Brazilian Journal of Microbiology (2021) 52:1315–1325
https://doi.org/10.1007/s42770-021-00520-4

CLINICAL MICROBIOLOGY - RESEARCH PAPER

Highly specific and sensitive detection of the *Mycobacterium tuberculosis* complex using multiplex loop-mediated isothermal amplification combined with a nanoparticle-based lateral flow biosensor

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Received: 17 January 2021 / Accepted: 27 April 2021 / Published online: 26 June 2021

Abstract

Tuberculosis (TB) is the deadliest infectious caused by *Mycobacterium tuberculosis* complex (MTBC). Because most TB cases occur within low-income populations, developing a specific, sensitive, cost-saving, and rapid point-of-care test for the early diagnosis of TB is important for achieving the WHO's End Tuberculosis Strategy. In the current study, a novel nucleic acid detection strategy that includes multiplex loop-mediated isothermal amplification combined with a nanoparticle-based lateral flow biosensor (mLAMP-LFB) was used to detect MTBC. The two sets of LAMP primers specific to the *IS6110* and *gyrB* genes of MTBC were successfully designed and validated for the detection of MTBC. The preferred reaction conditions for this assay were confirmed to be 65 °C for 40 min, and the amplification products could be visually identified through LFB within 2 min. The full assay process, including genomic DNA template extraction, LAMP reaction, and product detection, could be completed in 80 min. The limit detection of the assay was 100 fg of DNA in pure culture. The specificity of the assay was 100%, and it had no cross-reactions to other strains. Thus, the m-LAMP-LFB technology established in the present study was an objective, rapid, simple, and sensitive assay for MTBC identification, which could be applied in a clinical setting, especially in resource-constrained regions of the world.

Keywords Tuberculosis · *Mycobacterium tuberculosis* complex · Diagnosis · Loop-mediated isothermal amplification · Lateral flow biosensor limit of detection

Responsible Editor: Fernando R. Pavan

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Introduction

Tuberculosis (TB) is the deadliest infectious caused by *Mycobacterium tuberculosis* complex (MTBC) and remains one of the leading causes of death from a single infectious agent worldwide [1, 2]. According to a 2019 World Health Organization (WHO) report, there were an estimated 10.0 million new TB cases and nearly 1.5 million TB-related deaths worldwide [3]. Because most TB cases occur within poor economic backgrounds, developing a reliable, cost-saving, and rapid point-of-care test for the early diagnosis of TB is important for achieving the WHO's End Tuberculosis Strategy.

All MTBC agents evolved from a common ancestor, and the bacteria fall into phylogenetically distinct lineages and clades under the following species names: *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. orygis*, *M. suricattae*, *M. caprae*, *M. microti*, *M. canettii*, *M. pinnipedii*, and *M. mungi*. Among them, *M. tuberculosis*, *M. africanum*, and *M. bovis* are considered human-pathogenic agents [1, 4, 5]. Mycobacterial culture tests were previously considered the gold standard for TB diagnosis [6]. However, these tests are time-consuming and laborious and require a long time (6–8 weeks) to identify MTBC isolates [7]. In recent decades, nucleic acid amplification tests have become the new gold standards for rapid TB diagnosis. GeneXpert (Xpert MTB/RIF assay) provides an unprecedented level of automation for detecting MTBC isolates in clinical application and has been endorsed by the WHO [8, 9]. Other PCR-based methods, such as multiplex PCR and real-time PCR, have also been used to identify TB [10, 11]. However, these techniques require expensive facilities and skilled operating personnel, which may not be readily available in resource-constrained regions. Hence, a highly accurate, rapid, sensitive, and cost-effective assay for the detection of MTBC is essential for the prevention and treatment of the spread of TB disease.

To overcome the shortcomings of the above methods, loop-mediated isothermal amplification (LAMP), a rapid, sensitive, and low-cost detection method, has been developed and used to identify many pathogens, such as *Staphylococcus aureus*, *Enterococcus faecalis*, and *Brucella* [12–14]. In previous reports, LAMP has also been used for MTBC detection [15, 16]. LAMP products can be identified by agarose gel electrophoresis, turbidimetry, and color changes [15–17]. However, these detection methods are not specific to target genes and easily cause false positive or negative outcomes. To address these defects, a nanoparticle-based lateral flow biosensor (LFB), a visual, simple, and target-specific detection method, was successfully developed and used for identifying LAMP products [18, 19].

In the present report, mLAMP-LFB technology was established for the rapid and visual detection of MTBC strains by highly specific targeting of the *IS6110* and *gyrB* genes. The amplification conditions, such as the reaction temperature, time, and template concentration, were optimized in pure cultures, and the specificity and feasibility were confirmed with clinical samples.

Materials and methods

Materials and instruments

Acidic Roche medium was purchased from Shandong Haozhong Chemical Technology Co., Ltd. (Shandong, China). Bacterial genomic DNA extraction kits (QIAamp DNA Mini Kits; Qiagen, Hilden, Germany) were obtained from Qiagen (Beijing, China). Isothermal amplification kits, biotin-14-dCTP, and colorimetric indicators (malachite green, MG) were purchased from Bei-Jing HaiTaiZhengYuan. Co., Ltd. (Beijing, China). A lateral flow biosensor was obtained from Beijing Haitai Zhengyuan Technology Co., Ltd. (Beijing, China). Anti-Dig, anti-FAM, and biotin-BSA were obtained from Abcam Co., Ltd. (Shanghai, China). A real-time turbidimeter (LA-500) was purchased from Eiken Chemical Co., Ltd. (Japan).

