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Microfluidic method for rapid turbidimetric detection of the DNA of *Mycobacterium tuberculosis* using loop-mediated isothermal amplification in capillary tubes

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Abstract We describe a microfluidic method for rapid isothermal turbidimetric detection of the DNA of *Mycobacterium tuberculosis*. Loop-mediated isothermal amplification is accomplished in capillary tubes for amplifying DNA in less than 15 min, and sensitivity and specificity were compared to conventional loop-mediated isothermal amplification (LAMP). The method can detect as little as 1 pg mL^{-1} DNA in a sample. Results obtained with clinical specimens indicated 90 % sensitivity and 95 % specificity for microfluidic LAMP in comparison to culture methods. No interference occurred due to the presence of nonspecific DNAs. The findings demonstrate the power of the new microfluidic LAMP test for rapid molecular detection of microorganisms even when using bare eyes.

Keywords Microfluidic-LAMP · M. tuberculosis · Capillary tube

Introduction

Nucleic acid isothermal amplification technologies are the powerful molecular methods for detection of microorganisms [1]. The most known nucleic acid isothermal amplification is loop-mediated isothermal amplification of DNA (LAMP) technology [2]. LAMP has attracted wide considerable interests for molecular diagnosis of nucleic acid targets because of its special properties including the significant specificity, sensitivity, rapidness, and its high clinical performance in the practice [3]. LAMP occurs with an incubation time at a temperature between 60 – 65 °C. Its main reaction included a thermophilic DNA polymerase, with the strand displacement activity, betaine, and the primers for targeting DNA template [4]. The amplification process are conducted under a constant thermal condition and a more efficiently amplification achieved at a single temperature [5].

LAMP result can be assessed by its turbidity or via an extrinsic fluorescence of the cauliflower-like DNAs stained with a fluorescent dye [6]. The isothermal amplification process conventionally performed in a microtube; however, microfluidic chambers could be improved rapidness of the amplification process. These characteristics made various technological approaches adapted with this amplification technology for several applications [7]. For instance, the conventional LAMP assays were reported for detection of known infectious agents via targeting a specific region of the desired gene [8]. Also, two-step reverses transcription (RT)-LAMP assays were developed for identifying RNA of the pathogen as the viability marker [9]. Recently, microfluidic LAMP assay was introduced as a rapid and portable identification test [10]; however, the microfluidic LAMP would be useful in the point-of-care for diagnostic purposes [11].

On the other hand, the molecular detection of *M. tuberculosis* in clinical specimens is important as a clinical diagnostic tool [12]. The assays for identifying mycobacterial pathogens require detection of the physical characteristics via the acid-fast staining and the microscopic studying, physiological specifications via the growth on defined media, or the biochemical analysis according to the membrane lipid composition [13]. These analyses require highly concentrations of *Mycobacteria* in the specimen and they need a long time [14].

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The assays detect nucleic acids of mycobacteria were preferred because of their sensitivity and their relative speeds in the measurements [15]. Advances in the miniaturization of nucleic acid amplification technologies have allowed designing numerous rapidly molecular assays for the detection of microorganisms [16]. In this study, we aimed the employment of glass capillaries in a microfluidic LAMP for the rapid molecular detection of *M. tuberculosis* DNA with bare eyes. Sensitivity, specificity and consumed time for the microfluidic LAMP were compared to the conventional LAMP and the culture.

Materials and methods

Chemicals and instruments

The isothermal conditions for LAMP reactions in microtubes and glass capillaries were provided via an oven (Labtron, Iran; http://www.labtronco.com). The large fragment of Bst DNA polymerase was purchased from New England Biolabs (USA; https://www.neb.com). Betaine and $MgSO_4$ solutions were prepared from Sigma-Aldrich (http://www.sigmaaldrich.com). Also, Oligonucleotides were synthesized and desalted by Sigma-Aldrich. SYBR Gold nucleic acid gel staining solution (10000X) was purchased from Molecular Probes (USA; http://www.lifetechnologies.com/ir/en/home/brands/molecular-probes.html). GeneRuler™ DNA Ladder Mix was from Thermo Scientific (USA; http://www.thermoscientificbio.com). Glass capillaries were purchased from Yancheng Huida Medical Instruments Co., Ltd. (China; http://www.chinahuida.cn/eng/product/10.html). Glass plain slides were from T& Q Industries (China; http://www.tq-medical.com). λ-DNA was also purchased from Takara Bioscience Inc. (Japan; http://www.takara-bio.com). Qiaquick DNA extraction kit was from Qiagen (Germany; www.qiagen.com). The lids for capping the capillaries (Vinyl Caps for Capillary Cell) were commercially available through TA Instruments (USA; www.tainstruments.com).

