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Next-generation diagnostic test for dengue virus detection using an ultrafast plasmonic colorimetric RT-PCR strategy

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HIGHLIGHTS

- An ultrafast PPT-RTcPCR is developed for DENV RNA detection.
- The limit of detection of DENV2-specific PPT-RTcPCR was 1.6 copies/\textmu L.
- The clinical performance of PPT-RTcPCR was comparable to the reference RT-qPCR.
- The PPT-RTcPCR has the potential for point-of-care detection of infectious pathogens.

GRAPHICAL ABSTRACT

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ABSTRACT

The current global COVID-19 pandemic once again highlighted the urgent need for a simple, cost-effective, and sensitive diagnostic platform that can be rapidly developed for distribution and easy access in resource-limited areas. Here, we present a simple and low-cost plasmonic photothermal (PPT)-reverse transcription-colorimetric polymerase chain reaction (RTcPCR) for molecular diagnosis of dengue virus (DENV) infection. The assay can be completed within 54 min with an estimated detection limit of 1.6 copies/\textmu L of viral nucleic acid. The analytical sensitivity and specificity of PPT-RTcPCR were comparable to that of the reference RT-qPCR assay. Moreover, the clinical performance of PPT-RTcPCR was evaluated and validated using 158 plasma samples collected from patients suspected of dengue infection. The results showed a diagnostic agreement of 97.5% compared to the reference RT-qPCR and demonstrated a clinical sensitivity and specificity of 97.0% and 100%, respectively. The

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simplcity and reliability of our PPT-RTcPCR strategy suggest it can provide a foundation for developing a field-deployable diagnostic assay for dengue and other infectious diseases.

1. Introduction

Global attention has been placed on the pandemic of the coronavirus disease 2019 (COVID-19) for the past three years. Most health resources, such as human resources, hospital care, laboratory testing, and public health services, were dedicated to COVID-19, posing a significant threat to the health system of all countries worldwide. In contrast, other illnesses, like dengue fever, suffered a substantial delay in diagnosis and hospital admissions, especially in low-income countries with a more fragile health system [1,2]. Dengue fever is the world’s fastest-growing mosquito-borne disease caused by dengue virus (DENV), an RNA virus belonging to the family Flaviviridae [3]. In the past few decades, dengue infection has spread rapidly within tropical and subtropical countries, placing nearly a third of the global human population at risk [4–9]. Nowadays, it is one of the most significant public health problems in many resource-limited countries, menacing human life in different aspects and causing immense pressure on the medical management system [10–12]. More importantly, the combined impact of the COVID-19 pandemic and dengue epidemics can potentially cause devastating consequences for patients in hyper-endemic regions with multiple serotypes of DENV [9,12]. Without effective vaccines and specific treatment, diagnosing DENV infection in its early stages is critical to improve clinical outcomes and prevent disease transmission [13]. A simple, cost-effective, and sensitive diagnostic test for use in the field is essential for disease control, especially in remote areas with limited access to healthcare providers and facilities. However, diagnostic tools with such capabilities have yet to be readily available.

Biosensors are versatile analytical devices that convert biological responses into processable and measurable signals. Since their invention in 1962, biosensors have been extensively researched and developed for their potential applications in medical diagnosis [14]. Traditional serological and virological methods have been integrated into biosensing platforms to improve diagnostic efficiency and accuracy [15]. Immunosensors are well-established biosensors that utilize antibodies as the recognition element and detection of specific antibody-antigen interaction [16]. Although immunoassay techniques, such as enzyme-linked immunosorbent assay (ELISA), are simple and convenient, detecting viral antigens at the early stage of infection is still challenging because of the low level of viral proteins in serum [17–19]. Moreover, interpreting serological results is still challenging due to a broad antigenic cross-reactivity to different DENV serotypes and other flaviviruses [20–22].