Microbial strains and genomic template preparation

Thirty strains were employed in the present study, including the MTB reference strain H37Rv (ATCC 27,294), *M. bovis* Bacillus Calmette-Guerin (BCG) strain, *M. africanum*, *M. bovis*, 10 isolated *M. tuberculosis* strains, 9 nontuberculous mycobacteria (NTBM), and 7 *nonmycobacterial* pathogens (Table 2). Genomic DNA was extracted from all culture strains with QIAamp DNA extraction kits according to the manufacturer's instructions and quantified with a NanoDrop ND-2000 (Beijing, China). The genome of H37Rv (ATCC 27,294) was serially diluted (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg) for sensitivity detection.

Design and synthesis of mLAMP assay primers

The two sets of LAMP primers were designed with Primer Explorer V5 (http://primerexplorer.jp/e/; Eiken Chemical Co., Ltd., Japan) based on the target genes *IS6110* (GenBank Accession No. JX627755.1) and *gyrB* (GenBank Accession No. NC_000962.3). All primers were analyzed with the basic local alignment search tool (BLAST). The positions of the primer pairs are displayed in Fig. 1, and the sequences and modifications are shown in Table 1. All of the oligomers were synthesized and purified by TsingKe Biotech Co., Ltd. (Beijing, China) with HPLC purification grade.

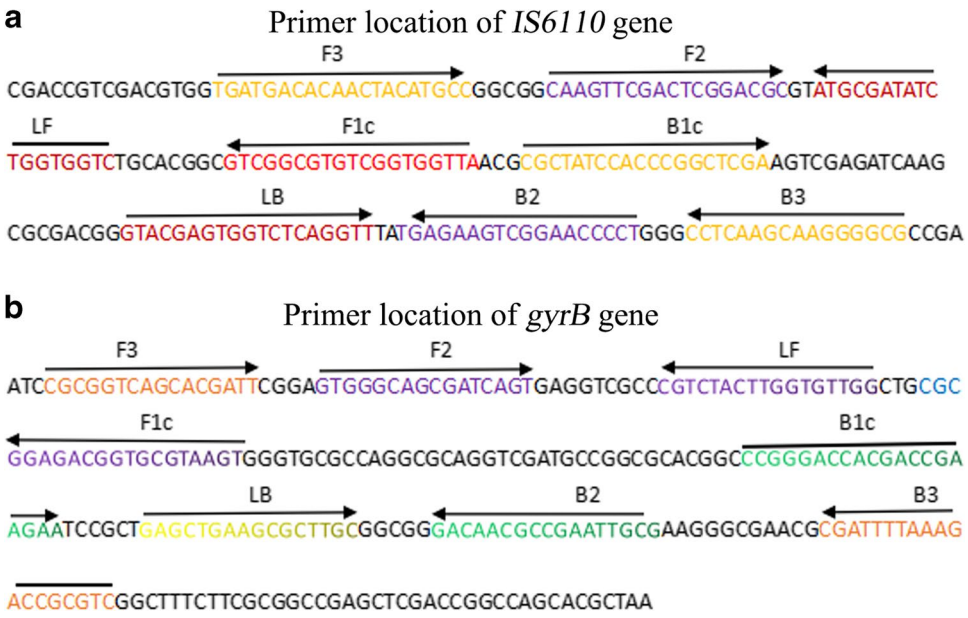

Fig. 1 Location and sequence of the *IS6110* (**a**) and *gyrB* (**b**) genes used to design loop-mediated isothermal amplification primers. The nucleotide sequences of the sense strand of the *IS6110* and *gyrB* genes are shown in the diagram. Right arrows and left arrows indicate sense and complementary sequences, respectively

Preparation of lateral flow biosensor

The LFB strips were designed as previously described with some modifications [20]. In brief, the sample pad, conjugate pad, NC membrane, and absorbent pad were assembled in an orderly manner on a backing card. Anti-FAM, anti-Dig, and biotin-BSA were immobilized on NC membranes to form the test line (TL1) (*IS6110* gene), test line 2 (TL2) (*gyrB*), and control line (CL), with each line separated by 5 mm. SA-PNPs (dye streptavidin-coated polymer nanoparticles) were deposited on the conjugate pad of the strip. The assembled biosensors were packed in a plastic box.

Standard LAMP reaction

The *IS6110*- LAMP or *gyrB*-LAMP reactions were carried out in 25 µl amplification mixtures. In brief, each reaction contained 0.4 µM each of outer primer F3 and B3, 0.8 µM each of loop primer LF* and LB, 1.6 µM each of inner primer FIP* and BIP, 1 µl of *Bst* DNA polymerase (8 U), 12.5 µl of 2 × reaction buffer, 1 µL of amplification indicator (MG), and 1 µl of DNA template.

The mLAMP reaction was performed in a 25 µl amplification system as follows: each reaction contained 0.2 µM outer primers *IS6110*-F3, *IS6110*-B3, *gyrB*-F3, and *gyrB*-B3; 0.4 µM loop primers *IS6110*-LF*, *IS6110*-LB,

Table 1 LAMP primers used in this study

Primer name	Sequence and modifications	Length	Gene
F3	5′-CGCGGTCAGCACGATT -3′	17 nt	*IS6110*
B3	5′-GACGCGGTCTTTAAAATCG -3′	20 nt	
FIP*	5′-FAM-ACTTACGCACCGTCTCCGCG-GTGGGCAGCGATCAGT-3′	38 nt	
BIP	5′-CCGGGACCACGACCGAAGAA-CGCAATTCGGCGTTGTC-3′	39 nt	
LF*	5′-Biotin-CCAACACCAAGTAGACG-3′	18 nt	
LB	5′-GAGCTGAAGCGCTTGC-3′	17 nt	
F3	5′-TGATGACACAACTACATGCC-3′	21 nt	*gyrB*
B3	5′-CGCCCCTTGCTTGAGG-3′	17 nt	
FIP*	5′-Dig-TAACCACCGACACGCCGACG-CAAGTTCGACTCGGACGC-3′	40 nt	
BIP	5′-CGCTATCCACCCGGCTCGA-AGGGGTTCCGACTTCTCA-3′	39 nt	
LF*	5′-Biotin-GACCACCAGATATCGCAT-3′	19 nt	
LB	5′-GTACGAGTGGTCTCAGGTT-3′	20 nt	