Sample collection and processing

Bacillus Calmette Guerin (BCG), *Mycobacterium tuberculosis* subsp. Tuberculosis strain TB-9521 (i.e., MTT9521), and sputum samples were analyzed in this work. Samples serially diluted from the BCG and MTT9521 (Pasture Institute in Iran and Golestan University of Medical sciences, respectively) with the nuclease free water were used as the standards for determining the limit of detection (LOD). During the DNA extraction, 100 μL of the dilutions was boiled up to 95 °C for 5 min and then the supernatants were used in the microfluidic LAMP or the conventional LAMP. Fifty sputa were obtained and verified by the culture analysis. The sputa were ready for the DNA extraction using Qiaquick DNA extraction kit.

Oligonucleotides

Four oligonucleotide primers contained forward inner primer (FIP), backward inner primer (BIP), F3, and B3 were designed (Table 1) and synthesized for amplifying 16S rDNA of *Mycobacterium tuberculosis* as the DNA target sequence (Gene Bank Accession No. KC503894).

Table 1 Oligonucleotide primers for targeting *Mycobacterium tuberculosis* 16 s rDNA by LAMPF3

Name	Composition	Position	Sequence
F3	5' F3 3'	642 – 658	5' CTGGCTCAGGACGAACG 3'
B3	5' B3 3'	837 – 852	5' GCTCATCCCACACCGC 3'
FIP	5' F1c F2 3'	F2 : 670 – 700 F1c : 731 – 745	5' CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT 3'
BIP	5' B1 B2c 3'	B1 : 760 – 771 B2c : 803 – 823	5' TCGGGATAAGCCTGGACCACAAGACATGCATCCCGT 3'

Conventional LAMP reaction

Conventional LAMP mix in total volume of 25 μL were contained 2.5 μL from 10X Bst DNA polymerase reaction buffer, 8 mM $MgSO_4$, 0.8 M betain, 1.4 mM each of dNTPs, 1.6 mM FIP, 1.6 mM BIP, 0.2 mM F3, 0.2 mM B3, 8 U Bst DNA polymerase and 0.4 ng from the extracted DNA template [17]. The master mix was dispensed in each microtube. For the negative control and the blank reactions, 0.4 ng of λ-DNA and the equal volume of the distilled water were added to the LAMP mix, respectively. The solutions were mixed by pipetting and then spinning down, immediately. Then the reaction mixes were kept in an oven and incubated at 60 °C for 15, 30, 45, and 60 min [18].

Microfluidic LAMP

LAMP microreactor device

The 75 mm × 25 mm × 3.5 mm microfluidic device (Fig. 1) was consisted of three parts: two glass plain slides and a glass capillary (1 mm thick of two glass plain slides in the top and the bottom and 1.5 mm from the outer diameter of a capillary tube in the middle of slides). The various layers were contained of glass plain slides in the top and the bottom respectively. Glass capillaries in the middle of them were bonded with a cyanoacrylate as silicon-based glass glue at room temperature. Before bonding of the parts to fabrication of designed microreactor device, glass capillaries were bended 90° with regard to the horizontal axis using indirectly heating for the preparation of a single inlet port for loading a sample to the microreactor. Total volume of the reaction chamber was approximately 25 μL. The inner diameter of both the inlet hole and the outlet hole was 1.2 mm, which enables adding the reactants into the device via a micropipette. Upon injection, the reaction mix was simply pushed through the channel due to the pressure generated by the pipette and the capillary force. The device was then incubated at 60 °C-oven for preparing the isothermal condition. For determining the reaction turbidity, the glass plain slide at the bottom of the device had a black surface. The turbidity was produced within a microfluidic LAMP reaction due to magnesium-pyrophosphate complexes precipitated in the reaction solution. Vinyl Caps were used for closing two ends of a glass capillary.