Alternatively, nucleic acid amplification technology (NAAT) has been explored as a molecular diagnostic tool for numerous viruses and contributed significantly to healthcare [23,24]. Compared to traditional virus isolation, these methods have achieved more sensitive, accurate, and rapid diagnosis [25]. In particular, NAAT-based reverse transcription polymerase chain reaction (RT-PCR) remains the gold standard for detecting many RNA viruses [26–28]. Owing to its ability to amplify a specific sequence exponentially, RT-PCR is currently the most sensitive and specific detection method of viral RNA [29]. However, most RT-PCR assays must be performed in special laboratories or facilities. Moreover, despite their established efficiencies, developing RT-PCR-based detection methods to point-of-care (POC) applications is hindered by its lengthy detection time, sophisticated instrumentation, and high energy consumption [30,31]. Furthermore, although isothermal nucleic acid amplification technologies can be applied in resource-limited settings, they require complex primer design and may have low sensitivity [32,33].

In recent years, photothermal metallic nanoparticles and films that provide low-energy light-based platforms for temperature control have shown great potential for developing a new generation PCR [34–37]. The light-to-heat conversion by plasmonic nanomaterials, known as the plasmonic photothermal (PPT) effect, can be utilized as an efficient heat source for rapid thermocycling [36,38]. Therefore, portable PPT-based PCR devices have been fabricated by combining the photothermal effect of plasmonic nanostructures to circumvent the critical limitations of conventional PCR, such as the need for sophisticated instrumentation, high energy consumption, and lengthy detection time [33,39–41]. However, these devices still rely on gel electrophoresis or fluorescent signal detection to analyze the PCR amplification products, requiring additional post-processing or expensive equipment [42,43]. As a result, it overshadows the advantages of plasmonic thermocycling and impedes its further development and application in remote locations. Previously, we developed a novel PPT-based PCR coupled with colorimetric assay (PPT-cPCR) for simple, fast amplification and sensitive detection of nucleic acids [44]. Based on the photocatalytic activity of double-stranded DNA-SYBR Green I dye (dsDNA-SGI) complex, 3,3′,5′-tetramethylbenzidine (TMB) can be oxidized upon blue light emitting diode (LED) illumination after PPT-based amplification, resulting in the color change of the mixture from colorless to blue color that can be visually distinguished with the naked eye. Based on this platform, a PPT-RcPCR was developed for DENV detection, and its performance was evaluated. The analytical sensitivity, specificity, and clinical performance of the assay were assessed using RNA extracted from tissue culture fluid (TCF) and human clinical samples (e.g., plasma), respectively, and compared with the reference real-time RT-qPCR recommended by the Centers for Disease Control and Prevention [28]. The results showed that the DENV-specific PPT-RcPCR provided a simple and sensitive nucleic acid detection platform with a rapid readout at a low cost, which can be significant in resource-limited areas.

2. Materials and methods

A detailed description of materials, instruments, synthetic methods, PPT-RcPCR optical device setup, cells and viruses, clinical specimens, and statistical analysis can be found in the Supplementary Material.

2.1. In vitro transcribed RNA preparation

The analytical performance of the DENV-specific PPT-RcPCR assay was first assessed using in vitro-transcribed (IVT) RNA. Briefly, the partial sequence of the E gene of the DENV-2 NGC strain (nt 1453 to 1550; GenBank accession AF038403) was synthesized and cloned into the pGEM-3Z vector downstream of the T7 promoter sequence. The resulting plasmid pGEM-3Z-DENV was linearized by Sall restriction digestion and subsequently used as the template for in vitro transcription using an mMESSAGE mMACHINE™ T7 Transcription Kit (Invitrogen™, USA). Residual RNA was removed using TURBO Dnase (Invitrogen™, USA). Single-use IVT RNA aliquots were placed at ~80 °C. Ten-fold serial dilutions of the IVT RNA (1 to 10⁶ copies/μL) were used to determine the analytical sensitivity of the PPT-RtPCR. The concentration of the IVT RNA was calculated as described previously [45].