IS6110-FIP*, 5′-labeled with FAM when used in LFB assay; *IS6110*-LF*, 5′-labeled with biotin when used in LFB assay; *gyrB*-FIP*, 5′-labeled with Dig when used in the LFB assay; *gyrB*-LF*, 5′-labeled with biotin when used in the LFB assay; *FAM*, carboxyfluorescein; *Dig*, digoxigenin; *nt*, nucleotide; *mer*, monomeric unit

Table 2 Bacterial strains used in this study

No	Bacteria	Strain no. (source of strains)	No. of strains	m-LAMP-LFB result	
				IS6110	*MBP64*
1	*M. tuberculosis* H37Rv	ATCC27294	1	P	P
2	*M. bovis* BCG	GZCDC	1	P	P
3	MTBC isolate strain	GZCDC	10	P	P
4	*M. africanum*	ATCC25420	1	P	P
5	*M. bovis*	ATCC19210	1	P	P
6	*M. marinum*	ATACC927	1	N	N
7	*M. gordon*	ATCC14470	1	N	N
8	*M. phlei*	ATCC11758	1	N	N
9	*M. xenopi*	ATCC19250	1	N	N
10	*M. abscess*	ATCC19977	1	N	N
11	*M. chelonae*	ATCC14472	1	N	N
12	*M. aureus*	ATCC23366	1	N	N
13	*M. avium*	ATCC25291	1	N	N
14	*M. flavum*	ATCC43999	1	N	N
15	*Streptococcus pneumoniae*	Isolate strains (GZCDC)	1	N	N
16	*Staphylococcus aureus*	Isolate strains (GZCDC)	1	N	N
17	*Escherichia coli*	Isolate strains (GZCDC)	1	N	N
18	*Bacillus cereus*	Isolate strains (GZCDC)	1	N	N
19	*Listeria monocytogenes*	Isolate strains (GZCDC)	1	N	N
20	*Salmonella*	Isolate strains (GZCDC)	1	N	N
21	*Klebsiella pneumoniae*	Isolate strains (GZCDC)	1	N	N

MTBC, *Mycobacterium tuberculosis* complex; *ATCC*, American Type Culture Collection; *GZCDC*, Guizhou Provincial Centers for Disease Control and Prevention; *BCD*, Bacillus Calmette-Guerin; *P,* positive; *N,* negative

gyrB-LF*, and *gyrB*-LB; 0.8 μM inner primers *IS6110*-FIP*, *IS6110*-BIP, *gyrB*-FIP*, and *gyrB*-BIP; 1 μl of *Bst* DNA polymerase (8 U); and 0.4 mM biotin-14-dCTP; and 1 μl of DNA template.

Detection of LAMP products

Three monitoring technologies, including colorimetric indicators (MGs), real-time turbidimeters, and LFBs, were applied to analyze the LAMP amplicons. When using MG, the LAMP products cause the color to change from colorless to light green, indicating positive reactions. However, the products remain colorless, indicating negative results. With LFB detection, the appearance of the control line and test line 1 indicated a positive reaction for *IS6110*-LAMP; the appearance of the control line and test line 2 indicated positive results for *gyrB*-LAMP amplification; the appearance of the control line, test line 1, and test line 2 indicated positive results for m-LAMP amplification; and the appearance of only the control line indicated negative results or blank control amplifications.

Temperature optimization of the LAMP assays

Reaction temperature is important for LAMP amplification. The optimal amplification temperature was tested and ranged from 63 to 70 °C, with 1 °C interval for 60 min. The LAMP products were analyzed with a real-time turbidimeter (LA-500). Turbidity > 0.1 was regarded as a positive result.

Sensitivity of the LAMP-LFB assays

The limit of detection (LoD) of each LAMP reaction (*IS6110*-LAMP-LFB, *gyrB*-LAMP-LFB, and mLAMP-LFB) was detected using serial dilutions of template genomic DNA from reference strains (H37Rv) (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per microliter). The LAMP amplifications were carried out as described above, and the LAMP products were analyzed with MG and LFB. Three replicates were detected for each dilution.

Optimization of the reaction time of the MTBC-mLAMP-LFB assay

The optimal amplification time for the MTBC-mLAMP-LFB assay was tested by increasing the reaction time from 30 to 60 min at 10 min intervals. The MTBC-mLAMP-LFB products were determined using LFB. Each reaction time was tested at least three times.

Specificity analysis of the MTBC-mLAMP-LFB assay

The specificity of the MTBC-mLAMP-LFB was determined with DNA templates from H37Rv (ATCC 27,294), the Bacillus Calmette-Guerin (BCG) strain, *M. africanum*, *M. bovis*, 10 isolated MTBC strains (GZCDC), 11 nontuberculous mycobacteria (NTBM), and 7 *nonmycobacterial* strains (Table 2). The LAMP reactions were carried out as described above, and the products were tested using LFB. All examinations were carried out at least three times.

Feasibility of the MTBC-mLAMP-LFB assay with clinical samples

To verify the feasibility of the MTBC-mLAMP-LFB assay, sixty suspected MTBC infection specimens were collected from the Guizhou Provincial Center for Disease Control and Prevention. These clinical samples were simultaneously detected with traditional cultivation, sputum smear microscopy, GeneXpert MTB/RIF, and mLAMP-LFB methods for MTBC identification.

