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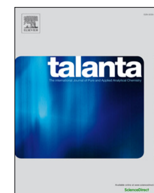

Rapid discriminative detection of dengue viruses via loop mediated isothermal amplification

Jong-Gil Kim[a], Seung Hoon Baek[a], Seungrok Kim[a], Hae In Kim[a], Seung Woo Lee[a], Le Minh Tu Phan[a], Suresh Kumar Kailasa[a,b], Tae Jung Park[a,*]

[a] Department of Chemistry, Institute of Interdisciplinary Convergence Research, Research Institute of Halal Industrialization Technology, Chung-Ang University, 84 Heukseok-ro, Dongjak-gu, Seoul 06974, Republic of Korea

[b] Department of Applied Chemistry, S. V. National Institute of Technology, Surat 395007, Gujarat, India

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ABSTRACT

Dengue virus (DENV) is one of the life-threatening viruses to the human. In this study, we have designed specific novel primers for rapid discriminative detection of DENV-1, DENV-2, and DENV-4 by real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) reaction. The effect of parameters such as reaction temperature and magnesium sulfate was investigated on the RT-LAMP reaction for detection of DENV RNA. Under the optimal conditions, this method is able to differentiate and to detect DENV within 25 min, exhibiting detection limit of 3.5 copies/μL. Importantly, the novel specific primers-based RT-LAMP assay did not react with other viruses, suggesting the selectivity of the method towards DENV RNA. The RT-LAMP reaction products are easily visualized with naked-eye when irradiated them under UV light at 365 nm. Amplification products could be visualized directly for color changes. This method provides a facile, and accurate molecular amplication technique for the rapid discriminative detection of dengue viruses. The RT-LAMP platform can be used as a promissing diagnostic tool for discriminative detection of DENV without aid of complicated protocols or sophisticated equipment.

1. Introduction

The dengue virus is a single-stranded positive-sense RNA, which belongs to the family *Flaviviridae*, genus *Flavivirus*. Dengue virus exists as four genetically different serotypes, i.e., DENV-1, DENV-2, DENV-3, and DENV-4 with 11 kb positive-stranded RNA genome [1,2]. Generally, it is transmitted to humans via various mosquito vectors namely Aedes aegypti (A. aegypti), and A. albopictus, A. polynesiensis, A. Pseudo scutellaris [3,4]. It causes either severe dengue fever or dengue hemorrhagic fever or dengue shock syndrome, which also shows a spectrum of clinical illnesses (dengue fever, dengue hemorrhagic fever and dengue shock syndrome) [5]. The World Health Organization reports reveal that 390 million dengue cases have been registered in every year in worldwide [6], which causes even life-threatening to humans [7]. In view of this, a facile, rapid, and reliable diagnostic tool is essentially required for descriminative detection of DENV RNA, which helps to implement effective treatment for dengue virus infections.

Various molecular biology techniques including virus isolation, serological (hemagglutination inhibition, and immunoglobulin G (IgG) and immunoglobulin M (IgM) specific DENV antibodies based enzyme-linked immunosorbent assay (ELISA)), and molecular diagnostic (reverse-transcription polymerase chain reaction (RT-PCR) and real-time PCR) techniques have been applied to diagnosis of dengue infections [8,9]. Further, NS1 Ag strip® test kit was also used for non-quantitative dengue detection that can useful to control DENV transmission [10]. Even though these diagnostic tools are effectively identified dengue virus, unfortunately these exhibit several limitations such as specific equipment with high level technical skills, cross-reactivity, and expensive nucleic acid amplification, which severely limit their wider accessibility for diagnosis of dengue infections [11].

Recently, loop-mediated isothermal amplification (LAMP) has revolutionized as one of the promising biomolecular technique for the rapid diagnosis of various microbiological infections including dengue disease since its discovery by Notomi's group in 2000 [12,13]. Further, reverse transcription loop-mediated isothermal amplification (RT-LAMP) has proven to be a single-step biomolecular technique for nucleic acid amplification, in which reverse transcriptase was used for the generation of complementary DNA from RNA at a constant temperature [14]. This technique is considered as a rapid and sensitive tool for effective diagnosis of various microbial diseases, which requires 4–6

* Corresponding author.
E-mail address: tjpark@cau.ac.kr (T.J. Park).