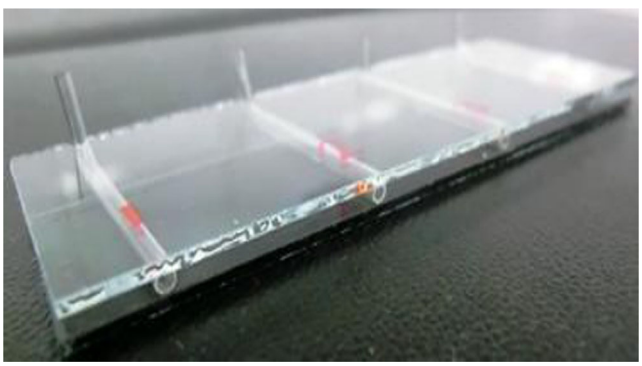

Fig. 1 The microreactor device contained glass capillaries for microfluidic LAMPs

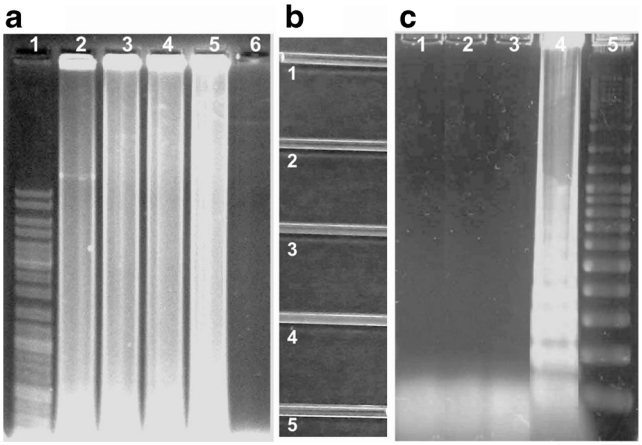

Fig. 2 **a** Gel-electrophoresis results of microfluidic LAMP. Lane 1: DNA ladder, 100–10000 bp (Fermentas); microfluidic-LAMP reactions after 15 min (*lane 2*), 30 min (*lane 3*), 45 min (*lane 4*), and 60 min (*lane 5*); lane 6: negative result for microfluidic-LAMP reaction. **b** Turbidimetric microfluidic-LAMP results after 15 min (*capillary 1*), 30 min (*capillary 2*), 45 min (*capillary 3*), and 60 min (*capillary 4*); *capillary 5*: negative result after 60 min; **c** Gel-electrophoresis results of conventional-LAMP reactions after 15 min (*lane 2*), 30 min (*lane 3*), 45 min (*lane 4*), and 60 min (*lane 5*); lanes 1: DNA ladder, 100–10000 bp

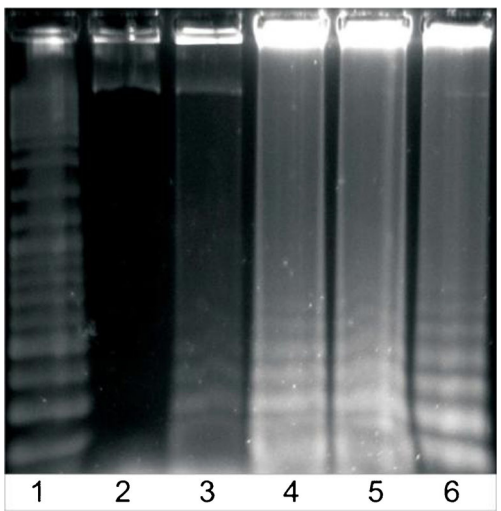

Fig. 3 Gel-electrophoresis results of microfluidic-LAMP tests with 5 μL (*lane 2*), 10 μL (*lane 3*), 15 μL (*lane 4*), 20 μL (*lane 5*), and 25 μL (*lane 6*) total volumes contained 1 pg·mL^{-1} specific DNA in the reactions at 60 °C for 15 min. Lane 1: DNA ladder, 100–10000 bp