Results

Confirmation of *IS6110*- and *gyrB*-LAMP assays

To verify the most effective of the three sets of primers for *IS6110*-LAMP or *gyrB*-LAMP, LAMP reactions were performed at 65 °C for 60 min with genomic DNA from H37Rv strain pure cultures as templates. The two most effective sets of primers were selected using real-time turbidity (LA-500), which has been used as a future experimental primer (data not shown). Then, to confirm the amplification with the two sets of LAMP primers (Table 1; Fig. 1), *IS6110*-LAMP, *gyrB*-LAMP, and mLAMP amplicons were analyzed based on color changes and the LFB method. The color changed from colorless to brilliant green for positive LAMP amplifications but remained colorless for negative amplifications (Fig. 2a, c, and e). In LFB detection, the appearance of the control line (CL) and test line 1 (TL1) in the biosensor indicated positive results of *IS6110*-LAMP, the appearance of the control line (CL) and test line 2 (TL2) indicated positive results of *gyrB*-LAMP, and the appearance of only the control line (CL) indicated the blank control (Fig. 2b, d, and f). Thus, these results indicated that the two sets of LAMP primers for *IS6110*-LAMP and *gyrB*-LAMP amplification were suitable for the MTBC-mLAMP-LFB assay.

Optimal reaction temperature

To identify the optimal reaction temperature, the LAMP reaction was conducted from 60 to 67 °C (1 °C interval) with 10 pg per reaction of H37Rv (ATCC 27,294) genomic DNA as a template. The *IS6110*- and *gyrB*-LAMP amplifications were determined through real-time turbidity (LA-500). The results suggested that the amplification temperatures of *IS6110*-LAMP and *gyrB*-LAMP ranged from 64 to 67℃ and 65 to 70℃, respectively (Fig. 3). Therefore, these results confirmed that 65 °C was the most suitable reaction temperature for the MTBC-mLAMP-LFB assay.

Sensitivity of the *IS6110*-LAMP-LFB, *gyrB*-LAMP-LFB, and mLAMP-LFB assays

The LoD of each LAMP assay was determined with serially diluted DNA template (range from 100 ng to 1 fg). LAMP amplifications were performed as described above, and the products were analyzed using MG reagents and LFB methods. The results demonstrated that as little as 100 fg of DNA template could produce sufficient amplicons for each detection (Fig. 4). More importantly, the LoD of mLAMP for simultaneously detecting the *IS6110* and *gyrB* genes was also 100 fg of DNA template per reaction (Fig. 5).

Optimization of reaction time for MTBC-mLAMP-LFB assay

To optimize the reaction time for the MTBC-mLAMP-LFB assay of the isothermal amplification stage. Four amplification times (30, 40, 50, and 60 min) were tested at the optimal amplification temperature of 65 °C. The results demonstrated that the LoD of the MTBC DNA template (100 fg per reaction) was identified when the mLAMP reaction lasted 40 min (Fig. 6). Therefore, a reaction time of 40 min was regarded as an optimal amplification for the MTBC-mLAMP-LFB assay.

Specificity of the MTBC-mLAMP-LFB assay

M. tuberculosis H37Rv, *M. africanum*, *M. bovis*, Bacillus Calmette-Guerin (BCG), 6 isolated MTBC, 6 nontuberculous mycobacteria, and 7 *nonmycobacterial* strains were tested with mLAMP-LFB technology. Only MTBC strains showed positive results, and a positive result was not observed when genomic DNA was extracted from non-MTBC strains (Fig. 7). Therefore, the mLAMP-LFB was highly specific for MTBC assessment.