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primers for the specific recognition of 6–8 distinct regions of the target DNA [15,16]. It has an ability to amplify near about 10^9 copies within 1.0 h, which avoids nonspecific binding and provides high specificity for point-of-care diagnostic assays of various viral diseases. As a result, this technique has been used for the detection of dengue virus in various clinical samples [17–21].

In LAMP assay, the post-amplication process is required for the identification of LAMP DNA products, leading to either cross-reaction or non-specific identification of LAMP products. To overcome these difficulties, significant efforts have been made on LAMP technique to enhance the selectivity and sensitivity for point-of-care testing of viral infections [22,23]. These drawbacks were effectively resolved by using intercalating dyes (SYBR Green and EvaGreen), turbidity analysis, and agarose gel electrophoresis, respectively. Further, the use of intercalating dyes as fluorescent probes has proven to be an efficient platform for the visualization of LAMP DNA products with high sensitivity, which could be observed with naked-eye [18].

In this study, we explore the use of novel primer sequences in RT-LAMP technique for discriminative detection of three dengue viruses (DENV-1, DENV-2, and DENV-4). Importantly, the RT-LAMP assay products of DENV-1, DENV-2, and DENV-4 were easily visualized with naked-eye when irradiated them under UV light at 365 nm. This method exhibits several advantages, i.e., high sensitivity, selectivity and rapidity (30 min), which signifies that it can be used as a promising technique for rapid, accurate and discriminative detection of DENV viruses in clinical samples.

2. Experimental

2.1. Materials

Viral lysates and purified viral RNA of DENV types were obtained from Korea Center for Disease Control (Cheongju, Korea) and Korea Bank for Pathogenic Viruses (Seoul, Korea). Viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). DENV specific primers were procured from GenoTech (Daejeon, Korea). 10X Isothermal Amplification Buffer II, magnesium sulfate, dNTP Mix, *Bst* 3.0 DNA Polymerase and AMV Reverse Transcriptase were purchased from New England Biolabs (Ipswich, MA, USA). EvaGreen fluorescent DNA stain was purchased from Bio-Medical Science (Seoul, Korea). Ultrapure water was obtained from Welgene (Gyeongsan, Korea). UV–visible spectrophotometer (Optizen NANO-Q, Mecasys, Daejeon, Korea) was used for the estimation of RNA concentration.

2.2. Design of RT-LAMP primers

To screen each type of DENV, the full sequence of DENV was obtained from Genbank (National Center for Biotechnology Information). The Genbank numbers of three dengue viruses (FJ687475, KP406804, and KP406806) were provided by Korea Center for Disease Control and Korea Bank for Pathogenic Viruses (Cheongju, Korea). The specific primers that specifically amplified the DENV RNA sequences were designed by using a Primer Explorer V5 program from the Eiken website (http://primerexplorer.jp/e/). The primers that amplify specifically were identified using the basic local alignment search tool (BLAST). All designed primers were obtained from GenoTech Corporation.

2.3. RT-LAMP reaction

The RT-LAMP reaction was performed by mixing the following solutions, i.e., 1.0 µL of each inner primer (FIP and BIP, 1.6 µM), 1.0 µL of outer primer (F3 and B3, 0.2 µM), 3.5 µL of deoxynucleotide solution mixture (1.4 mM, dATP, dCTP, dGTP and dTTP), 2.5 µL of 10X isothermal amplification buffer II (1 ×), 1.5 µL of $MgSO_4$ (8 µM), 1.0 µL of *Bst* 3.0 DNA polymerase (320 U/mL), 0.5 µL AMV reverse transcriptase (200 U/mL), 0.5 µL of EvaGreen (1 ×), 9.5 µL of ultrapure water, 2.0 µL of a purified viral RNA solution of DENV in 25.0 µL PCR tube. The reaction was carried out at different temperatures ranging from 60° to 72°C for 40 min. To visualize the RT-LAMP products, the above solutions were treacted with 20 µL of Evagreen and then color changes were observed under UV light illumination at 365 nm. In order to know the best conditions for RT-LAMP assay, we have investigated the effects of reaction temperature and magnesium sulfate concentration on RT-LAMP reaction for effective DENV detection. The sequence amplification was carried out by using the Real-Time Thermal Cycler (MiniOpticon™ Detector, Bio-Rad, Hercules, CA, USA) and confirmed by gel electrophoresis, which was checked using ChemiDoc (ChemiDoc™ MP Imaging System, Bio-Rad).

