Smartphone-based point-of-care multiplexed-genes detection of Mycobacterium tuberculosis on a low-cost paper/polymer hybrid microfluidic device

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Tuberculosis (TB) caused by mycobacterium tuberculosis (M.tb) bacteria is one of the leading causes of death worldwide, with the highest disease burden occurring in developing countries. The existing diagnostic methods either have low sensitivity and specificity or need bulky and expensive instruments, which limit their broad application, especially in resource-limited settings. To address this need, we have developed a paper/polymer hybrid microfluidic platform integrated with loop-mediated isothermal amplification (LAMP) technique for rapid, sensitive, specific, and instrument-free multiplexed-genes detection of M.tb.

Keywords: Tuberculosis; Mycobacterium tuberculosis; Microfluidic devices; Multiplexed-gene detection; Point-of-care detection.

I. INTRODUCTION

Tuberculosis is a major cause of morbidity and mortality [1, 2] accounting for an estimated were 5.8 million cases and almost 1.5 million deaths worldwide. Approximately 90% of TB-related deaths occurred in developing countries [3]. An estimated one-third of the population of the world is latently infected with *M.tb* [4]. Over a lifetime, approximately 1 in 10 of these individuals will develop active TB with higher risks among those with HIV, diabetes, or inadequate nutrition, or who smoke [5]. Despite enormous burden of TB, case-detection rates remain low [6, 7]. Widely used traditional methods to diagnose TB include sputum smear microscopy (SSM), culture of *M.tb*, and tuberculin skin testing (TST) [7, 8]. However, these existing diagnostic methods either have low sensitivity and specificity or need bulky and expensive instruments, which limit their broad application, especially in resource-limited settings. Additionally, it is challenging for these methods to distinguish active and latent TBs. For instance, TST has a very low specificity and cannot distinguish *Mycobacterium bovis* (*M.bovis*) bacillus Calmette-Guérin (BCG) vaccination or exposure to environmental nontuberculous mycobacteria from *M.tb* infection [9]. Therefore, there is a great demand for developing simple and low-cost molecular tests to detect *M.tb* DNA for the specific diagnosis of active and latent TB at the point of care.

Loop-mediated isothermal amplification (LAMP) that allows highly sensitive, efficient and rapid DNA amplification under isothermal conditions [10-13] is a promising candidate for detection of *M.tb*. [14-18]. In particular, LAMP targeting the ESAT-6 gene may have high potential to distinguish *M.tb* infection from *M. bovis* BCG or nontuberculous mycobacteria exposure [19]. But the use of UV light for LAMP detection has safety concerns and low excitation efficiency. In addition, microfluidic devices has unique advantages in low-reagent consumption, fast assays, low cost, and high portability for biomedical applications [20-32]. The integration of LAMP on microfluidic devices provides a new platform for rapid and low-cost disease diagnostics [21, 24, 25, 29, 33, 34] especially in low-resource settings.

Herein, we developed a simple and cost-effective paper/ polydimethylsiloxane (PDMS) hybrid microfluidic platform integrated with LAMP for rapid, sensitive, specific, and instrument-free diagnostics of TB through multiplexed-gene detection of two *M.tb* genes, ESAT-6 and16s-rRNA. The paper in the device serves as a 3D substrate for the storage of LAMP reagents and ESAT-6 and 16s-rRNA genes DNA primers. We fabricated a portable battery-powered heater and a DarkBox to allow heating and visualization at low cost, respectively. The limits of detection (LODs) of M.tb of 5 and 15 DNA copies per LAMP zone for ESAT-6 and 16s-rRNA were successfully achieved. The results are observable by the naked eye and can be imaged by a smartphone camera under a portable blue LED light pen within 30 minutes without using any expensive and bulky instruments. This paper/polymer hybrid microfluidic platform has great potential for low-cost accurate diagnostics TB and other infectious diseases in low-resource settings.

II. EXPERIMENTAL SECTION

A. Chemicals and Materials

A LAMP DNA amplification kit was purchased from New England BioLab (Ipswich, MA). LAMP primers (Integrated DNA Technologies, Coralville, IA) targeting *M.Tb* are shown in Table 1. Concentrations of DNA samples were determined via a NanoDrop spectrophotometer (Sigma-Aldrich, St. Louis, MO).

