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Virology

Development of a pan-serotype reverse transcription loop-mediated isothermal amplification assay for the detection of dengue virus



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ABSTRACT

During dengue outbreaks, acute diagnosis at the patient's point of need followed by appropriate supportive therapy reduces morbidity and mortality. To facilitate needed diagnosis, we developed and optimized a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay that detects all 4 serotypes of dengue virus (DENV). We used a quencher to reduce nonspecific amplification. The assay does not require expensive thermocyclers, utilizing a simple water bath to maintain the reaction at 63 °C. Results can be visualized using UV fluorescence, handheld readers, or lateral flow immunochromatographic tests. We report a sensitivity of 86.3% (95% confidence interval [CI], 72.7–94.8%) and specificity of 93.0% (95% CI, 83.0–98.1%) using a panel of clinical specimens characterized by DENV quantitative reverse transcription–polymerase chain reaction. This pan-serotype DENV RT-LAMP can be adapted to field-expedient formats where it can provide actionable diagnosis near the patient's point of need.

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1. Introduction

Dengue is one of the fastest growing global health problems today, caused by a single-stranded, positive-sense enveloped RNA virus that belongs to the Flaviviridae family (Trent et al., 1989; Rico-Hesse, 2003). The human immune system recognizes 4 antigenically distinct serotypes of dengue virus (DENV) (i.e., DENV1–4) (George and Lum, 1997; Gubler, 1998). Symptoms can range from mild fever, headaches, myalgia, and rashes (Henchal et al., 1983; Trent et al., 1990) to severe disease characterized by plasma leakage and hemorrhage. Severe dengue can be fatal, especially without intensive medical care. The virus is transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes and is endemic in most tropical and subtropical areas where these vectors are found. An estimated 390 million DENV infections are believed to occur each year, resulting in 96 million symptomatic cases (Bhatt et al., 2013). Neither vaccines nor antiviral treatments are available to reduce DENV-associated morbidity or mortality. The only available treatment option is supportive bed rest, fluids, and symptomatic relief with analgesics.

Diagnosing dengue is important for guiding appropriate supportive care and for alerting the physician to disease-specific warning signs that may require hospitalization; however, it is not possible to make an accurate differential diagnosis of dengue based on clinical features

alone, as many symptoms of dengue resemble those of other diseases, such as malaria, chikungunya, measles, influenza, and rickettsial infections (Teles et al., 2005; Shu and Huang, 2004). Traditional laboratory techniques for dengue diagnosis include viral isolation followed by indirect immunofluorescence assay or serological assays such as plaque reduction neutralization test, hemagglutination inhibition, and IgM antibody capture enzyme-linked immunosorbent assay (Teles et al., 2005; Shu and Huang, 2004; Kao et al., 2005). Some of these techniques can take days or weeks to complete. Furthermore, serological methods require convalescent samples demonstrating an increase in antibody titer from pre-exposure samples in order to make a definitive diagnosis, making it impossible to confirm a diagnosis in the acute stage, limiting the value of serological assays for informing patient management.

Reverse transcription–polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) can be used to identify DENV RNA, which enables diagnosis during the acute phase of dengue infection. This can be an advantage because it facilitates earlier detection of dengue (within 1–7 days post onset of symptoms), before an immune response has been mounted and serology-based diagnostics can detect IgM or IgG levels (typically detectable 5–14 days post onset of symptoms). However, PCR equipment is expensive, requires trained personnel, and is best suited to diagnostic reference laboratories. Other nucleic acid detection strategies based on isothermal amplification such as nucleic acid sequence–based amplification (NASBA) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) have been developed to address these

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challenges and move molecular assays closer to point-of-care use (Jittmittraphap et al., 2006; Neeraja et al., 2015).