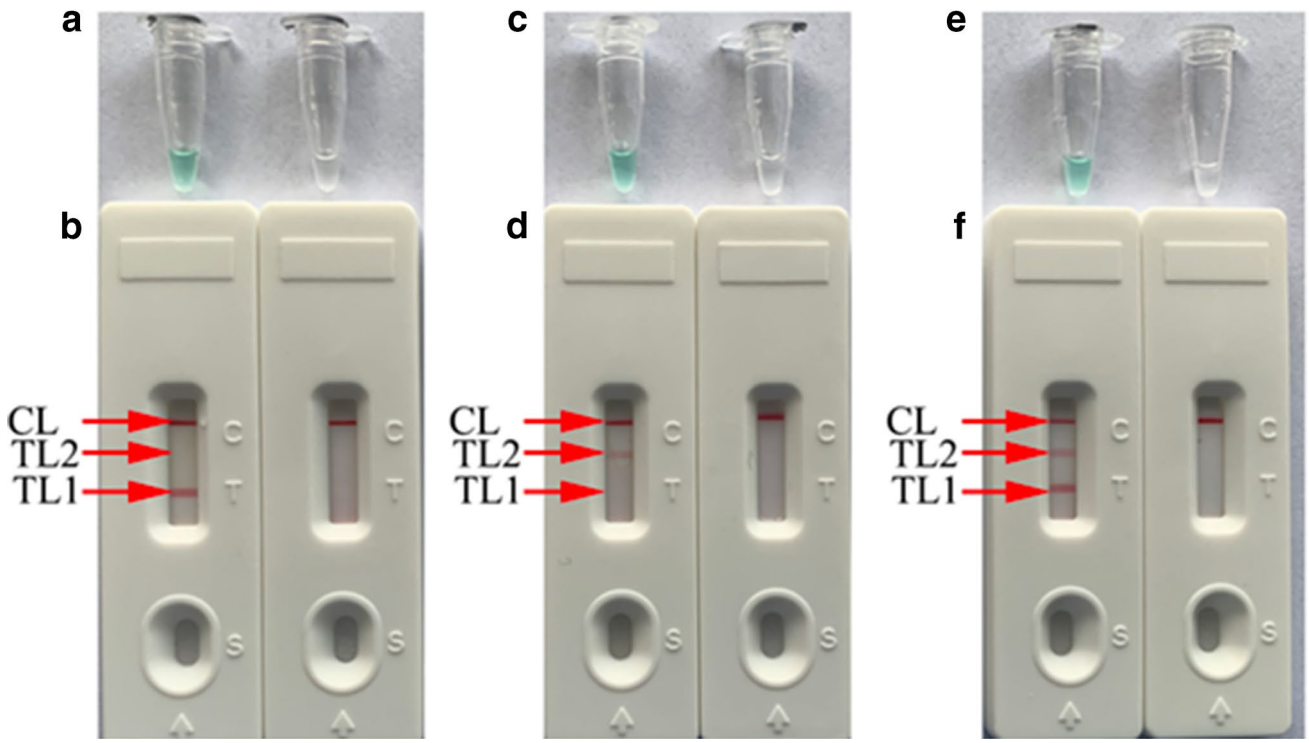

Fig. 2 Confirmation of m-LAMP technology for identifying MTBC strains. The *IS6110*-, *gyrB*-, or mLAMP reactions, containing 10 ng of genomic DNA template (H37Rv, ATCC27294), were amplified at a constant temperature of 65℃ for 1 h, and the LAMP products were analyzed with visual detection reagents (**a**, **c**, **e**) and a lateral flow biosensor (**b**, **d**, **f**). Double distilled water (DW) was used as a negative control (NC). The color changed from colorlessness to brilliant green, indicating positive LAMP amplifications, while the negative amplifications remained colorless. CL and TL1 appeared as crimson red bands, indicating positive results of the *IS6110*-LAMP products; CL and TL2 appeared as crimson red bands, indicating positive results of the *gyrB*-LAMP products; and CL, TL1, and TL2 appeared as three red bands, indicating positive results of mLAMP amplification

Evaluation of MTBC-mLAMP-LFB technology for testing clinical samples

To further demonstrate the mLAMP-LFB technique as a valuable method for the detection of MTBC, a total of 60 clinical sputum samples from suspected MTBC-infected patients were collected from the Guizhou Provincial Center for Disease Control and Prevention (GZCDC). The results indicated that 43 of 60 had been verified as MTBC-positive samples with traditional cultivation. The results of the MTBC-mLAMP-LFB assay were consistent with the cultivation detection. Nevertheless, only 37 and 41 clinical samples were identified as MTBC-positive through sputum smear microscopy and GeneXpert detection, respectively (Table 3). The true positive rate and true negative rate for the MTBC-mLAMP-LFB assay were both 100%, while they were 86.04% and 100% for sputum smear microscopy and 95.35% and 100% for GeneXpert, respectively. Thus, the results indicated that MTBC-mLAMP-LFB technology could represent a feasible tool for detecting MTBC strains.

Discussion

According to the 2018 WHO global tuberculosis report (WHO, 2018), tuberculosis remains one of the top 10 causes of death in many developing countries, and a considerable number of TB patients remain in developed areas [3, 21]. Developing better TB diagnostic methods is essential for achieving "The End TB Strategy." Conventional pathogenic diagnosis techniques, including culture-based techniques and sputum smear microscopy, are time-consuming and lack sensitivity, respectively [22]. For these reasons, many patients miss the optimal treatment period or drugs, resulting in drug resistance. Along with the continuous advancement of molecular detection technology, PCR-based methods, such as real-time PCR and GeneXpert, have been extensively applied in clinical practice [23, 24]. In 2013, GeneXpert MTB/RIF became the first molecular TB test endorsed by the WHO and has been a considerable investment in its extended application [25, 26]. However, the major drawback of these methods is their relatively high consumption and