TABLE 1. LAMP PRIMER SEQUENCES FOR ESAT-6 GENE OF M.TB

Primers	Sequences (5'-3')
FIP	CGCTGCGAGCTTGGTCATGTCACGTCCATTCATTCC
BIP	TAGCGGTTCGGAGGCGTACGTTGTTCAGCTCGGTAG
F3	CAAGCGCAATCCAGGG
B3	GCTTCGCTGATCGTCC

Sponsors: NIH, NSF, CPRIT.

Primers	Sequences (5'-3')
FL	CTGCTTCCCCTCGTCAAG
BL	AAATGGGACGCCACGG

B. *Microfluidic Platform Design and Fabrication*

Fig. 1 shows the design of the hybrid microfluidic platform. The microfluidic device consists of three layers, two PDMS layers and one glass layer. The top PDMS layer serves mainly to cover the LAMP reaction wells. These wells consist of 4 inlet reservoirs and 8 outlet reservoirs (each 1.0 mm diameter, depth 1.5 mm), and microchannels (100 µm width, 100 µm depth). The middle PDMS layer, that serves to deliver reagents and for on-chip LAMP reactions, includes 4 inlet reservoirs (diameter 1.0 mm, depth 1.5 mm), 8 LAMP zones (diameter 2.0 mm, depth 1.5 mm), and microchannels. The bottom layer is a glass slide (75 mm × 25 mm) that functions as structural support. A Whatman No. 1 chromatography paper disk with a (2.0 mm diameter) was cut by a laser cutter (Epilog Zing 16, Golden, CO) and was placed inside each LAMP zone as a 3D storage substrate for ESAT-6 and 16s-rRNA gene LAMP primers, forming a paper/polymer hybrid microfluidic device.

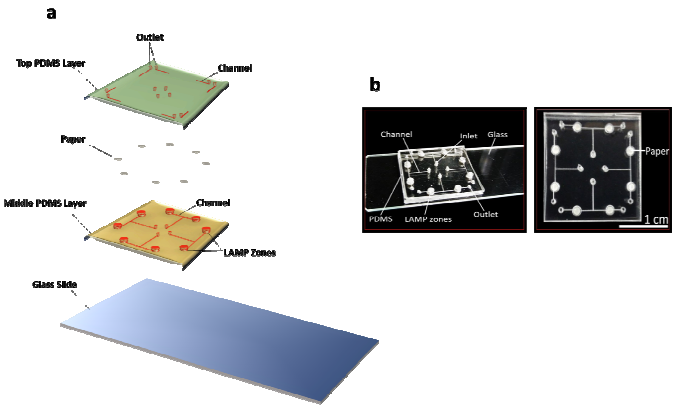

Fig. 1. The structure of the PDMS/paper hybrid microfluidic device. (a) Illustration of the different layers of the hybrid microfluidic chip. Each LAMP zone contains a chromatography paper disk preloaded with LAMP primers. (b) Overhead and oblique photographs of the hybrid microfluidic device for TB diagnosis.

C. *On-Chip LAMP procedures*

As shown in Fig. 2a, the LAMP reaction mixtures with or without an *M.tb* DNA sample were prepared by following the manufacturer's protocol and introduced from each inlet to their corresponding reaction zones using a pipette for sample test and negative control (NC). Afterward, the inlet and outlets were sealed with Epoxy glue to prevent evaporation during on-chip LAMP reactions and the biochip was incubated at 65 °C for 30 min for LAMP reactions then at 80 °C for 5 min for the termination of on-chip LAMP reactions by using a 3D-printed portable battery-powered heater (devised by our laboratory as described previously) [13].

To facilitate the application of our device in resource-poor settings, we devised a DarkBox using small cardboard boxes and black trash bags (Fig. 2b) at a cost of less than 10 cents. A blue LED light pen was embedded through the roof of the DarkBox. A section of laser protection goggles (190-490 nm) was used as a filter to remove interfering light from the excitation source, allowing the chip image to be captured. A smartphone was used to take a photo of the chip. The images were processed with the ImageJ software (NIH) to determine the mean gray value (MGV) for more quantitative analysis.