3. Results and discussion

3.1. Optimization of RT-LAMP conditions

To achieve the best amplification signal of RT-LAMP reaction, the effect of parameters, i.e., reaction temperature and magnesium sulfate concentration was investigated. Fig. S1 shows the RT-LAMP assay for the detection of dengue viruses (DENV-1, DENV-2, and DENV-4) at various temperatures in the range of 60 – 72 °C. It can be observed that the temperatures of 69.7, 65.0 and 66.5 °C were found to be optimal RT-LAMP reaction temperatures for the detections of DENV-1, DENV-2, and DENV-4, respectively.

It is well-known that magnesium sulfate acts as a cofactor during RT-LAMP reaction, which can reduce the charge interactions between double-stranded DNAs. Magneisum is essentially needed to proceed with RT-LAMP reaction, however, the lower or higher concentration of magnesium can be strongly influenced on the RT-LAMP reaction as well as reaction products [24]. In view of this, we have investigated the effect of magnesium sulfate concentration (6.0–10.0 mM) on RT-LAMP reaction at optimal reaction magnesium sulfate concentration (Fig. S2 in Supporting Information). The best RT-LAMP reaction was observed using magnesium sulfate concentrations at 6.0 mM for DENV-1 and DENV-4, and at 8.0 mM for DENV-2. Based on these observations, the RT-LAMP assays were performed for the detection of DENV-1, DENV-2 and DENV-4 at optimul reaction conditions.

3.2. Sensitivity of RT-LAMP assay for the detection of dengue viruses

After RNA extraction, the RNA concentration was estimated by using UV–visible spectrophotometer. The extracted RNA was diluted 10-times with ultrapure water and used as a template for RT-LAMP reaction. The copy number was calculated based on the amplicon size and the measured concentration of extracted RNA from dengue viruses. The amplicon sizes of DENV-1, DENV-2 and DENV-4 are 180 bp, 198 bp and 203 bp, respectively. Analytical efficiency of the method was evaluated by plotting calibration graphs between cycle threshold (Ct) values and $\log_{10}$ RNA copy number (Fig. 1a–c). Since the concentration of RT-LAMP products was efficiently estimated by measuring the Ct values of real-time PCR, which is equal to the time-to-positive parameter [25]. As a result, good linearities were observed between cycle threshold (Ct) values and concentration of dengue viruses ranging of $6.23 \times 10^3 - 6.23 \times 10^9$, of $7.91 - 7.91 \times 10^9$ and of 5.98×10^2–5.98×10^9 copies/µL for DENV-1, DENV-2, and DENV-4, respectively (Fig. 1a-c). The method exhibits detection limits of 33.00, 3.55 and 9.06 copies/µL for DENV-1, DENV-2, and DENV-4, respectively. This method was effectively discriminated against three dengue viruses, and the analysis was completed within 25 min (the maximum amplification time was within 5 min), signifying the potentiality and versatility of the method for rapid diagnosis of dengue diseases with high degree.

