR machines (e.g., ABI 7700). It is also relatively lightweight and field-deployable. The SmartCycler can run up to 16 reactions concurrently, and can display results in a multitude of ways on the attached computer monitor in real-time. The entire PCR protocol takes approximately one hour and this speed can be a major advantage. Quick diagnosis can enable the caregiver to eliminate other potential causes for the observed symptoms, and tailor the treatment appropriately. There was no loss of performance as a result of the drying down process. In addition, the dry-format of the assay enables easy storage and transportation of the assay at ambient temperatures. Furthermore, accelerated stability testing has determined that the assay can be safely stored at room temperature for over one year establishing a long shelf life. Sophisticated laboratory facilities, reliable refrigeration, or highly trained personnel may not be available in many parts of the world where dengue is most prevalent and its diagnosis most necessary. With half of the world's population living in regions where they are susceptible to dengue fever, the need for sensitive and rapid diagnostic assays is a global medical concern. We have evaluated a qRT-PCR dengue assay that has been developed for use in field conditions. In addition, it can detect dengue effectively and with high specificity. Thus, the assay can become a particularly useful tool for dengue diagnosis throughout the world.

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REFERENCES

1. Rico-Hesse R, 2003. Microevolution and virulence of dengue viruses. *Adv Virus Res 59:* 315–341.
2. Trent DW, Manske CL, Fox GE, Chu MC, Kliks SC, Monath TP, 1989. The molecular epidemiology of dengue viruses, genetic variation and microevolution. Kurstk E, ed. *Applied Virology Research, Virus Variation and Epidemiology*. Plenum, NY: Plenum Press.
3. WHO, *Strengthening Implementation of the Global Strategy for Dengue Fever and Dengue Haemorrhagic Fever, Prevention and Control*. Report of the informal consultation. Geneva, Switzerland: World Health Organization, 1999.
4. Gubler DJ, 1998. Dengue and dengue hemorrhagic fever. *Clin Microbiol Rev 11:* 480–496.
5. George R, Lum L, 1997. Clinical spectrum of dengue infection. Gubler DJ, Kuno G, eds. *Dengue and Dengue Hemorrhagic Fever*. Wallingford, United Kingdom: CAB International, 89–113.
6. Henchal EA, McCown JM, Seguin MC, Gentry MK, Brandt WE, 1983. Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay. *Am J Trop Med Hyg 32:* 164–169.
7. Rigau-Perez JD, Gubler DJ, 1997. Dengue: a literature review and case study of travelers from the United States, 1986–1994. *J Travel Med 4:* 65–71.
8. Halstead SB, 1988. Pathogenesis of dengue: challenges to molecular biology. *Science 239:* 476–481.
9. Rigau-Perez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, Vorndam AV, 1998. Dengue and dengue haemorrhagic fever. *Lancet 352:* 971–977.
10. Teles FR, Prazeres DM, Lima-Filho JL, 2005. Trends in dengue diagnosis. *Rev Med Virol 15:* 287–302.
11. Shu PY, Huang JH, 2004. Current advances in dengue diagnosis. *Clin Diagn Lab Immunol 11:* 642–650.
12. Kao CL, King CC, Chao DY, Wu HL, Chang GJ, 2005. Laboratory diagnosis of dengue virus infection: current and future perspectives in clinical diagnosis and public health. *J Microbiol Immunol Infect 38:* 5–16.
13. Callahan JD, Wu SJ, Dion-Schultz A, Mangold BE, Peruski LF, Watts DM, Porter KR, Murphy GR, Suharyono W, King CC, Hayes CG, Temenak JJ, 2001. Development and evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. *J Clin Microbiol 39:* 4119–4124.
14. Houng HS, Chung-Ming Chen R, Vaughn DW, Kanesa-thasan N, 2001. Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1–4 using conserved and serotype-specific 3′ noncoding sequences. *J Virol Methods 95:* 19–32.
15. Houng HS, Hritz D, Kanesa-thasan N, 2000. Quantitative detection of dengue 2 virus using fluorogenic RT-PCR based on 3′-noncoding sequence. *J Virol Methods 86:* 1–11.
16. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV, 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol 30:* 545–551.
17. Lanciotti RS, 2003. Molecular amplification assays for the detection of flaviviruses. *Adv Virus Res 61:* 67–99.
18. Henchal EA, McCown JM, Seguin MC, Gentry MK, Brandt WE, 1983. Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay. *Am J Trop Med Hyg 32:* 164–169.
19. Cheng ZJ, Hu LH, Fu WR, Li YR, 2007. Rapid quantification of hepatitis B virus DNA by direct real-time PCR from serum without DNA extraction. *J Med Microbiol 56:* 766–771.