2.2. Nucleic acid extraction

For analytical sensitivity analysis, viral RNA was extracted from TCF containing 1.15 × 10⁶ focus forming unit (FFU)/mL of DENV2 and subjected to 10-fold serial dilutions (10⁴ to 10⁶) in nuclease-free water. For analytical specificity analysis, viral RNA was extracted from TCF samples containing DENV1, DENV4, ZIKV, CVB3, or FIPV. The archived human plasma samples were used for nucleic acid extraction without...
Fig. 1. Workflow of the PPT-RTcPCR platform. (A) Workflow with a timeline for PPT-RTcPCR platform combining PPT-based RT-PCR with colorimetric detection. (B) TMB-based colorimetric readout. In a negative sample, no specific amplification occurs. Without the formation of the dsDNA-SGI complex, TMB cannot be oxidized, resulting in a minor color change. In a positive sample, amplicons increase exponentially. TMB can be further oxidized by the dsDNA-SGI complex, resulting in a noticeable color change from colorless to blue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. Characterization and photothermal performance of PMNs. (A) TEM images of PMNs with a core-shell structure. (B) High-angle annular dark-field (HAADF) image and elemental mapping of PMNs (C) Ultraviolet-visible (UV-Vis) spectrum of PMNs. (D) Magnetization curve of PMNs. (E) The temperature profile of the solution with or without PMNs upon illumination. (F) Effect of PMN concentration on heating and cooling ramp rate. The optimal ramp rate was observed at 16 OD PMNs. (G) Representative temperature profile for PPT-based RT-PCR. The thermocycling began at 50 °C (5 min), followed by 40 cycles of 90 °C (0 s) and 60 °C (8 s). (H) Enlarged temperature profile for isothermal reverse transcription. (I) Enlarged temperature profile for PCR amplification.
further dilution. All samples were extracted using a MagMAX™-96 Viral RNA isolation kit (Applied Biosystems, USA) by KingFisher Flex System (Thermo Fisher, USA). After the extraction steps, nucleic acids were eluted with 90 μL nuclease-free water. All nucleic acids were stored at −80 °C for further use.

2.3. DENV-specific PPT-RTcPCR description and optimization

The DENV-specific PPT-RTcPCR was developed as described previously with some modifications [28,45]. Target DENV2 RNA was first amplified through a PPT-based RT-PCR in the presence of plasmonic magnetic nanoparticles (PMNs). The reaction mixture contained 5 μL of 2 × reaction mix, 0.2 μL of SuperScript™ III RT/Platinum™ Taq Mix, 0.1 μL of 50 μM forward (5′-CAG GCT ATG GCA CYG TCA CGA T-3) and reverse (5′-CCA TYT GCA GCA RCA CCA TCT C-3) primers, 2 μL of PMNs with optical density (OD) of 80, 1 μL of viral nucleic acid, 1.6 μL of nuclease-free water, and 20 μL of mineral oil. The thermocycling protocol began at 50 °C for 5 min, followed by 40 cycles between 90 °C (0 s) and 60 °C (8 s). After the PPT-based RT-PCR, 10 μL of the colorimetric solution containing 2-(N-morpholino) ethanesulfonic acid (MES) buffer (8 μL, 0.1 M), 40 mM TMB (1 μL) and 80 × SGI (1 μL), was added to the reaction solution. Utilizing the magnetic properties of PMNs, the particles were collected with a magnet before blue light LED (300 mA, 14 V) irradiation for 2 min. With the photocatalytic activity of the dsDNA-SGI complex, TMB was oxidized under the excitation of blue light LED by adjusting the pH value of the solution to around 5 [44,46]. The absorbance of the resulting mixture was measured by NanoDrop™ OneC Microvolume UV–Vis Spectrophotometer (Thermo Scientific™).

The reference real-time DENV2 RT-qPCR was performed as described previously with modifications [28]. Briefly, the reaction mixture contained 5 μL of 2 × reaction mix, 0.2 μL of SuperScript™ III RT/Platinum™ Taq Mix, 0.1 μL of 50 μM forward and reverse primers, 0.18 μL of 10 μM probe (5′-FAM-CTG ATG GCA CYG TCA CGA T-3), 1 μL of viral nucleic acid, and 3.42 μL of nuclease-free water. The RT-qPCR was performed using CFX Opus 96 Real-Time PCR system (Bio-Rad) with the following protocol: 50 °C for 30 min, 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. For the reference real-time RT-PCR, the samples with Ct values larger than 37 would be considered negative since the amplification results were difficult to ascertain.