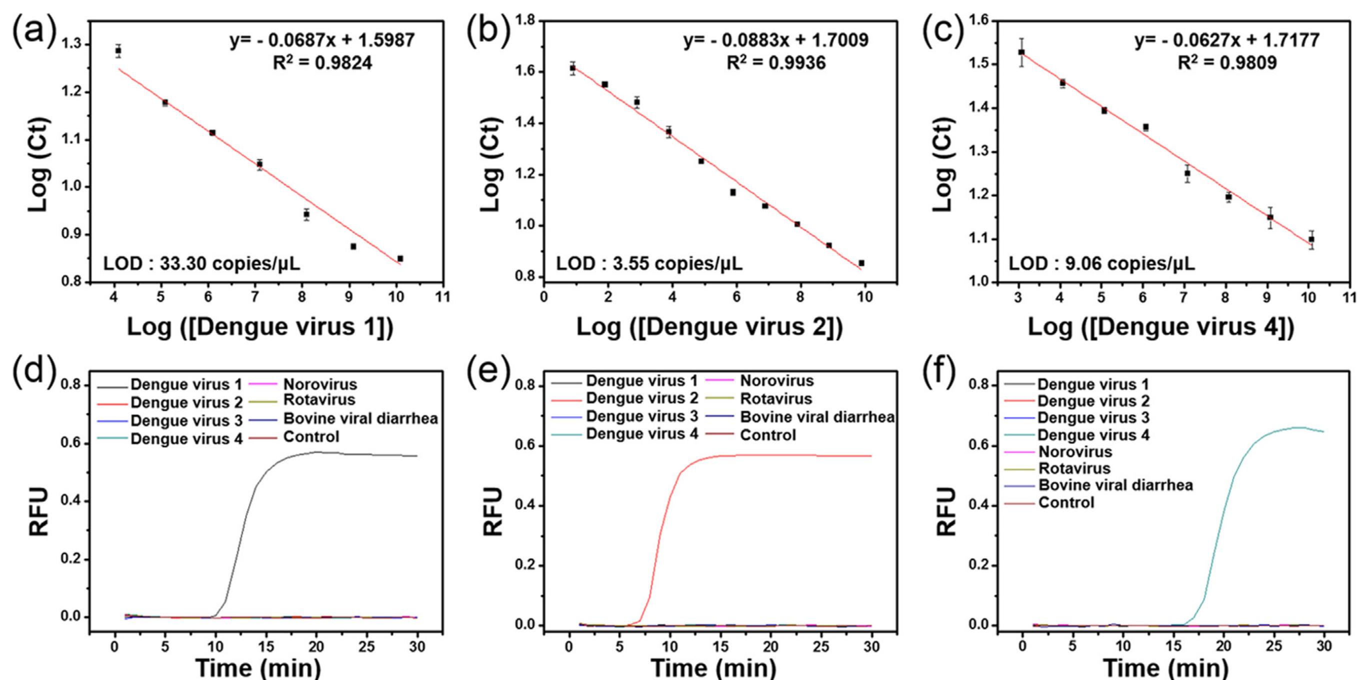

Fig. 1. Sensitivity of RT-LAMP assay for the detection of (a) DENV-1, (b) DENV-2, (c) DENV-4 using newly designed primer sets. Specificity of RT-LAMP assay for the discriminative detection of (d) DENV-1, (e) DENV-2, (f) DENV-4 using newly designed primer sets, respectively.

3.3. Specificity of RT-LAMP for the detection of dengue virus

In order to evaluate the specificity of RT-LAMP assay for the detection of three dengue viruses, the RT-LAMP reaction was carried out separately by using newly designed primers with RNA of various viruses (DENV 1–4, norovirus, rotavirus and bovine viral diarrhea) at optimal reaction conditions. As shown in Fig. 1d–e, the RT-LAMP reaction was proceeded only with RNAs of DENV-1, DENV-2 and DENV-4 and not with RNAs of other viruses (DENV-3, norovirus, rotavirus and bovine viral diarrhea), confirming the specificity of the designed primers for the discriminative detection of three dengue viruses via RT-LAMP assay. Furthermore, we have also confirmed the specificity of the method for the specific amplification of designed primers with RNAs of three dengue viruses. As shown in Fig. S3 of Supporting Information, the RT-LAMP reaction was specifically proceeded only with RNAs of DENV-1, DENV-2 and DENV-4 using the newly designed primer sets. To monitor RT-LAMP assay, EvaGreen dye was added to the above RT-LAMP reaction products and observed their color changes with naked-eye. EvaGreen dye is more effective than SYBR Green I, because SYBR Green I can affect as an inhibitor into the reaction [20,26]. It can be observed that the RT-LAMP reaction products of DENV-1, DENV-2 and DENV-4 RNAs exhibit green fluorescence whereas others show orange and yellow fluorescence under UV light illumination at 365 nm (Fig. 2). In a day light, the bright green colors were observed only with the RT-LAMP reaction products for DENV-1, DENV-2 and DENV-4 RNAs (Fig. S4 in Supporting Information). The other non-fluorescent 7 samples showed the specificity and selectivity of the RT-LAMP reactions. These observations revealed that the specific binding of newly designed primers with RNA of DENV, suggesting the potentiality of the method for accurate recognition of DENV. RT-LAMP products can be detected by monitoring the increase in fluorescence by adding a fluorescence dye to the RT-LAMP reaction mix.