Here, we have explored a DENV diagnostic solution using the RT-LAMP technology. The technology was originally developed by Eiken Chemical Company (Notomi et al., 2000) and is based on the principle of strand displacement. Briefly, *Bst* polymerase has both polymerase and strand displacement activity and can be used to amplify nucleic acids using a series of primers that initiate a specific stem loop structure following binding to a target sequence (Notomi et al., 2000; Nagamine et al., 2002). This structure allows the enzyme to polymerize nucleic acids continuously at permissive temperatures beyond 57–65 °C. A variety of methods can be used to visualize the amplified DNA product or the magnesium pyrophosphate by-product. The DNA amplicon can be labeled with an intercalating agent such as SYBR Green and visualized using the naked eye or under UV light. Alternatively, the magnesium pyrophosphate causes turbidity, which can be visualized by the naked eye or using a turbidimeter (Mori et al., 2001). Another benefit of this assay is that it does not require expensive instrumentation and takes less than an hour to perform, making LAMP and RT-LAMP suitable for diagnostic applications in low-resource settings. The technology has been adapted to detect a variety of microorganisms ranging from bacteria such as *Mycobacterium* (Iwamoto et al., 2003); parasites such as *Plasmodium* (Sattabongkot et al., 2014); and viruses such as human immunodeficiency virus (Curtis et al., 2008, 2009), severe acute respiratory syndrome-coronavirus (Hong et al., 2004), and hepatitis B virus (Nagamine et al., 2001, 2002). RT-LAMP has also been evaluated for diagnosing other members of the Flaviviridae family, including yellow fever virus (Kwallah et al., 2013), Japanese encephalitis virus (Parida et al., 2006; Toriniwa and Komiya, 2006), West Nile virus (Parida et al., 2004), and tick-borne encephalitis virus (Hayasaka et al., 2013).

In this study, we developed an RT-LAMP assay to detect all 4 serotypes of DENV in a single reaction. Other groups have explored this concept, with several assays detecting the 4 serotypes of DENV in separate reactions (Neeraja et al., 2015; Parida et al., 2005). Some groups have developed pan-serotype DENV RT-LAMP assays (Teoh et al., 2013), and others have expanded this concept to a pan-flaviviral assay that detects DENV1–4, Japanese encephalitis virus, and West Nile virus in a single reaction (Li et al., 2011). We report a pan-serotype DENV RT-LAMP assay that has been optimized and evaluated with an extensive panel of clinical samples obtained from febrile patients in South America. We have additionally explored a method of reducing assay variability in a DENV quantitative RT-LAMP (qRT-LAMP) that uses a quencher to reduce false positives. Finally, we have also examined methods for streamlining sample preparation and evaluated visualization of the qRT-LAMP product using lateral flow immunochromatographic tests (ICTs) and a handheld fluorescence reader. Together, this represents a complete assay, from sample to result, which can be used to identify DENV with high accuracy in low-resource settings.

2. Methods

2.1. Human use statement

The procedures applied in this study were done in accordance with the ethical standards of the Naval Medical Research Center (NMRC) Institutional Review Board and with the Helsinki Declaration of 1975, as revised in 1983. Study protocols were approved by the NMRC Institutional Review Board (NMRC.D.2000.0006 and NMRC.2005.0007) in compliance with all applicable federal regulations governing the protection of human subjects. Clinical serum samples of DENV viremic patients or those determined to have other febrile illness were collected during ongoing febrile surveillance studies (years 1996–2005) at the Naval Medical Research Unit-6 Peru at regional sites in Piura, Tumbes, Madre de Dios, and Iquitos. The samples were deidentified and shipped to the NMRC. Additional serum samples from DENV-negative individuals were obtained from a dengue vaccine trial at the NMRC and a

blood bank at the Walter Reed Army Medical Center, both in the United States. All of these specimens were obtained with institutional review board approval.

2.2. Reference methods

Tissue culture–derived laboratory virus stocks propagated in Vero African green monkey kidney cells at the NMRC were used as positive controls. The following DENV strains were used to cover all 4 serotypes: DENV1, WP74; DENV2, 16803; DENV3, CH53489; and DENV4, 341750. RNA extraction for these DENV stocks as well as clinical serum samples was performed using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. RNA was eluted in 60 µL of nuclease-free water. Samples were characterized as positive or negative for DENV using a qRT-PCR assay, modified from one previously described (McAvin et al., 2005). Briefly, the sequences of the qRT-PCR primers used were as follows: DENV forward: 5'-GGTTAGAGGAGACCCCTC-3', DENV reverse: 5'-CAGAGATCCTGCTGTCTC-3', probe: 5'-FAM-CAGCATATTGACGCTGGGA-TAMRA3'. We utilized the TaqMan® EZ RT-PCR core reagents kit (Life Technologies, Carlsbad, CA, USA) with a final volume of 25 µL, with the following reaction concentrations: 0.7 µmol/L forward primer, 0.7 µmol/L reverse primer, 0.27 µmol/L probe, 0.2 mmol/L dNTP, 4 mmol/L Mn(OAc)₂, 1X ABI Buffer, 2.5 U rTth Polymerase, and 0.25 U AmpErase. The reaction was held at 50 °C for 2 minutes and 60 °C for 30 minutes, followed by 40 cycles of denaturation at 94 °C for 20 seconds and polymerization at 61 °C for 45 seconds. To quantify RNA copies, the DENV 3'UTR was cloned into the PCR_Script AMP vector (Stratagene, La Jolla, CA, USA), and the transcript was transcribed *in vitro* using the Riboprobe T7 Transcription kit (Promega, Madison, WI, USA). RNA was quantified using a spectrophotometer, and the RNA copy number was calculated. This RNA standard with known copy numbers was serially diluted and run alongside all qRT-PCR reactions to enable quantification of RNA copies in the reaction.