3. Results and discussions

3.1. Overall workflow from sample to signal

The overall workflow of DENV nucleic acid detection using the PPT-RTcPCR platform, from sample collection to target detection, is depicted in Fig. 1. Firstly, the viral RNA extracted from plasma samples of patients suspected of dengue infection was subjected to DENV-specific PPT-RTcPCR reaction. In this system, PMNs were employed as nano-sized heaters for homogeneous heating. When an infrared (IR)-LED is illuminated, the reaction temperature can be easily and precisely controlled by tuning the light intensity. The heating protocol begins with an isothermal process for the reverse transcription of RNA into complementary DNA (cDNA), followed by two-step thermocycling for cDNA denaturation and specific target extension. Next, amplicons are visually detected using a TMB oxidation-based colorimetric strategy by adding the colorimetric solution containing MES buffer, SGI, and TMB. The amplified dsDNA is the reactant for forming the photocatalyst, triggered only when the SGI is intercalated in the dsDNA. Upon blue light LED irradiation, singlet oxygen is generated by energy transfer from SGI to dissolved oxygen in the solution, which is responsible for the subsequent TMB oxidation. The resulting solution changes from colorless to blue color, making amplicon generation visible. Without viral RNA, there would be no specific RT-PCR amplification, no formation of dsDNA-SGI complex, and thereby no oxidation of TMB so that the solution would remain colorless. Although this is a simple strategy without the requirement of complex instrumentation, there are only a few reports using a combination of the TMB oxidation method with PCR detection, much less applying it for clinical assays. Such a simple design enables our device to be developed into a POC diagnostic system.

3.2. Characterization of the PPT-RTcPCR platform

The PMNs were used as nano-sized heaters since they provide efficient PPT-based thermocycling and convenient magnetic separation. The particle had a magnetic iron oxide core enclosed by a plasmonic Au shell (Fig. 2A). Iron oxide cores prepared by solvothermal reaction were first functionalized with an amine-terminated surface by organosilane. Then, small AuNPs were attached to the core surface via electrostatic assembly, followed by the growth of an Au shell. To increase the stability of PMNs and minimize the nonspecific interaction during amplification, PMNs were finally modified with methoxy-polyethylene glycol (mPEG)-thiol. The elemental mapping images indicated the core was entirely encapsulated by the shell (Fig. 2B). In addition, PMNs showed a strong and broad plasmon wavelength, matching the peak wavelength of IR-LED used in our device (Fig. 2C). Magnetic measurements also confirmed that PMNs had good magnetic properties after shell coating with a magnetic moment of 4.75 emu/g (Fig. 2D).

Using PMNs in the PPT-based optical device (Fig. S1), the temperature of the particle-containing solution increased dramatically under IR-LED illumination, while there was no noticeable temperature change in the solution without PMNs (Fig. 2E). By adjusting the PMN concentration, optimal thermocycling efficiency was achieved with 16 OD PMNs, corresponding to the heating and cooling rates of 8.22 ± 0.24 °C/s and 5.27 ± 0.14 °C/s, respectively (Fig. 2F). In this optimal condition, the temperature profile, including an isothermal process (5 min at 50 °C) and 40 cycles between 90 °C (0 s) and 60 °C (8 s), was completed within 20 min (Fig. 2G, H, and I). Testing with DENV2 RNA, we confirmed that the influence of PMNs on amplification was negligible by commercial PCR equipment. Our thermocycling protocol produced sufficient amplicons even at low target concentrations in our device (Fig. S2). To increase the signal-to-noise ratio for the subsequent colorimetric detection, the related parameters, such as the pH value, the
concentration of SGI and TMB, the LED power, and the irradiation time, were further optimized (Fig. S3). As a result, the colorimetric detection was completed within 4 min, including the time for PMNs collection (<1 min), blue light irradiation (2 min), and signal measurement (<1 min). Taking advantage of ultrafast PPT-based thermocycling and simple colorimetric strategy, the overall assay time was less than 54 min (30 min for commercial RNA extraction, <20 min for thermocycling, and <4 min for signal detection).