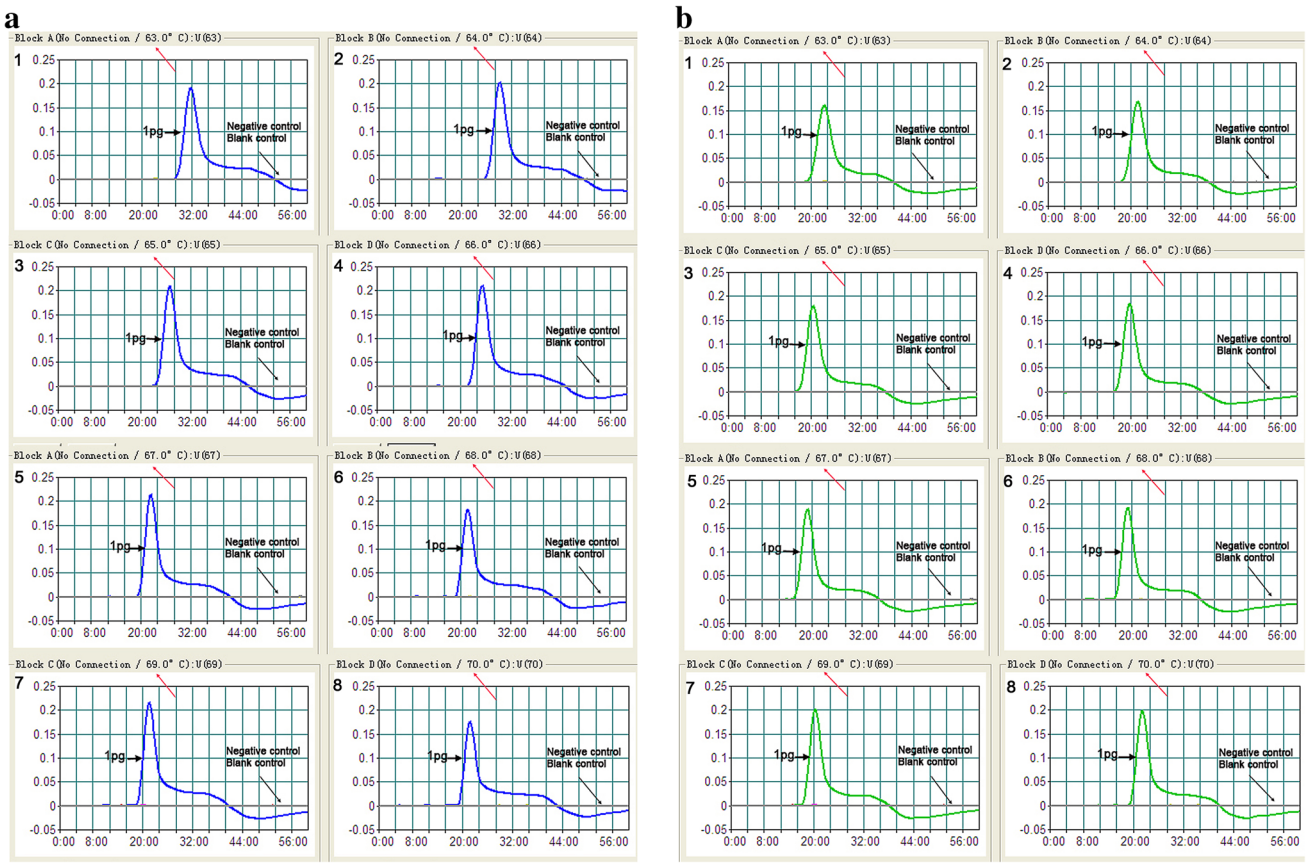

Fig. 3 Optimization of reaction temperature for mLAMP detection. LAMP amplification for the assessment of *IS6110* (**a**) and *gyrB* (**b**) was determined by real-time measurement of turbidity (LA-500), and the corresponding curves of DNA concentrations are marked in the graphs. Turbidity > 0.1 was considered a positive result. Eight kinetic graphs were obtained at various reaction temperatures (63–70 °C, 1 °C intervals) with the target DNA template from the H37Rv strain (ATCC27294) at the level of 10 ng per reaction. **a** Graphs from 2 (64℃) to 5 (67℃) showed robust amplification and **b** graphs from 3 (65℃) to 8 (70℃) showed robust amplification

device costs, which makes them unaffordable in most developing countries with a high TB burden. To expand the availability of molecular testing for TB, developing a low-cost, reliable, simple, rapid, sensitive, specific detection technique to identify MTBC is essential for the therapy and management of TB patients.

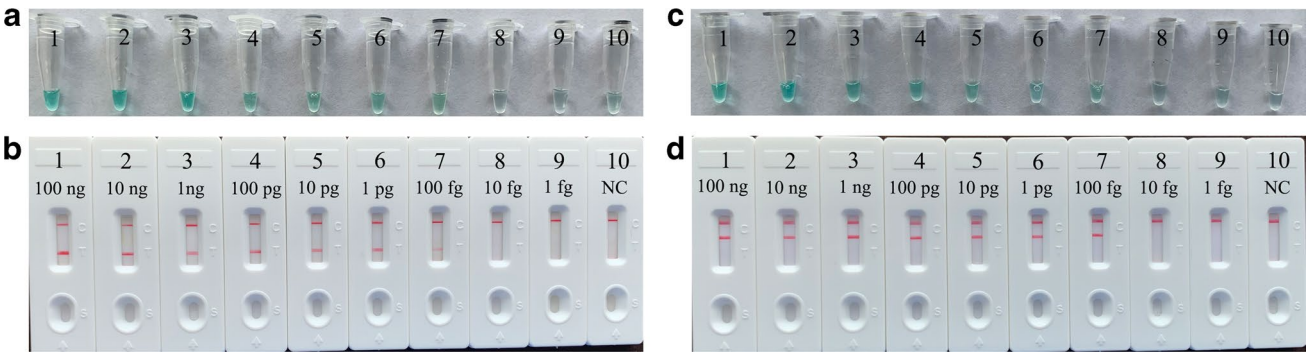

Fig. 4 Sensitivity analysis of *IS6110*-LAMP-LFB (**a**, **b**) and *gyrB*-LAMP-LFB (**c**, **d**) techniques with serial dilutions of DNA template extracted from the H37Rv strain (ATCC27294). Serial dilutions (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per microliter) of H37Rv strain genomic DNA were used as templates, and distilled water (DW) was used as a negative control (NC). The amplification products were analyzed with MG and LFB. The LoD of *IS6110*-LAMP was 100 fg per reaction, which was the same as the LoD of the *gyrB*-LAMP assay

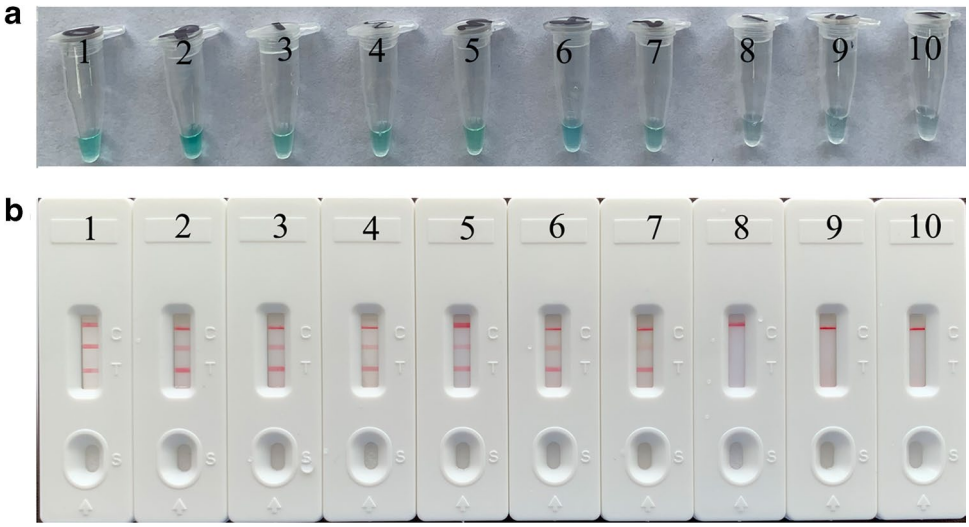

Fig. 5 Sensitivity analysis of the MTBC-mLAMP-LFB assay with serial dilutions of genomic DNA extracted from the H37Rv strains. Two sets of primers targeting the *IS6110* and *gyrB* genes were simultaneously added to a single reaction system, and the LoD of mLAMP for detecting the *IS6110* and *gyrB* genes was analyzed with MG (**a**) and LFB (**b**) techniques. The genomic DNA was serially diluted (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per microliter) and carried out with standard LAMP and distilled water (DW) as a negative control. The LoD of the m-LAMP-LFB assay was 100 fg per reaction