2.3. RT-LAMP design and reaction conditions

RT-LAMP primers were designed against the conserved regions of DENV using Eiken's PrimerExplorer version 4 (<http://primerexplorer.jp/e/>) utilizing DENV2 genome (GenBank accession number FM210242.2) as the initial template. These primers were then aligned with DENV1, 3, and 4, and degenerate primers were manually created to improve sequence consensus. These included 2 outer primers (F3 and B3), 2 inner primers (forward inner primer [FIP] and backward inner primer [BIP]), and 1 loop primer (LoopB). These primers were then aligned with up to 36 published strains each of DENV1, DENV3, and DENV4. Six additional primers were added to broaden detection of these DENV strains: FIPdegen1/3, FIPdegen4, BIPdegen1/3, BIPdegen4, B3degen1/3, and B3degen4.

The RT-LAMP reactions were carried out in a 25-µL volume with the following concentrations: 20 pmol/L FIP, FIPdegen4, BIP, and BIPdegen4; 30 pmol/L FIPdegen1/3, BIPdegen1/3, and LoopB; 2.5 pmol/L F3 and B3 (Integrated DNA Technologies, Coralville, IA, USA); 1X ThermoPol Buffer (New England Biolabs, Ipswich, MA, USA); 0.4 mol/L Betaine (Sigma-Aldrich, St. Louis, MO, USA); 1.4 mmol/L each dNTP (Life Technologies, Grand Island, NY, USA); 8 mmol/L total MgSO₄ (Sigma-Aldrich); 16 U *Bst* DNA Polymerase (New England Biolabs); 2.4 U AMV RT (Life Technologies, Grand Island, NY); and 3 µL of template. Reactions were held at 63 °C for 60 minutes unless otherwise indicated. A positive control template corresponding to 10⁶ copies/reaction DENV RNA was used in RT-LAMP reactions, unless otherwise stated.

2.4. UV fluorescence-based readout

Completed reactions were run on a 2% agarose gel containing 1X SybrSafe (Life Technologies). The RT-LAMP product banding pattern

was also compared against those from true positives. To visualize RT-LAMP product in the tubes, 400X SybrSafe was added. The agarose gels and tubes were visualized using an E-Gel Imager system (Life Technologies). Fluorescence in reaction tubes was also measured using the PicoFluor™ Model number 8000-004 (Turner Biosystems, Sunnyvale, CA, USA).

2.5. Lateral flow immunochromatographic tests

The method of visualizing RT-LAMP product on lateral flow ICTs was adapted from previous reports (Puthawibool et al., 2010; Ding et al., 2010). A home-made ICT strip, which had streptavidin on the detection zone and the mouse anti-FITC antibody conjugated with colloidal gold in the sample pad of the strip, was used for detection of the LAMP product. For visualizing the RT-LAMP product on this system, the reaction was performed as described above except with the addition of Biotin-11-dUTP (Life Technologies) and FITC-12-dUTP both to a final concentration of 10 μmol/L. Following a LAMP reaction, the mixture was heated to 100 °C for 10 minutes and then cooled on ice for 5 minutes. Approximately 0.5 μL of the biotinylated RT-LAMP product was applied to the binding pad of the lateral flow ICT. Running buffer (200 μL of 0.1% Tween 20) was added to facilitate capillary flow, and the lateral flow ICTs were visually inspected after 30 minutes. The presence of a visible purple line was considered reactive.

2.6. Sequence-specific detection

Sequence-specific detection was performed as above, but incorporating a FAM-labeled–LoopB primer instead of the unlabeled primer. To reduce visualization of nonspecific amplification, 60 pmol/L of Black Hole Quencher (BHQ) 1–labeled Antisense LoopB primer was added to the RT-LAMP reaction tube following isothermal amplification. Both the FAM- and BHQ-labeled primers were obtained from Integrated DNA Technologies. The tubes were quenched for approximately 2 hours at room temperature, followed by visualization using a UV source as described above.