3.3. Evaluation of analytical sensitivity and specificity of DENV-specific PPT-RTcPCR

The limit of detection (LoD) for PPT-RTcPCR was investigated using serial dilutions of DENV2 IVT RNA and compared to the reference real-time RT-qPCR. The mean OD$_{650}$ of nine negative samples was 0.39 ± 0.046, resulting in a threshold of OD$_{650}$ 0.53 (three-fold standard deviation of the blank sample) for positivity. As a result, the detection limit was estimated to be approximately 1.9 copies/μL for PPT-RTcPCR, comparable to the reference real-time RT-qPCR (2.1 copies/μL) (Fig. 3A). The amplification signals generated by PPT-RTcPCR increased as the target concentration increased and reached the plateau when the concentration of RNA was higher than 10$^4$ copies/μL, which may be limited by the concentration of SGI and TMB (Fig. 3A). In addition, samples containing 1 copy/μL DENV IVT RNA resulted negative since the amplification signals were lower than the threshold for PPT-RTcPCR and reference RT-qPCR. Consequently, a good linear relationship was obtained when the target concentration ranged from 1 copy/μL to 10$^4$ copies/μL with a correlation equation of $A = 0.47 + 0.21 \log C$ (where $A$ is the absorbance at 650 nm and $C$ is the target concentration, $R^2 = 0.99$).

Ten-fold serial dilutions of the viral RNA extracted from normal human serum samples spiked with DENV2 were used to assess the analytical sensitivity in a more clinically relevant setting. As shown in Table S1 and Fig. 3B, PPT-RTcPCR exhibited analytical sensitivity comparable to the reference real-time RT-qPCR. The PPT-RTcPCR detected all samples containing 11.5 FFU/mL of DENV particles as positive, whereas none of the samples containing 1.15 FFU/mL were detected as positive. Thus, a good linearity ($R^2 = 0.99$) was obtained between 1.15 × 10$^4$ FFU/mL and 11.5 FFU/mL with the $A = 0.44 + 0.19 \log C$ correlation equation, indicating PPT-RTcPCR have similar analytical performance compared to the reference RT-qPCR. The calculated LoD based on DENV2 titers was approximately 3.42 FFU/mL.

The analytical specificity of PPT-RTcPCR was evaluated by testing viral RNA extracted from TCF containing DENV1, DENV4, ZIKV (a flavivirus), CVB3 (a picornavirus), and FIPV (a coronavirus). All reactions were negatives with absorbance signals under the threshold, indicating a high analytical specificity of PPT-RTcPCR (Table S2 and Fig. 3C).

3.4. Validation of the clinical performance of DENV-specific PPT-RTcPCR

To determine the diagnostic performance of DENV-specific PPT-RTcPCR, a panel of 158 human plasma samples collected from clinically suspected dengue patients was tested and compared with those of the reference real-time RT-qPCR assay that was run side-by-side. Using the predefined absorbance threshold of OD$_{650}$ 0.53, PPT-RTcPCR detected DENV2 positive in 125 out of 131 positively confirmed samples (Table S3), whereas the reference RT-qPCR detected all 131 samples positive. PPT-RTcPCR and the reference RT-qPCR assays determined all 27 negative samples as true negatives (Table S4, Fig. 4A, and B). Thus, the clinical sensitivity and specificity of PPT-RTcPCR were 95.4% (95% CI, 90.4%–97.9%) and 100% (95% CI, 87.5%–100%), respectively. To