3.4. Evaluation of the present method

To evaluate the performance of RT-LAMP assay for DENV detection, we have provided sequences of the newly designed primers used in the study and already used primers in the literature [27] in Table 1. The present method requires only four primer sets for each DENV detection whereas the reported method needed six primer sets, which explores that the significance and simplicity of the method for the rapid and discriminative detection of three DENV. The four primers were used that were more effective and economical than the six primer set to obtain the best performance and specific and sensitive data. To order to estimate the rapidity of the method, we have measured the amplification signals of RT-LAMP reactions using newly designed primer sets and conventional primer sets (Fig. 3). It can be noticed that the maximum intensity of the amplification signal was achieved within 15 min by

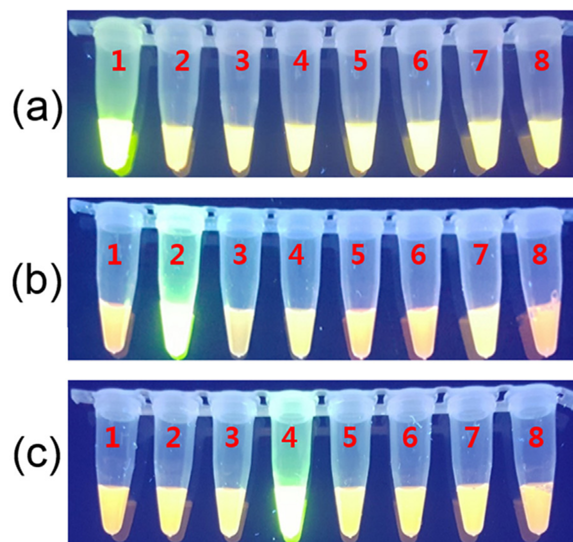

Fig. 2. Visualization of RT-LAMP reaction products of DENV RNA under UV light illumination at 365 nm. Amplification of (a) DENV-1, (b) DENV-2, and (c) DENV-4 using conventional PCR with EvaGreen. DENV-1 (1), DENV-2 (2), DENV-3 (3), DENV-4 (4), norovirus (5), rotavirus (6), bovine viral diarrhea (7), and deionized water (8) were used for the amplifications templates, respectively. Green fluorescent signals represent their specific detections for the targets.

Table 1
Comparison of the newly designed primer sets with the conventional primer sets of the reported method for the detection of DENV *via* RT-LAMP assay.

This study				Reported method (Parida et al. [27])		
Dengue virus	Genome position	Primers	Sequence (5′→3′)	Genome position	Primers	Sequence (5′→3′)
DENV-1	1247–1426	F3	AGACACTGCATGGGACTTTG	10469–10667 (199 bp)	F3	GAGGCTGCAAACCATGGAA
	(180 bp)	FIP	TGCGGTTCCAAAAACCTGGTGT-GTTCCATAGGAGGGGTGTTC		FIP	GCTGCGTTGTGTCTTGGGAGGTTTT CTGTACGCATGGGGTAGC
		B3	AGAGTGACGTGCTCCTTGAA		B3	CAGCAGGATCTCTGGTCTCT
	Four primers	BIP	ATGGGGTCTTGTTCAGCGGTG-AGCCATGTCAGCAGAATCC	**Six primers**	BIP	CCCAACACCAGGGGAAGCTGTTTTTTT GTTGTTGTGCGGGGG
		–	Not required		FLP	CTCCTCTAACCACTAGTC
		–	Not required		BLP	GGTGGTAAGGACTAGAGG
DENV-2	3089–3286 (198 bp)	F3	GTTAAAAGTTGCCACTGGC	10449–10659 (211 bp)	F3	TGGAAGCTGTACGCATGG
	Four primers	FIP	CCAGCGAGATTCTTTGGAATTATCA-GTCACACACTCTTTGGAGC	**Six primers**	FIP	TTGGGCCCCCATTGTTGCTGTTTT AGTGGACTAGCGGTTAGAGG
		B3	ACTGTAGTTCCTTTGCAGAA		B3	GTGCCTGGAATGATGCTG
		BIP	CAGTGTCACAACACAATAACAGACC-CCATCTCAAGTTTGCCTAGA		BIP	GGTTAGAGGAGACCCCCCCAATTTT GGAGACAGCAGGATCTCTGG
		–	Not required		FLP	GATCTGTAAGGGAGGGG
		–	Not required		BLP	GCATATTGACGCTGGGA
DENV-4	615–817	F3	ACCGAACCTGAGGACATTGA	10289–10517 (229 bp)	F3	CTATTGAAGTCAGGCCAC
	(203 bp)	FIP	CGTTCCCCACTCTGAGTGCATG-TTGCTGGTGCAATCTCACG		FIP	TGGGAATTATAACGCCTCCCGTTTTTT CCACGGCTTGAGCAAACC
		B3	CCCTCTGAGCATGTTTCCAA		B3	ACCTCTAGTCCTTCCACC
	Four primers	BIP	GAAGCGCTCAGTAGCCCTAACA-CCCCTTCCGATGACATCCA	**Six primers**	BIP	GGTTAGAGGAGACCCCTCCCTTTT AGCTTCCTCCTGGCTTCG
		-	Not required		FLP	GGCGGAGCTACAGGCAG
		-	Not required		BLP	TCACCAACAAAACGCAG