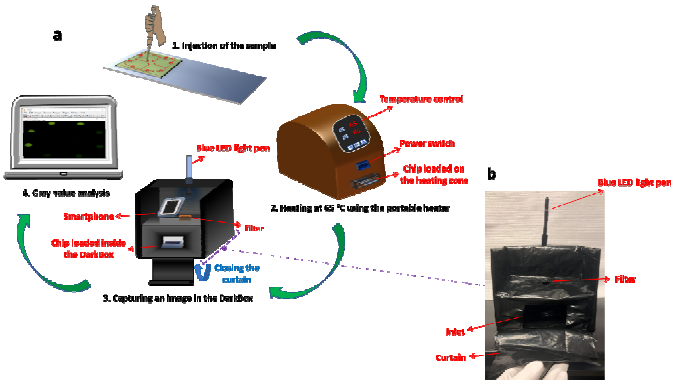

Fig. 2. (a) Schematic of the smartphone-based instrument-free TB diagnosis system for POC detection of *M.tb*. (b) A photograph of the DarkBox. A section of the trash bag acts as a curtain and is closed to cover the inlet of the DarkBox after the chip is inserted.

III. Results and Discussion

A. *Instrument-free visualization of the on-chip LAMP result in the DarkBox using a blue LED light pen*

Calcein fluorescence, quenched by manganese ions, is the usual detection reagent for LAMP reactions. Following LAMP reactions, a calcein pyrophosphate ion byproduct complexes with manganese ions producing bright fluorescence [10, 33]. This allows instrument-free detection of from the LAMP zones under a light pen using the naked eye or a smartphone camera. UV light is usually applied as an excitation source for visualization of LAMP products. However, UV light evokes maximum excitation wavelength of calcein of only 495 nm [35]. To attempt to improve the excitation signal for on-chip LAMP product visualization, in the current study we compared the emission spectra of the UV to that of a commercial blue LED light pen using a portable spectrometer system. Our system consisted of a light pen, optical fibers, a detector, and a computer in which emitted light is sent to the detector via optical fibers, and absorbance data are sent to the computer via a USB cable and recorded using Ocean View software. The excitation and emission spectra of calcein were scanned using a spectrophotometer. The emission spectra of the UV and blue LED light pens and the excitation/emission spectra of calcein are shown in Fig. 3b. The maximum emission wavelength of the blue LED light pen (λ_{max} = 465 nm) was closer to the maximum excitation wavelength of calcein (λ_{max} = 495 nm) than that of the UV light pen ((λ_{max} = 406 nm). The purple (I) and green (II) overlap zones within the range of both UV and blue LED light wavelengths that excite calcein. The green (II) zone area is much greater than the purple (I) zone area, suggesting that a blue LED light pen is a more appropriate excitation source for calcein. To confirm whether blue LED light is a better excitation source for calcein, the on-chip LAMP purified DNA *M.tb* plus ESAT-6 primers reaction

mixtures were imaged under UV and blue LED light pens and analyzed by ImageJ. As shown in Fig. 3 (c-e), the LAMP products generated stronger fluorescence under the blue LED light than under the UV light pen. Moreover, the signal-to-noise (S/N) ratios were 6.87 and 4.02 for blue LED and UV light, respectively. The S/N ratio for LAMP results under blue LED light pen was 71% higher than under UV light pen. Therefore, a blue LED light pen was used for on-chip LAMP detection of *M.tb*.

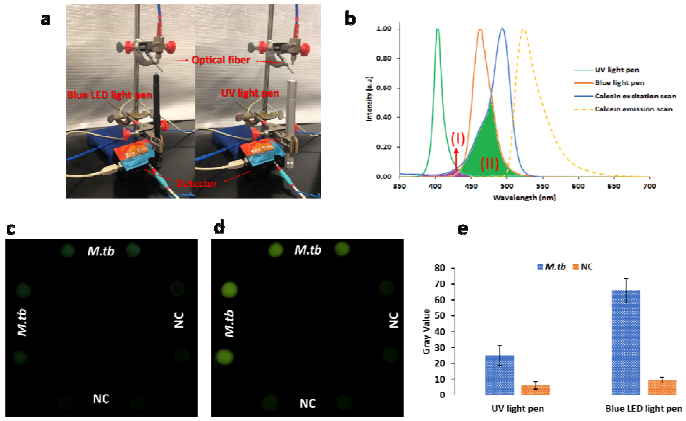

Fig. 3. (a) Setup for the measurement of the emission spectra of the portable UV and blue LED light pens. (b) The emission spectra of UV and blue LED light pens and the excitation/emission spectra of calcein. On-chip LAMP products of extracted *M.tb* DNA imaged by a smartphone camera under the UV (c) and blue LED (d) light pens in the DarkBox using ESAT-6 primer sets. (e) MGVs of the LAMP products under UV and blue LED light pens measured by ImageJ.