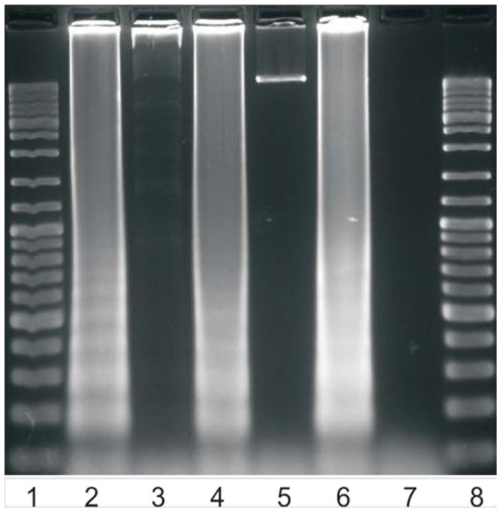

Fig. 4 Gel-electrophoresis results of specificity test for microfluidic-LAMP reactions. Lanes 1 and 8: DNA ladder, 100–10000 bp; microfluidic-LAMP results after 15 min amplification at 60 °C contained *M. tuberculosis* DNA template (*lanes 2, 4, and 6*), human DNA template (*lane 3*), λ-DNA template (*lane 5*), and blank test with *E. coli* DNA template (*lane 7*)

Microfluidic LAMP reaction

Master mixes contained 1 pg·mL^{-1} specific DNA for a microfluidic LAMP reaction was prepared similar to the conventional LAMP [17]. Total volume of 5, 10, 15, 20, 25 μL of the master mixes were separately transferred to each capillary channels. Microfluidic LAMPs were incubated in an oven at 60 °C for 15, 30, 45, and 60 min.

Specificity and LOD of microfluidic LAMP

To validate specificity of the microfluidic-LAMP reactions, BCG-DNA and MTTB9521-DNA (as the specific DNA templates) and E.coli-DNA, λ-DNA, and human genomic DNA (as non-specific DNA templates) were used in the microfluidic-LAMP tests. For LOD validation, various concentrations (10 ng·mL^{-1}, 1 ng·mL^{-1}, 0.1 ng·mL^{-1}, 0.01 ng·mL^{-1}, and 0.001 ng·mL^{-1}) of the specific DNAs were tested in a total 10-μL microfluidic-LAMP reaction.

SYBR Gold staining of LAMP products

Gel electrophoresis was performed on a 2 % (w/v) agarose gel with 0.5 X Tris-borate-EDTA, pH 8.3 (TBE buffer) as gel and electrophoresis buffer. The gels were stained using SYBR Gold nucleic acid gel stain according to the manufacturer's instruction. The amount of DNA loaded into the gel was adjusted to the yield approximately the equal brightness in all lanes.

Statistical analysis

True positives (TP) and true negatives (TN) were determined by the culture results and the LAMP results with the false positives (FP) and the false negatives (FN) attributed to the findings from the microfluidic-LAMP assays. The sensitivities and the specificities were deter-

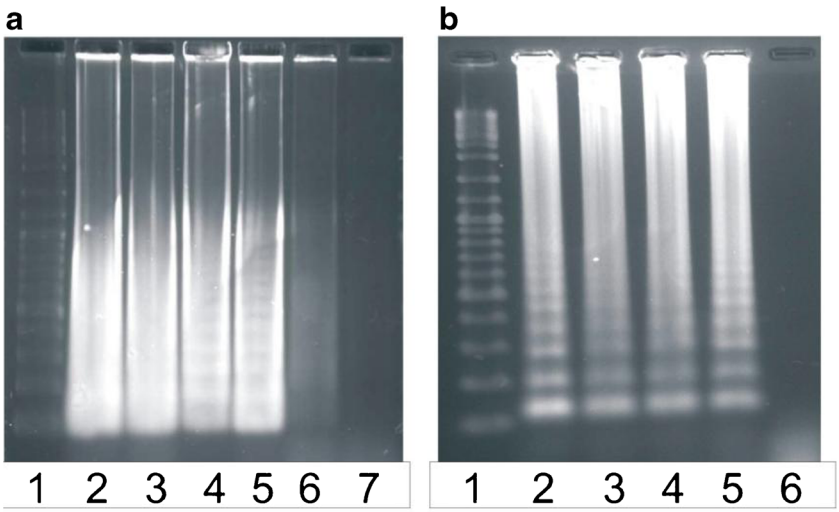

Fig. 5 **a** Gel-electrophoresis results of sensitivity test (or detection limit; LOD) for microfluidic-LAMP reactions. Lane 1: DNA ladder, 100–10000 bp; microfluidic-LAMP results after 15 min at 60 °C contained 10 ng (*lane 2*), 1 ng (*lane 3*), 0.1 ng (*lane 4*), 0.01 ng (*lane 5*), 0.001 ng (*lane 6*), and 0.0001 ng (*lane 7*) of *M. tuberculosis* DNA. **b** Gel-electrophoresis results of sensitivity test (detection limit) for conventional LAMP reactions. Lane 1: DNA ladder, 100–10000 bp; conventional LAMP results after 15 min at 60 °C contained 10 ng (*lane 2*), 1 ng (*lane 3*), 0.1 ng (*lane 4*), 0.01 ng (*lane 5*), and 0.001 ng (*lane 6*) of *M. tuberculosis* DNA