In the current study, a novel nucleic acid amplification method, multiplex loop-mediated isothermal amplification (mLAMP) combined with a nanoparticle-based lateral flow biosensor (LFB), was applied for the detection of the *M. tuberculosis* complex. LAMP technology relies on autocycling strand displacement DNA synthesis performed by *Bst* DNA polymerase [27, 28], which can be conducted under isothermal conditions ranging from 60 to 65 °C and has high specificity for six primers that recognize eight distinct regions on the target DNA. Moreover, under thermal cycling, the LAMP reaction time is shorter than that of PCR technology. The *IS6110* gene, which belongs to a family of ISs of the IS3 category, has been widely applied to PCR detection in the *M. tuberculosis* complex genome, and this process is believed to confer higher sensitivity and specificity [15]. However, in recent decades, several clinical investigations have found that some *M. tuberculosis* strains lack the *IS6110* gene, especially from Southeast Asian areas [7, 16]. Therefore, it is necessary to use other potential target genes for detecting MTBC. The *gyrB* gene encodes the subunit B protein of DNA gyrase (topoisomerase type II) and is a valuable subsidiary nucleic acid marker in studies performed to detect MTBC [29, 30]. In the current study, the MTBC-mLAMP-LFB assay targeting the *IS6110* and *gyrB* genes was successfully developed.

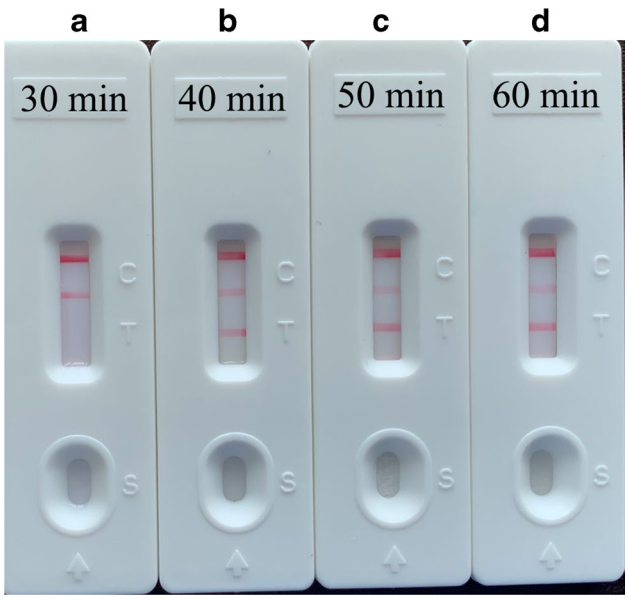

Fig. 6 Optimal amplification time for the MTBC-mLAMP-LFB assay. Four different amplification times (**a** 30 min, **b** 40 min, **c** 50 min, and **d** 60 min) were evaluated at 65℃. The genomic DNA was 100 fg per reaction. The best sensitivity was observed when the reaction lasted for 40 min (**b**)

The MTBC-mLAMP-LFB assay was able to detect 100 fg of genomic DNA per reaction (Figs. 4 and 5). However, this process represented a type of ideal reaction state. To better fit clinical applications, the artificial sputum contaminated with a pool of bacteria, including non-tuberculous bacteria, could be used for identification of the minimal amount of *M. tuberculosis* DNA with the novel method for further evaluation. mLAMP-LFB detection of the target *IS6110* and *gyrB* genes identified MTBC with 100% specificity, which was verified using genomic DNA templates from *M.*