using newly designed primer sets whereas conventional primer sets required longer time (~30 min) to generate amplification signals. Notably, the newly designed primer sets were effectively produced the amplification signals with high intensity than the conventional primer sets, revealing the potential utility of the newly designed primer sets to speed up RT-LAMP reaction. In addition, gel electrophoresis was also studied for the confirmation of RT-LAMP reaction products using four newly designed primer sets and six conventional primer sets for each virus (Fig. 4). Remarkably, the newly designed primer sets allow an effective separation of RT-LAMP reaction products of DENV RNA. Further, it was noticed that the RT-LAMP reaction products of DENV RNA using conventional primer sets were not well separated due to the

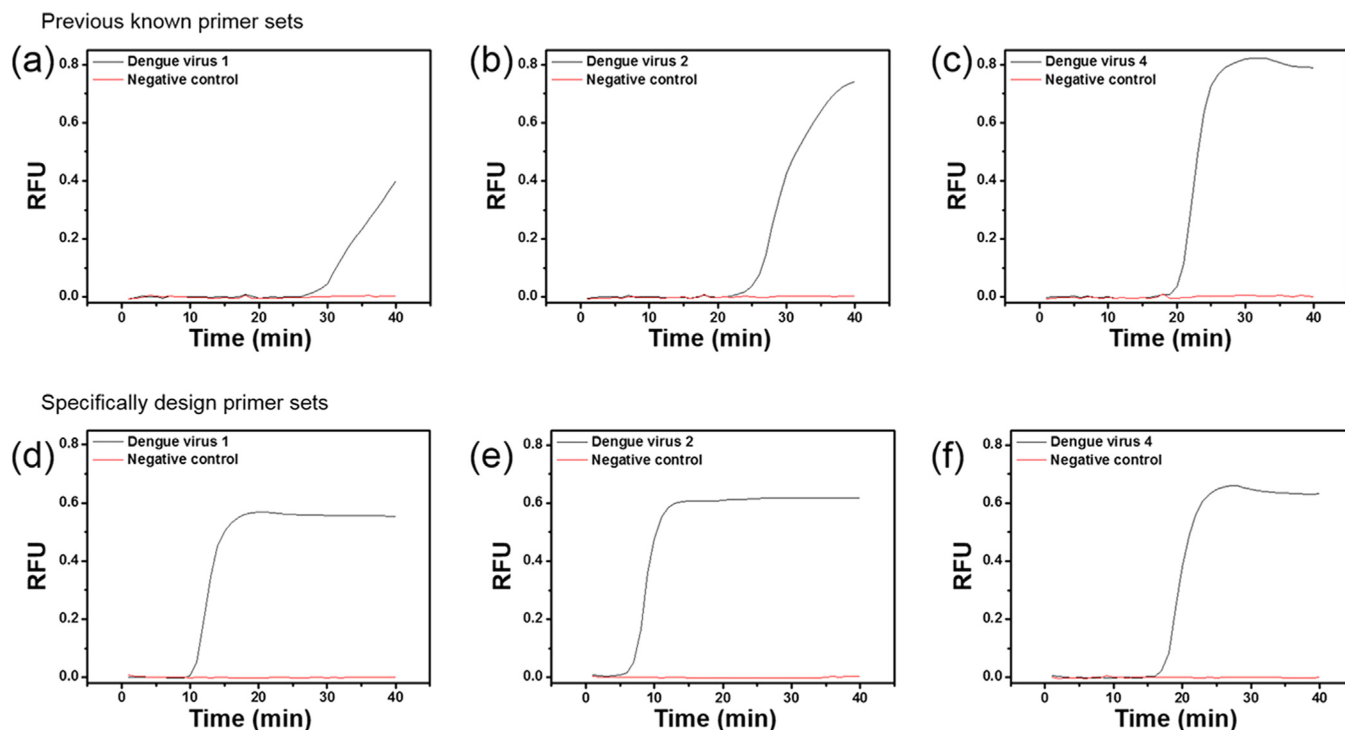

Fig. 3. Measurement of amplification signals of RT-LAMP reaction products of (a) DENV-1, (b) DENV-2, and (c) DENV-4 RNA using previous known primer sets at 63 °C for 40 min. Measurement of amplification signals of RT-LAMP reaction products (d) DENV-1, (e) DENV-2, and (f) DENV-4 RNA using newly designed primer sets at 65 °C for 40 min. Negative control indicates the reaction without template.