SUPPLEMENTARY TABLE 1

Dengue clinical sample assay results

Patient no.	Day of viremia*	Date sample collected (month/day/year)	Ct value	Dengue serotype
Dengue positive clinical samples†				
1	0	5/28/2005	19.18	DEN-3
2	0	1/27/1996	25.69	DEN-2
3	0	7/8/2005	27.19	DEN-1
4	0	4/13/2005	38.94	DEN-1
5	1	7/7/2005	18.00	DEN-3
6	1	6/27/2005	19.99	DEN-1
7	1	6/14/2000	20.09	DEN-2
8	1	4/18/2001	21.06	DEN-2
9	1	4/27/2005	21.13	DEN-1
10	1	7/26/2005	22.04	DEN-3
11	1	4/22/2005	22.29	DEN-1
12	1	1/23/2002	22.62	DEN-2
13	1	3/8/2000	22.94	DEN-4
14	1	2/6/2002	23.73	DEN-2
15	1	6/13/2005	23.98	DEN-1
16	1	5/10/2002	24.17	DEN-1
17	1	5/3/2002	24.22	DEN-1
18	1	5/23/2001	25.14	DEN-2
19	1	1/11/1996	25.17	DEN-2
20	1	7/8/2005	26.05	DEN-1
21	1	2/4/2002	26.28	DEN-2
22	1	5/25/2002	26.34	DEN-1
23	1	5/13/2002	26.41	DEN-1
24	1	7/12/2005	27.17	DEN-3
25	1	5/13/2005	28.23	DEN-1
26	1	8/25/1995	30.85	DEN-2
27	2	5/20/2005	18.16	DEN-3
28	2	4/22/2005	18.21	DEN-1
29	2	5/13/2005	18.50	DEN-1
30	2	7/11/2005	20.51	DEN-3
31	2	2/6/2003	21.18	DEN-1
32	2	5/10/2005	21.54	DEN-3
33	2	3/31/2000	22.06	DEN-4
34	2	11/12/2002	22.94	DEN-1
35	2	8/12/2005	23.09	DEN-3
36	2	8/9/2005	23.18	DEN-3
37	2	7/3/2002	23.36	DEN-1
38	2	5/8/2002	24.60	DEN-1
39	2	9/2/2002	25.42	DEN-1
40	2	1/6/2003	25.83	DEN-1
41	2	6/14/2002	27.80	DEN-1
42	3	1/9/2003	0.00	DEN-1
43	3	5/13/2005	17.77	DEN-3
44	3	5/10/2005	18.89	DEN-3
45	3	6/10/2005	19.38	DEN-3
46	3	5/23/2005	22.19	DEN-3
47	3	7/27/2005	22.65	DEN-3
48	3	5/9/2005	22.74	DEN-3
49	3	7/4/2005	22.81	DEN-1
50	3	7/14/2005	22.89	DEN-3
51	3	5/27/2005	23.26	DEN-3
52	3	5/17/2002	23.61	DEN-2
53	3	5/18/2005	23.63	DEN-3
54	3	7/6/2005	23.69	DEN-1
55	3	5/18/2005	25.25	DEN-3
56	3	5/25/2005	25.26	DEN-3
57	3	2/1/2002	25.43	DEN-2
58	3	5/13/2005	25.99	DEN-3
59	3	7/18/2005	27.05	DEN-1
60	3	7/11/2005	27.88	DEN-3
61	3	5/10/2005	28.12	DEN-3
62	3	4/29/2002	29.37	DEN-2
63	3	5/24/2002	37.45	DEN-1
64	4	5/19/2005	22.06	DEN-3
65	4	6/7/2000	25.40	DEN-2
66	4	7/8/2005	25.70	DEN-1
67	4	5/10/2005	27.25	DEN-3
68	4	7/6/2005	30.98	DEN-1
69	6	2/19/2001	18.97	DEN-2
70	7	7/8/2005	22.26	DEN-3
71	8	7/18/2005	20.06	DEN-3
72	–‡	5/28/2005	21.57	DEN-3
73	–	5/13/2005	22.14	DEN-3
74	–	6/13/2001	23.98	DEN-2
75	–	6/7/2005	24.46	DEN-3
76	–	10/20/1995	32.56	DEN-2
77	–	8/11/2003	35.10	DEN-4
78	–	9/21/2001	35.31	DEN-4
79	–	4/30/2003	30.53	DEN-1
80	–	9/2/2003	32.23	DEN-1
81	–	6/4/2003	35.72	DEN-4
Dengue negative clinical samples				
82	NV§	–	0.00	–§
83	NV	–	0.00	–
84	NV	–	0.00	–
85	NV	–	0.00	–
86	NV	–	0.00	–
87	NV	–	0.00	–
88	NV	–	0.00	–
89	NV	–	0.00	–
90	NV	6/2/2006	0.00	–
91	NV	4/21/2006	0.00	–
92	NV	6/15/2006	0.00	–
93	NV	5/25/2006	0.00	–
94	NV	5/18/2006	0.00	–
95	NV	4/21/2006	0.00	–
96	NV	6/15/2006	0.00	–
97	NV	3/23/2006	0.00	–
98	NV	7/27/2006	0.00	–
99	NV	6/1/2006	0.00	–
100	NV	5/25/2006	0.00	–
101	NV	7/13/2006	0.00	–
103	NV	5/25/2006	0.00	–
104	NV	5/18/2006	0.00	–
105	NV	6/2/2006	0.00	–
106	NV	8/4/2006	0.00	–
107	NV	3/16/2006	0.00	–

* Day of viremia when sample taken [date sample collected – date of onset].
† Dengue virus isolated.
‡ – = viremia date not available.
§ Normal human sera samples.
NV = no virus.