B. On-chip LAMP detection of M.tb

We tested the feasibility of the PDMS/paper hybrid microfluidic device to detect *M.tb* by using purified bacterial genomic DNA. The LAMP primers specific for genes encoding *M.tb* ESAT-6 and 16s-rRNA were pre-loaded in each corresponding LAMP zones along with chromatography paper substrate. Due to a high surface-to-volume ratio of paper, it is an ideal 3D porous substrate for the storage of DNA primers. Fluorescent byproducts produced by LAMP reactions were observable by the naked eye or imaged by a smartphone camera as described previously. This endpoint visualization step is easy and does not require any special expensive and bulky equipment, offering POC detection of *M.tb* in the field and low-resource settings.

Bright green fluorescence was observed in images of samples containing *M.tb* DNA incubated with both ESAt-6 and 16s-rRNA primers captured by a smartphone camera (Figs. 4a, b) while the negative control showed a weak background. An approximately 5-fold greater MGV was observed for the *M.tb* LAMP reaction than for negative control reaction using either ESAT-6 or 16s-rRNA primers in images processed using ImageJ (Figs. 4c, d). Moreover, strong fluorescence was observed in LAMP zones for *M.tb* using both ESAT-6 and 16s-rRNA primers simultaneously (Fig. 4e), but not for the negative control (Fig. 4f).

The detection sensitivity of the proposed microfluidic platform was investigated by testing different *M.tb* DNA template samples at concentrations of 15, 5, and 1 copies per well. Strong fluorescence of the LAMP products was observed with a DNA template as low as 5 and 15 copies per reaction well using ESAT-6 and 16s-rRNA primers, respectively. The LODs of the on-chip LAMP method for *M.tb* detection were 5 and 15 DNA copies per detection well for ESAT-6 and 16s-rRNA primers, respectively.

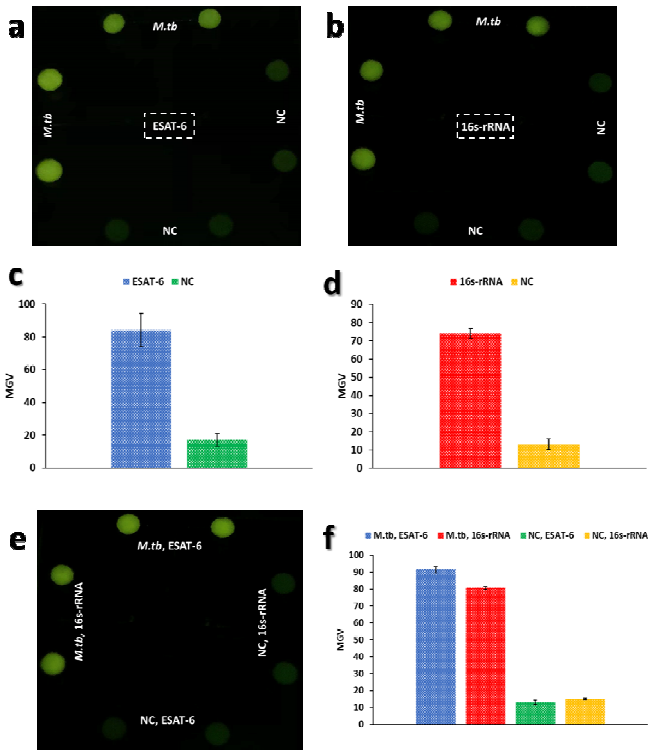

Fig. 4. On-chip LAMP detection of extracted *M.tb* DNA by a smartphone camera under the portable blue LED light pen in the DarkBox using ESAT-6 (a) and 16s-rRNA (b) regions primers. (c) MGV of the LAMP products using ESAT-6 and 16s-rRNA (d) regions primers measured by ImageJ (e) Multiplexed-genes detection of extracted *M.tb* DNA using both ESAT-6 and 16s-rRNA regions primers simultaneously on a single chip and (f) MGV of the LAMP products using both ESAT-6 and 16s-rRNA primers simultaneously on a single chip.

IV. CONCLUSIONS

We developed a low-cost paper/PDMS hybrid microfluidic device for rapid and instrument-free POC multiplexed-gene detection of *M.tb*. Simultaneous targeting of *M.tb* ESAT-6 and 16s-rRNA enabled highly sensitive and specific for *M.tb* detection. We demonstrated that a simple blue LED light pen excitation source is superior to a UV source for visualization of the LAMP products. The hybrid microfluidic biochip combines the advantages of high liquid control efficiency of PDMS and high porosity of paper for LAMP primer storage.

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