![Fig. 4. Clinical validation of PPT-RTcPCR platform. (A) Heat map depicting the result of PPT-RTcPCR on 131 positive samples and 27 negative samples. (B) The absorbance of the clinical testing results. The predefined threshold of 0.53 and a ROC optimal threshold of 0.51 were used to classify the samples. (C) ROC curve analysis of the clinical results. The AUC was 0.99.](image-url)
rule out the possibility that the threshold value calculated based on the 3σ principle makes for the differences in sensitivity observed between PPT-RTcPCR and the reference RT-qPCR, the optimal threshold value was recalculated using receiver operating characteristic (ROC) analysis. As shown in Fig. 4C, the optimal threshold was 0.51 with an area under the curve (AUC) of 0.99 [47, 48]. Note that the ROC optimal threshold is highly correlated with the sample set, and in this study, decreasing the threshold from 0.53 to 0.51 resulted in higher sensitivity. With the ROC optimal threshold, the analytical and clinical performance of PPT-RTcPCR were re-evaluated. The calculated LoD for viral nucleic acid and DENV2-spiked human sera were approximately 1.6 copies/PPT-RTcPCR were re-evaluated. The calculated LoD for viral nucleic acid and DENV2-spiked human sera were approximately 1.6 copies/PPT-RTcPCR were re-evaluated. The calculated LoD for viral nucleic acid and DENV2-spiked human sera were approximately 1.6 copies/μL and 2.69 FFU/mL, respectively. Given these results, the PPT-RTcPCR identified 127 of 131 positive samples as true positives and determined all 27 negatives as true negatives showing the clinical sensitivity and specificity of 97.0% (95% CI, 92.4%–98.8%) and 100% (95% CI, 87.5%–100%), respectively, compared to the reference RT-qPCR (Table 1). There were four samples tested as false negatives by PPT-RTcPCR, which can be potentially attributed to the extremely low concentration of target RNA with the colorimetric signal being below the detection limits of the spectrophotometer. The overall agreement value between the PPT-RTcPCR and reference RT-qPCR was 97.5% (95% CI, 83.4%–99.7%; κ = 0.92), indicating that PPT-RTcPCR possessed excellent diagnostic accuracy.

4. Conclusion

In the fight against emerging infectious diseases, developing a cost-effective platform and validating its use in clinical settings is urgently demanded. In this study, we developed a PPT-RTcPCR to detect DENV in clinical samples based on our previous system and validated its diagnostic performance. Utilizing the ultrafast PPT-based thermocycling combined with the colorimetric assay, the PPT-RTcPCR platform can complete the detection of DENV2 RNA within 54 min exhibiting high sensitivity with the LoD of 1.6 copies/μL for viral nucleic acids and 2.69 FFU/mL for DENV-spiked in human serum, comparable with the reference RT-qPCR. Validating the DENV-specific PPT-RTcPCR assay using 158 clinical plasma samples demonstrated excellent diagnostic performance with clinical sensitivity and specificity of 97.0% and 100%, respectively, and 97.5% agreement with the reference RT-qPCR. Compared to most reported diagnostic platforms (Table S5), PPT-RTcPCR is more straightforward and cost-effective, eliminating the need for complicated design or sophisticated equipment unsuitable for POC diagnostic assay while maintaining ultrahigh sensitivity comparable to real-time RT-qPCR. Although current PPT-RTcPCR involves several operations, such as adding the colorimetric solution after amplification, which makes the workflow relatively complicated, this issue could be addressed using microfluidic chips and automated systems. Moreover, taking advantage of the PPT and magnetic properties, PMNs have the potential to be applied for virus lysis and nucleic acid extraction and purification. Thus, integrating nucleic acid extraction into PPT-PCR or PPT-RTcPCR can significantly facilitate on-site detection. Taken together, the results obtained from this study will serve as the basis for the development of a field-deployable diagnostic assay along with an all-in-one integrated device containing extraction, amplification, and detection capabilities for sensitive on-site nucleic acid detection not only for DENV but also for other important infectious diseases such as influenza viruses and highly pathogenic coronaviruses.

CRediT authorship contribution statement


Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341565.

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