Table 2 Clinical performance of microfluidic LAMP in comparison to the culture and the conventional-LAMP

	Culture		Sensitivity (%)	Specificity (%)	Conventional-LAMP		Sensitivity (%)	Specificity (%)
	Pos.	Neg.			Pos.	Neg.		
MicrofluidicLAMP								
Pos.	9	2			10	0		
Neg.	1	38	90	95	0	40	100	100

mined as follows: sensitivity = TP/TP + FN and specificity = TN/TN + FP [18].

Results and discussion

Microfluidic-LAMP performance

Electrophoresis behavior of the microfluidic-LAMP results was characterized according to their ladder- shaped smears at the agarose gel. Positive results of the microfluidic LAMPs after 15 min incubation at 60 °C were seen (Fig. 2a). Also, positive results of the microfluidic LAMPs were seen via the turbidity encountered after 15 min incubation at 60 °C in the glass capillaries (Fig. 2b). Positive results of the conventional LAMPs after 60 min incubation at 60 °C were seen (Fig. 2c).

Total volume of the microfluidic-LAMP mix was set at 10 μL in the capillary tube; however, the conventional LAMP had not any positive band at 10 μL in a microtube (Fig. 3).

Specificity and sensitivity of microfluidic LAMP

Specificity of the microfluidic LAMP was checked using DNA templates isolated from BCG, *M. tuberculosis*, *E. coli*, human leukocytes, and λ-bacteriophage. Positive results of the microfluidic LAMPs were obtained via the specific targets (BCG and *M. tuberculosis* DNAs); however, the negative results of microfluidic LAMPs were seen from the non-specific DNA templates (such as *E. coli* DNA, human DNA, and λ-DNA) (Fig. 4).

Analysis of the gel electrophoresis shown fluorescent bands from the conventional LAMP with 0.01 ng specific DNA; however, 0.001 ng (1 pg) of the specific DNA was detected by a microfluidic-LAMP (Fig. 5).

Clinical performance of microfluidic LAMP

The clinical performance of the microfluidic LAMP was determined by comparing the test results to those by cultures and the conventional LAMPs. Included in the study were 50 sputa from 50 patients received by the laboratory for initial diagnosis. The overall results presented in Table 2 indicated that the capillary test had a sensitivity of 90 % and specificity of 95 % in comparison to those of the cultures. In addition, the sensitivity and the specificity of the microfluidic LAMPs were 100 % in comparison to those by the conventional LAMPs. Clinical sensitivity of the microfluidic LAMP was 90 % compared with 90.8 % of the culture; however, the sensitivity is dependent on major factors such as the analytical sensitivity of the assay, the sensitivity of the gold standard (culture), the distribution of positive samples, and the effects of sample heterogeneity, particularly in the specimens with low-positive results. Clinical specificity was 95 % compared to 98.9 % of the culture [19, 20]. This acceptable specificity seen here is because of using species-specific primers for *M. tuberculosis* 16S rDNA and efficiently amplified DNAs by LAMP with four specific primers; moreover, the sensitivities and the specificities of microfluidic LAMPs were equal to conventional-LAMPs.

Table 3 Figures of merit of comparable microfluidic LAMP methods for determination of *Mycobacterium tuberculosis* DNA

Microfluidic device material	Limit of detection	Detection method	Time (min)	Reaction volume (μL)	Cost	Portability	Reference
Polytetrafluoroethylene (PTFE)	10 bacteria	Fluorescent	50	10	Low	High	[25]
Polyacrylamide (PAA)	12 bacteria	Fluorescent	75	0.67	Low	High	[37]
Polymethylmethacrylate (PMMA)	270 copies/μL	Green color	30	5	Low	High	[38]
Glass capillary	1 $pg \cdot mL^{-1}$	Turbidity	15	10	Low	High	This study