3. Results

3.1. Assay characteristics

We designed four sets of LAMP primers to detect all serotypes and multiple strains of DENV in a single reaction. Among these, 1 set of primers was down-selected based on faster time to positivity in a pilot experiment (data not shown). Table 1 summarizes the primers used. The primers target a 3' untranslated region of DENV that is highly conserved between all 4 serotypes and multiple strains. Unlike traditional

LAMP assays, which require 4–6 primers, this pan-dengue RT-LAMP uses 11 primers, including 3 FIP, 3 BIP, and 3 B3 degenerate primers. Using a positive control template, the RT-LAMP reaction was tested within a range of temperatures (57–65 °C, Fig. 1A) using a simple water bath as the heat source. Nucleic acid amplification took place at all the temperatures tested, and subsequent experiments were performed at 63 °C. Next, various assay characteristics were systematically optimized, including primer, enzyme, and betaine concentrations, in order to achieve the fastest time to result (data not shown). Under optimized conditions, 10⁶ copies/reaction of DENV RNA was detectable in approximately 30 minutes when using gel electrophoresis or UV as a readout (Fig. 1B). The assay was designed to be reactive to all 4 serotypes of DENV, and this was confirmed using tissue culture–derived positive strains (Fig. 1C). Using a serial dilution of DENV2, we determined a limit of detection of 10³ copies/reaction where samples were detected reliably a majority (>50%) of the time, comparable to qRT-PCR (Fig. 2A). Fig. 2B illustrates how time to positivity is affected by the number of copies of DENV template present in the reaction. Based on these results, we selected a reaction time of 60 minutes to enable reliable detection of >10³ copies/reaction for all 4 DENV serotypes while minimizing nonspecific amplification. We also saw positive RT-LAMP reactions using DENV-positive samples that had been heat treated for 10 minutes at 100 °C. Boiling samples can eliminate the need for formal RNA extraction steps as has been previously reported with DNA-based pathogen targets (Mikita et al., 2014; Hopkins et al., 2013).

3.2. Clinical performance

A panel of 44 clinical specimens PCR confirmed as DENV positive was used to determine the clinical sensitivity of our optimized RT-LAMP reaction. The panel included DENV1, 2, and 3 naturally circulating in northern Peru at the time of sample collection. DENV-negative samples, including 17 human serum samples from individuals without fever symptoms as well as 40 samples from individuals presenting fever symptoms of unknown origin that tested negative for DENV by qRT-PCR, were used to determine clinical specificity (Fig. 3). Our optimized RT-LAMP assay demonstrated 86.3% sensitivity (95% confidence interval [CI], 72.7–94.8% using binomial confidence interval (Clopper and Pearson, 1934)) and 93.0% specificity (95% CI, 83.0–98.1%). The 6 false-negative samples consisted of 2 DENV1 and 4 DENV3 specimens. Overall, the assay demonstrated a 90.1% agreement with DENV qRT-PCR, underscoring the potential clinical utility of this assay.

3.3. Sequence-specific detection

As has been reported by others, we occasionally observed false-positive results when using our DENV RT-LAMP assay (Curtis et al.,

Table 1 RT-LAMP assay primers used for amplification of DENV.

Primer name	Type	Length	Sequence
FIP DENV1/3	Forward inner	49	GGTTATTCATCAGAGATCTGCTCTCTTTTTTTCGAATCGGAAGCTTCGT
FIP DENV2	Forward inner	49	GGTTATTCATCAGAGATCTGCTCTCTTTTATTCTTTGAGGGAGCTAAGC
FIP DENV4	Forward inner	49	TTCATTTTTCCAGAGATCTGCTCTCTTTTTTTCGAATCGGAAGCTTCGT
BIP DENV1/3	Reverse inner	49	AACGGAAAAAGACGGGTCAACCGTTTTCTTTGTCAGCTGTTGCACAGT
BIP DENV2	Reverse inner	49	AACGGAAAAAGGCGAGAAATACGCTTTTCTTTGTCAGCTGTTGCACAGT
BIP DENV4	Reverse inner	49	AACGGAAAAAGGTGGTTAGACCACCTTTTCTTCCACCAACCCTTGAGGGGT
F3	Forward outer	18	GTGGACCCGACAAGACAG
B3 DENV1/3	Reverse outer	18	GTGAGCAATCCTTTGAG
B3 DENV2	Reverse outer	18	TGCAGCATTCCAAGTGAG
B3 DENV4	Reverse outer	18	GAAAAAAGTCCGGTTGAG
LoopB	Reverse loop	19	GCGAGAGAAACCGCTGTC
FAM-LoopB	Reverse loop	20	[Fam] ^a -CGCGAGAGAAACCGCTGTC
Antisense LoopB	Reverse loop	20	GACACGCGGTTTCTCTCGCG-[Bhq] ^b

Final sequences for the optimized primer combination are given. The forward inner primers and backward inner primers contain a TTTT spacer as suggested in previous literature.