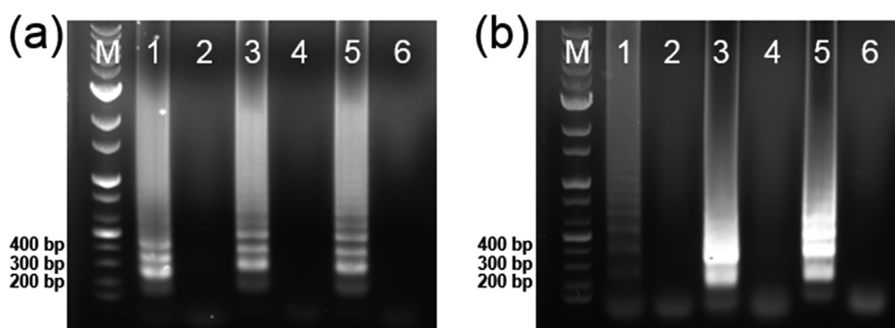

Fig. 4. Separation efficiency of RT-LAMP reaction products of DENV RNA using (a) newly designed four primers for each DENV detection and (b) already used six primers for each DENV detection. 1 kb DNA ladders (M); DENV-1 (lane 1); without DENV-1 (lane 2); DENV-2 (Lane 3); without DENV-2 (lane 4); DENV-4 (lane 5); without DENV-4 (lane 6). 1.2% agarose gel was used for the separation of RT-LAMP products.

Table 2
Comparison of present method with the reported methods for the detection of Dengue virus.

Analytical method	Detected entity	Limit of detection	Reaction time (min)	Reference
ELISA	Structure viral antigens	15 μg/mL	240	Young et al. [28]
Real-time RT-PCR	Viral RNA	250 RNA copies/mL	180	Sudiro et al. [29]
Fourplex Real-time RT-PCR	Viral RNA	100 PFU/mL	75	Johnson et al. [30]
Silicon Nanowire Biosensor	Peptide nucleic acid	1 fg/mL	30	Zhang et al. [31]
RT-LAMP	Viral RNA	1 PFU	60	Parida et al. [27] previous primers
RT-LAMP	DENV-1 RNA	33.00 copies/rxn	25	**This study**
RT-LAMP	DENV-2 RNA	3.55 copies/rxn	25	
RT-LAMP	DENV-4 RNA	9.06 copies/rxn	25	

dimerization of primers. Thus, the newly designed primers have shown high ability for specific interactions with RNA of DENV, favouring effective RT-LAMP amplication with moderative, which enhances the RT-LAMP assay for rapid diagnosis of dengue virus infections. Furthermore, as a result of the comparison of the dengue virus detection method reported by the present method, the RT-LAMP reactions using newly designed primer sets confirm within a short time as shown in Table 2 and Table S1.

4. Conclusions

In this work, we described the use of newly designed primer sets in RT-LAMP assay for rapid and discriminative detection of three dengue viruses (DENV-1, DENV-2, and DENV-4). This method shows good linearity between Ct values and DENV concentration in the range of 1.0×10^{0}–1.0×10^{10} copies/μL with detection limit of 3.5 copies/μL. Compared to the conventional primer sets-based RT-LAMP assay, this RT-LAMP assay exhibits several attractive features, i.e., (i) the maximum amplification signal was achieved within 5 min, (ii) requires only four primer sets for RT-LAMP reaction, (iii) the entire analysis was completed within 25 min, (iv) the RT-LAMP reaction products were well separated on gel electrophoresis, and (v) the RT-LAMP reaction products of DENV RNA are visualized with naked-eye. Thus, the use of newly designed primer sets was significantly enhanced the versatility of RT-LAMP assay for discriminative detection of three DENV, which can be useful for high-throughput point-of-care diagnosis of dengue infections.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2018.08.019.

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