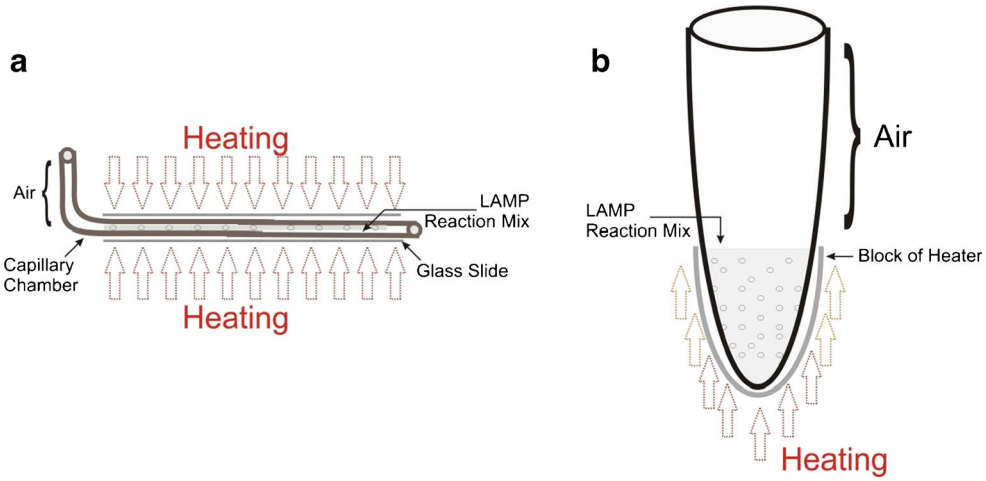

Fig. 6 Schematic comparison between capillary chamber and heating block for a LAMP reaction

Conclusions

Various kinds of molecular methods have been reported in attempts to overcome the shortcomings of the conventional microbial diagnostics particularly the detection of *M. tuberculosis* [21, 22]. Loop-mediated isothermal amplification was introduced as suitable technology for accelerating molecular detection tests [2]. LAMP technique has been specialized for amplifying nucleic acid targets within an isothermal condition (60–65 °C), which also offer the possibility of the miniaturized amplification with a low energy consumption. Hence, LAMP has been employed to achieve rapid and cost-effective diagnosis of pathogens in miniaturized containers [23, 24].

Recently, the miniaturized systems integrated with LAMP were developed for the rapid detection of pathogens. These micro-reactors utilized the rapid thermal conduction from increased surface-to-volume ratio, as well as the low sample/reagent consumption attributable to reduce the size of the container [25–27]. For this purpose, various approaches of microfluidic LAMPs were reported for indicating the superiority in terms of the speed, the miniaturization, the power consumption, and the cost beneficence [28–31]. Capillary tubes could also be applied for the microfluidic-LAMP reaction instead of some polymeric materials (e.g. polymethyl methacrylate (PMMA) or polydimethylsiloxane (PDMS)) reported previously. This consideration would be more important where the fabrication of microfluidic systems need some biocompatible polymers such as PMMA, PDMS or polyacrylic acid (PAA) [32–34]. Fabrications of polymeric microfluidics encountered with some problems such as preparation of the polymer molding solution, multilayer polymers, and penetration of the reaction mixtures in a polymer-based platform. Furthermore, fabrications of the microfluidic containers often were involved some surface treatments with the hydrophobic polymers for preventing the air bubble formations and improving the biocompatibilities, consequently [35, 36]; however, these limitations were not encountered in the capillary tubes for microfluidic LAMPs (Table 3).

Our findings confirmed the capillary tubes provided comfortable environments for the microfluidic LAMP analyzing with bare eyes; moreover, small volume of the microfluidic-LAMP reactants could efficiently be reacted for DNA amplification. In addition, capillary tubes provided a high aspect ratio (surface to volume ratio) for heating the microfluidic-LAMP reaction, thus it made the capillary tube be more suitably for receiving heat when compared with the microtubes in conventional LAMPs (Fig. 6). The development an easy-to-use and the robust non-polymeric microfluidic device for rapid detection based on microfluidic LAMP offered several advantages. These characteristics could be favorable in developing new generations of the rapid diagnostic assays for tuberculosis in future.

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Author contributions

P. Gill conceived and designed the experiments. A. Rafati performed the experiments. P. Gill analysed the data. P. Gill contributed reagents/materials/analysis tools. A. Rafati and P. Gill wrote the paper.