^a Oligo labeled with FAM (5 carboxy fluorescein).

^b Oligo labeled with BHQ.

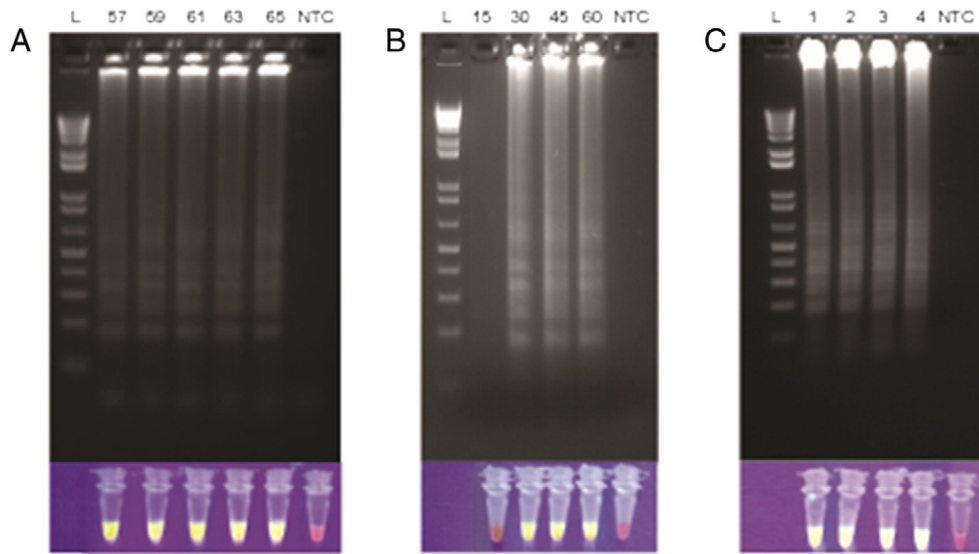


Fig. 1. Characteristics of a pan-dengue RT-LAMP assay. Amplicons generated via RT-LAMP were visualized via the incorporation of SybrSafe using agarose gel electrophoresis or UV illumination. A) 10^6 copies/reaction DENV1 at 57–65 °C, B) 10^6 copies/reaction DENV1 held at 63 °C for 15–60 min, C) 10^5 copies/reaction DENV1, 2, 3, or 4 held at 63 °C for 60 min. NTC = no template control.

2009). Precautionary measures were implemented to reduce risk of contamination from template, and these substantially reduced initial false-positive results. However, another kind of nonspecific amplification was also infrequently observed, which was distinguishable from contamination-derived false-positive results based on a different banding pattern on agarose gels (Fig. 5). It has been hypothesized that this is due to non-template-driven self-priming. Other groups were able to limit this phenomenon by attaching a fluorescent label to their loop primer, followed by the addition of a probe with a fluorescence

quencher (Curtis et al., 2009). As the increased number of primers in our pan-serotype assay may facilitate nonspecific amplification, we examined whether sequence-specific detection was a feasible solution. At the end of the RT-LAMP reaction, any unbound FAM-loop probe was quenched by BHQ-labeled probe (which consists of the reverse complement sequence of the FAM-loop probe with a BHQ modification.) We optimized this formulation and found no difference in the permissible temperature range, time to positivity, or number of serotypes detected when including a fluorescently labeled loop primer (Fig. 5A–C). In