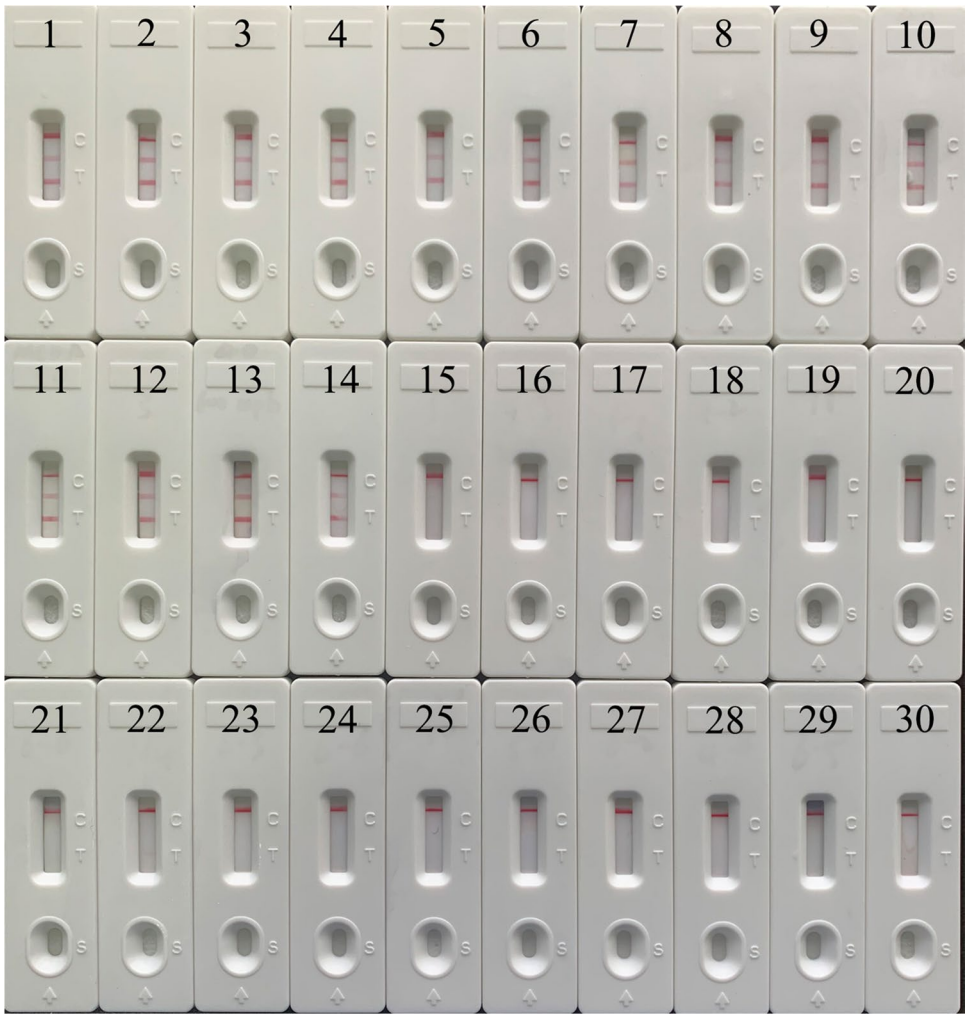

Fig. 7 Analytical specificity of the MTBC-mLAMP-LFB assay with different strains. The mLAMP reactions were performed using different genomic DNA as templates, and each of the amplification products was detected with the LFB method. Biosensor 1, H37Rv (ATCC27294); biosensor 2, *M. bovis* Bacillus Calmette-Guerin; biosensor 3, *M. africanum*; biosensor 4, *M. bovis*; biosensors 5–14, ten isolated MTBC strains from GZCDC; biosensors 15–23, nine NTBM strains from GZCDC (*M. marinum*, *M. gordon*, *M. phlei*, *M. xenopi*, *M. abscess*, *M. chelonae*, *M. aureus*, *M. avium*, and *M. flavum*, respectively); and biosensors 24–30, *S. pneumonia*, *S. aureus*, *E. coli*, *B. cereus*, *L. monocytogenes*, *salmonella*, and *K. pneumonia*

tuberculosis H37Rv, *M. africanum, M. bovis*, *M. bovis* BCG, 6 isolated *M. tuberculosis* strains, 9 NTBM, and 7 additional pathogenic bacteria strains. In addition, a nanoparticle-based lateral flow biosensor (LFB) detector was successfully applied to identify LAMP reaction products. According to previously reported agarose gel electrophoresis results, the MG and turbidity methods were equally appropriate for analyzing LAMP products [31, 32]. However, these methods are not specific for target genes and likely cause false-positive results. To address these shortcomings, a target-specific, easily operated, visual, and low-cost (approximately $2 USD) nanoparticle-based lateral flow biosensor (LFB) assay method was successfully developed and applied to identify MTBC-mLAMP products in this study.

Table 3 Comparison of conventional culture, sputum smear microscopy, GeneXpert, and mLAMP-LFB methods to detect *Mycobacterium tuberculosis* complex in clinical samples

Detection assay	Results	Gold standard method (Cultivation)		True positive rate (%)	True negative rate (%)
		+	-		
Sputum smear microscopy	+	37	0	86.04%	100%
	-	6	17		
Gene Xpert	+	41	0	95.35%	100%
	-	2	17		
mLAMP-LFB	+	43	0	100%	100%
	-	0	17		

The whole MCTB-mLAMP-LFB detection process, including DNA template extraction (~30 min), LAMP reaction (40 min), and LFB identification (~2 min), could be finished within 80 min. The running cost of one test, including DNA template extraction (~$1 USD), LAMP amplification kit (~$3.5 USD), and LFB detector (~$2 USD), is estimated to be $6.5 USD, which is less expensive than normal PCR-based methods.

In conclusion, the MTBC-mLAMP-LFB assay established in this study is a valuable, low-cost, rapid, and specific

approach for the detection of MTBC that could save diagnostic time and help determine the optimal treatments for patients in a timely manner. Furthermore, developing a low-cost diagnostic approach could reduce the patient's financial burden, especially in some resource-constrained areas.

Abbreviations MTBC: *M. tuberculosis* Complex; WHO: World Health Organization; ATCC: American Type Culture Collection; LAMP: Loop-mediated isothermal amplification; LFB: Lateral flow biosensor; MG: Malachite green; PCR: Polymerase chain reaction; LoD: Limit of detection; GZCDC: Guizhou Provincial Center for Disease Control and Prevention; FAM: Carboxyfluorescein; Dig: Digoxigenin; mer: Monomeric unit; nt: Nucleotide; CL: Control line; TL1: Test line 1; TL2: Test line 2; NC: Negative control; BC: Blank control; DW: Distilled water

Acknowledgements We acknowledge the medical staff at the Guizhou Provincial Center for Disease Control and Prevention for their cooperation in this study.

Author contribution XC and SL designed and conceived the experiments. XC, JH, and SL designed and analyzed the LAMP primers. XC, JH, ZX, XY, and YC performed the experiments. YC, WZ, WC, and HC collected and analyzed the data. XC and SL wrote and revised the manuscript. XC and SL revised the manuscript. All authors read and approved the final manuscript.

Funding This study was funded by grants from the Science and Technology Department of Guizhou Province (No. [2019]1186, [2019]2822), [2018]-5606, and [2018]-5767).

Data availability The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval The study was approved by the Human Ethics Committee of the Guizhou Provincial Center for Disease Control and Prevention and complied with the Declaration of Helsinki. All data/isolates were analyzed anonymously. The patients with tuberculosis included in the present research were given a subject information sheet, and they all provided written informed consent before participating in the study.

Competing interests The authors declare no competing interests.

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