Two Target Genes Based Multiple Cross Displacement Amplification Combined with Lateral Flow Biosensor for the Detection of Mycobacrterium Tuberculosis Complex

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Research Article

Abstract

Background: *Mycobacterium tuberculosis* complex (MTBC) causes tuberculosis (TB), which is a global public health problem that seriously endangers public health. Hence, development of a new and rapid method to detect MTBC is of great significance for prevention and treatment of TB.

Results: In this study, a multiple cross displacement amplification combined with nanoparticle-based lateral flow biosensor (MCDA-LFB) was developed to simultaneously detect two target genes (*IS6110* and *mpb64*) of MTBC. One suit of specific multiple cross displacement amplification (MCDA) primers, which was designed for *IS6110* and *mpb64* gene, respectively, was validated through using the genomic DNA extracted from reference strain H37Rv. The MCDA products were analyzed using real-time turbidity curve, colorimetric indicator (Malachite Green, MG) and LFB. The conditions of amplification temperature and time were optimized and the established MCDA-LFB method was applied to detect the sputum specimens and MTBC strains from clinical samples. The results show that two sets of MCDA primers for the *IS6110* and *mpb64* genes have detected MTBC validly. The MCDA reaction conditions were optimized at 67 °C for 35 min. The limit of detection of MCDA assay based on *IS6110* and *mpb64* genes was 100 fg of genomic DNA template per reaction. The specificity of MCDA-LFB detection was 100%, and no cross-reactions for non-MTBC strains detection. The positive rate of MCDA-LFB for the detection of MTBC strains was equal to that of semi-nested automatic real-time PCR (Xpert MTB/RIF), and had a higher positive rate than acid-fast staining (AFS) when used for the detection of sputum samples. The whole procedure of MCDA-LFB, including genomic template preparation, MCDA reaction and products analysis, was completed with 70 min.

Conclusion: The simplicity, rapidity, sensitivity and reliability of the MCDA-LFB based on *IS6110* and *mpb64* gene of MTBC developed in this study make it potentially significant for the prevention and treatment of TB.

Background

Tuberculosis (TB), a chronic infectious disease, is caused by *Mycobacterium tuberculosis* complex (MTBC), including *M. tuberculosis, M. bovis,M. bovis Bacillus Calmette* (BCG), *M. africanum, M. carinii, M. suricattae, M. orygis, M. microti, M. caprae, M. mungi, M. canettii,* and *M. pinnipedii* and *M. vole*. Especially the *M. tuberculosis*, *M. bovis* and *M. africanum* are highly pathogenic [1-4]. TB seriously endangers human health, which is a public health and social problem of global concern [5]. The World Health Organization (WHO) has made TB one of the key infectious diseases. There were estimated 10 million new TB cases and about 1.5 million TB-related deaths worldwide [5]. According to WHO estimated more than 2 billion people, approximately one-third of the global population, are latently infected with MTB [6, 7]. The detection and identification of MTBC is an important means for TB prevention and treatment. Therefore, developing a reliable, sensitive and rapid method for detection of MTBC is of great significance for controlling the current global pandemic [6, 8]. With the development of detection technology, there are increasing established methods for detection of MTB, mainly including sputum

smear microscopy, mycobacterial cultivation identification, molecular detection and other methods [8, 9]. Among the detection methods, it is deemed that sputum smear microscopy lacks sensitivity [10, 11]; mycobacterial culture has always been considered as a gold standard for MTBC diagnosis, but this method requires long processing time (more than 4 weeks) [10, 11]. The molecular methods are of high sensitivity, however, they need equip special instruments [10].

The multiple cross displacement amplification (MCDA) technique was based on isothermal strand-displacement polymerization reaction, with high specificity and sensitivity [12]. The target sequence has ten special primers spanning ten distinct regions and at a constant temperature to react [12, 13]. The real-time turbidity, agarose gel electrophoresis, colorimetric indicators and nanoparticles-based lateral flow biosensor (LFB) were selected for MCDA products analysis [12, 13], especially multiple cross displacement amplification combined with nanoparticles-based lateral flow biosensor (MCDA-LFB) make the products analysis simple and visual[13-15]. The MCDA technique was applied for the detection of *Salmonella spp*. strains and *Shigella spp* [13], *Vibrio parahaemolyticus* [14] and *Listeria monocytogenes* [15]. Our team also created the MCDA method for *Brucella spp* [16] and *Neisseria meningitidis* [17] detection successfully.