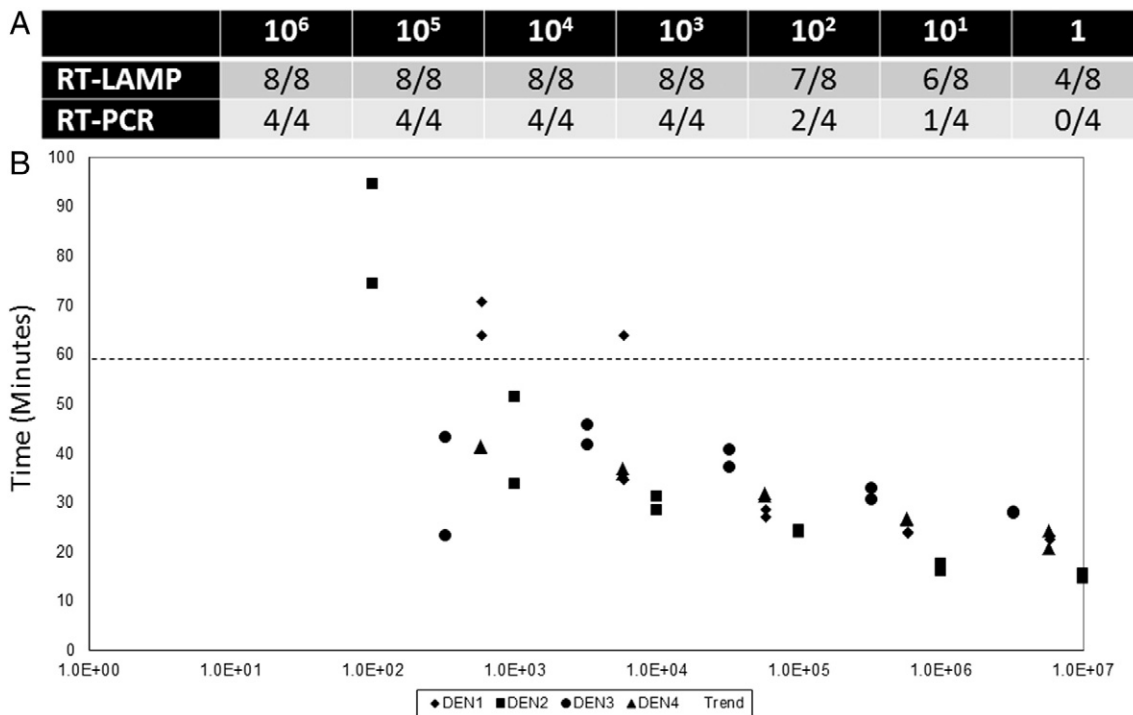


Fig. 2. Comparison of limit of detection between pan-serotype RT-LAMP and RT-PCR. Ten-fold limiting dilutions of DENV2 beginning at 10^6 copies/reaction were used to compare the limit of detection of our optimized pan-dengue RT-LAMP to an in-house RT-PCR assay.

		RT-PCR	
		+	-
RT-LAMP	+	38	4
	-	6	53
Total		44	57

% sensitivity = 38/44 = 86% % specificity = 53/57 = 93%

Fig. 3. Clinical sensitivity and specificity of RT-LAMP. Clinical specimens from subjects suspected of having dengue were tested in triplicate via RT-LAMP and RT-PCR. If 2 out of 3 reactions were positive, the sample was considered positive.

addition, this formulation was able to reduce the nonspecific amplification (Fig. 5D).

3.4. LAMP visualization using lateral flow ICTs and fluorescent readers

In our hands, RT-LAMP readout was not prone to operator error, and the positives and negatives were always clearly distinguishable. However, various methods to visualize RT-LAMP results including real-time PCR thermocyclers, agarose gel electrophoresis, turbidity measurements, and UV illumination are not practical in resource-limited settings. We thus explored the feasibility of determining

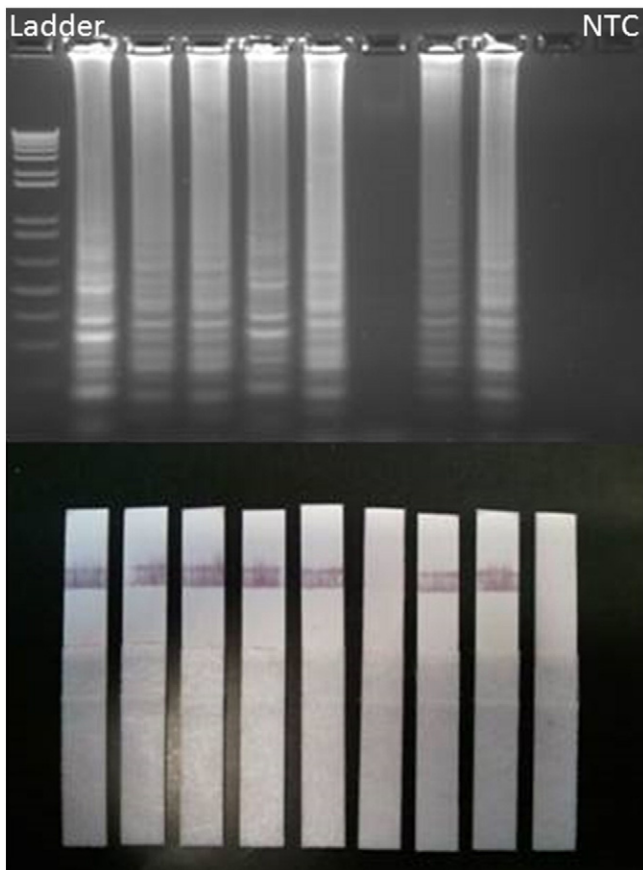


Fig. 4. Evaluation of pan-dengue RT-LAMP ICT. Eight DENV-positive clinical specimens each for DENV1, 2, and 3 were amplified via RT-LAMP and the product run on an agarose gel or applied to an immunochromatographic test. DENV2 results are shown. NTC = no template control.

RT-LAMP positivity via an inexpensive lateral flow ICT. We found 100% correlation between our agarose gel and ICT results when using 24 DENV1-, DENV2-, or DENV3-positive specimens. One specimen that was false negative when visualized via agarose gel electrophoresis was also negative via ICT (Fig. 4), suggesting that ICT has sensitivity comparable to other methods. Our data demonstrate that RT-LAMP results can be reliably determined without the aid of expensive or bulky equipment. We further evaluated the ability of our RT-LAMP assay to be visualized using a battery-operated handheld fluorescent reader and observed the kinetics of quenching over time. True-positive samples remain above a fluorescent threshold of 30 units following 1 hour of quenching.

4. Discussion

Diagnosing dengue accurately during acute infection is critical for informing patient management decisions. Lateral flow ICTs targeting the DENV nonstructural protein 1 (NS1) antigen have had some success in enabling early diagnosis at the point of need; however, nucleic acid tests (NATs) may detect DENV RNA earlier than NS1 antigen, and NATs can be more sensitive and specific. The primary disadvantage with PCR-based NATs involves the need for thermocyclers, which are often bulky and too expensive to be used in resource-limited settings. Commercially available PCR kits cost \$50–100/test and are generally unsuitable for high-throughput usage in developing economies (Dineva et al., 2007; Fiscus et al., 2006). In addition, thermocyclers can cost \$20,000–\$120,000, which may be prohibitive in many dengue-endemic countries (Dineva et al., 2007). Training personnel to operate the instrument and interpret results also requires resources typically only found in a reference laboratory. PCR is also subject to inhibition by substances such as heme compounds, heparin, and EDTA often present in a clinical samples and blood collection tubes, leading to false-negative results (Klein et al., 1997; Fredricks and Relman, 1998); removing PCR inhibitors can lead to increased overhead costs and turn-around time to results. Conversely, PCR is also often subject to reporting false positives from the presence of contaminating nucleic acids (Fredricks and Relman, 1998), necessitating the use of template-free “clean rooms” for preparing a master mix, which is impractical in the field.

To develop a diagnostic solution that overcomes these limitations, we developed a pan-DENV RT-LAMP assay. We demonstrate that the reaction can proceed continuously within a wide temperature range 57–65 °C, making it suitable for a water bath or electricity-free heater (LaBarre et al., 2011). In addition, we have explored various methods of visualizing the assay results. Electrophoresis gel confirmation of reaction products may be performed in a reference laboratory, but in the field, the reaction can be visualized in real time by measuring an increase in turbidity from magnesium pyrophosphate by-products (Mori et al., 2001). We propose a method involving the measurement of color change by the incorporation of SYBR Green adapted from a previous report, which was easier to read with the naked eye (Parida et al., 2005). We also explored visualization using lateral flow ICTs and demonstrated 100% concordance between RT-LAMP visualized on a gel versus those visualized using ICTs. This format is likely to be easier to read in resource-limited settings where a UV light source is not available. Finally, we explored the feasibility of using a handheld UV fluorescence reader, which when coupled with a quencher, can provide higher specificity detection of DENV. The handheld UV reader that we used was approximately \$500, and such readers could improve accuracy of readout as well as provide some level of quantitation to RT-LAMP results. LAMP has several other advantages over traditional PCR, including less sensitivity to inhibitors such as ethanol, isopropanol, EDTA, and sodium acetate (Mori et al., 2004; Kaneko et al., 2007). The use of loop-specific primers can reduce assay times to provide faster sample to result than PCR, and other groups have reported higher sensitivity (Nagamine et al., 2002). LAMP also does not require the template to be denatured

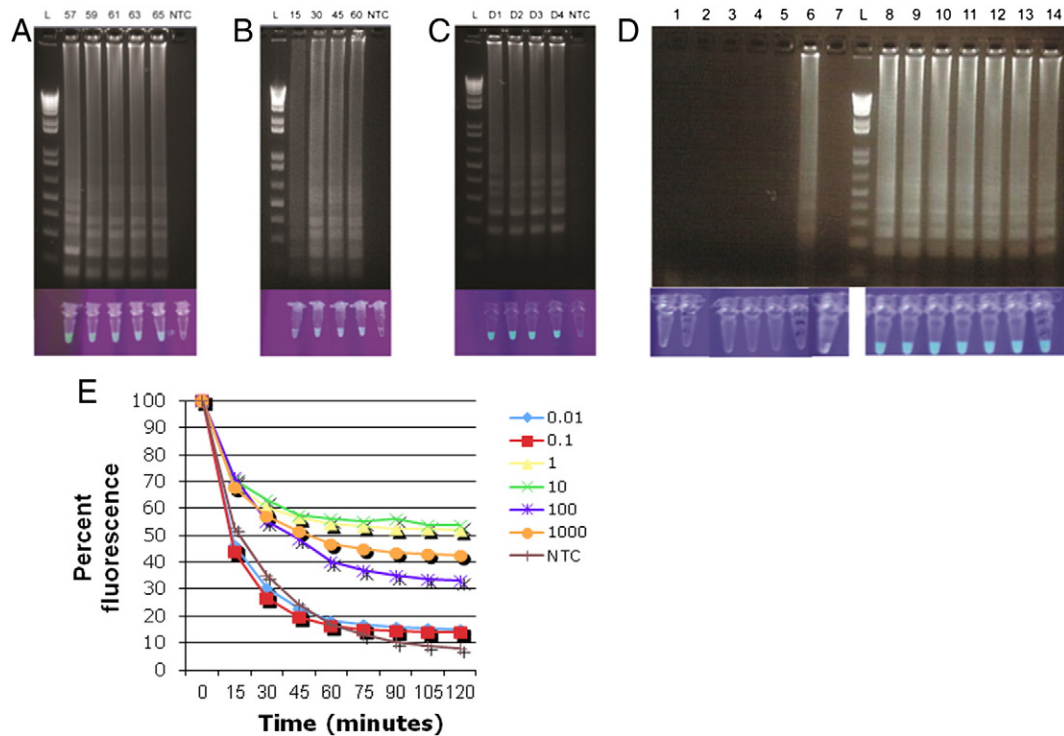


Fig. 5. Sequence-specific detection. Amplicons generated via sequence-specific RT-LAMP were visualized via agarose gel electrophoresis or UV illumination 90 minutes after the addition of a reverse complement loop quencher. A) 10^6 copies/reaction DENV1 at 57–65 °C, B) 10^6 copies/reaction DENV1 held at 63 °C for 15–60 minutes, C) 10^6 copies/reaction DENV1, 2, 3, or 4 held at 63 °C for 60 minutes, D) 7 replicates each of NTC (lanes 1–7) or DENV3 (lanes 8–14) after 60 minutes at 63 °C. Reactions were quenched for 2 hours prior to UV illumination. E. Quenching reaction measured using Picflour shows detection of RT-LAMP product following quenching. NTC = no template control.

(Nagamine et al., 2001), and contrasted with NASBA (a similar isothermal NAT), LAMP does not require a separate nucleic acid extraction step (Francois et al., 2011). This robustness further reduces the cost as well as time to result.

Like qPCR, qLAMP is amenable to multiplexing (Tanner et al., 2012), and we adapted it to detect all 4 serotypes of DENV in 1 reaction. Our data demonstrate that the use of degenerate primers is a feasible method by which to design assays for targets of interest even when sequence conservation is limited. The assay was able to detect a majority of DENV PCR-positive clinical samples obtained from South America (sensitivity of 86.3%), representing multiple circulating strains of DENV1–3; however, the clinical performance is limited with only 44 dengue-positive clinical specimens from 1 region and lack of inclusion of DENV4-positive specimens. The assay was also specific to DENV and did not react to other pathogens that cause similar fever-like symptoms. This demonstrates the assay's effectiveness for enabling differential diagnosis in a clinical situation with multiple strains and serotypes. Since this is a molecular assay, it will likely be able to detect DENV in mosquito samples and have further applications for vector surveillance. Due to the robustness of LAMP, it may be used without explicit sample preparation in applications where having false negatives for low copy number samples are acceptable and with integrated sample preparation where it is not. Acute DENV viremic patients have 10^3 – 10^{12} plaque-forming units/mL (Vaughn et al., 2000), and such high titers are detectable by LAMP without explicit sample preparation. The assay reagents can also be lyophilized for field expediency and provided in a single tube to which one may add hydration buffer and test sample. The assay can be run at a range of temperatures and can be performed using a water bath or other available heat source. We also envision the assay readout adapted to specific applications and present UV visualization, handheld fluorescence readers, and lateral flow ICTs as viable alternatives.

In conclusion, this work adds to the growing body of isothermal amplification assays available to detect DENV. More than previous reports,

we have explored all aspects of DENV detection from sample to result, with an emphasis on visualizing the RT-LAMP product, as readout is critical for ensuring accuracy. The assay can be adapted to use in dengue-endemic countries where it is most needed to reduce morbidity and mortality associated with this widespread disease.

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