In this study, the MCDA-LFB method for MTBC detection had been successfully established by our group. Two specific targets gene of the *IS6110* and *mpb64* were chosen for MTBC-MCDA detection, and 10 specifically primers were designed for the targets. The MCDA products were analyzed through using LFB, real-time turbidity and colorimetric indicator (Malachite Green, MG). Then the MCDA reaction conditions, including amplification temperature and time, were optimized. Subsequently, the sensitivity and specificity of the MTBC-MCDA-LFB technique were tested, and then the MCDA-LFB was applied to detect the sputum specimens and MTBC strains, which were from clinical samples.

Results

Confirmation and detection for MCDA Products

Disposable lateral flow biosensor (LFB) consist of test line 1 (TL1), test line 2 (TL2) and control line (CL). The anti-FITC Ab, anti-Dig Ab and biotin-BSA were sprayed onto the NC membrane for the lines. Firstly, *IS6110* gene (**Fig. 1 A**) and *mpb64* gene (**Fig. 1 B**) were examined by MCDA amplification and detected with LFB and colorimetric indicator (MG), respectively. Then both of the two target genes were detected at the same time (**Fig. 1 C**). The standard strain *M. tuberculosis* (H37Rv, ATCC 27294) genomic templates were used for MCDA assay.

Optimization of the Temperatures for MTB-MCDA Assay

To evaluate the optimum amplification temperature, *M. tuberculosis* (H37Rv, ATCC 27294) strain genomic templates were used as the positive control at a level of 100 pg per reaction and the reactions were

monitored by the real-time turbidity (LA-320C) method. Both *IS6110* gene and *mpb64* gene were detected (**Fig. 2**), and examined the effect fixed temperatures ranging from 63 °C to 70 °C with 1 °C intervals for MCDA amplification. The *M. avium* genomic templates were used as negative control. According to the generated typical kinetics graphs, at 67 °C, both the *IS6110* and *mpb64* genes amplification had the shortest time interval with higher turbidity and faster reaction times at 32 min and 28 min. Thus, the amplification temperature of 67 °C was applied to perform the remaining experiments in the study.

Optimization of the times for MTB-MCDA-LFB Assay

The four reaction times (20, 30, 40 and 50 min) were tested at 67 °C according to the standard MCDA conditions for the optimum time by *M. tuberculosis*-MCDA-LFB assay during reaction stage. The DNA level with 100 pg of *M. tuberculosis* genomic templates per reaction was displayed on three visible-red lines (TL1, TL2 and CL) on the LFB. The earliest was observed when the amplification lasted for 30 min at 67 °C (**Fig. 3**). So, the reaction time of 30 min was recommended as a reasonable reaction time for *M. tuberculosis*-MCDA-LFB assay.

Sensitivity of *M. tuberculosis*-MCDA-LFB method

The genomic DNA templates of MTB (H37Rv, ATCC 27294) were serially diluted (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg) for *M. tuberculosis*-MCDA sensitivity analysis. The limiting dilution of *M. tuberculosis* genomic DNA was evaluated by MCDA detection. The lowest limit of detection by *M. tuberculosis*-MCDA-LFB assay for singly *IS6110* gene (**Fig. 4 A**) or *mpb64* gene (**Fig. 4 B**) was found to be 10fg per reaction. Double targets gene were found to be 100fg per reaction (**Fig. 4 C**). As expected, CL, TL1 or/and TL 2 were observed on the biosensor, displaying the positive MCDA results for *IS6110* gene or/and *mpb64* gene. The double distilled water (DW) was the template for the blank control. Moreover, the analytical sensitivity of *M. tuberculosis*-MCDA by biosensor was consistent with colorimetric indicator analysis.

Specificity of MCDA-LFB for *M. tuberculosis* complex detection

Four MTBC strains, 12 NTM strains and 10 other bacteria strains were used to specificity of MCDA-LFB assay. When the genomic DNA, which were from the bacteria listed in **Table 1**, were used in the MCDA-LFB assay, only the DNA from MTBC strains showed positive results. Three red lines, including the TL1, TL2 and CL, appeared on the strips for the positive tests. The DNA from NTM and other bacteria strains presented negative results. Only the CL line appeared on the strips. DW as the template for BC represented negative result by MCDA-LFB assay (**Fig. 5**).

Application of MCDA-LFB assay for *Mycobacterium Tuberculosis* Complex Strains

The 34 culture strains of *Mycobacterium Tuberculosis* (MTB) were applied for MCDA-LFB and semi-nested automatic real-time PCR (Xpert MTB/RIF) detection (**Table 2**). The genomic DNA of the strains was amplification by MCDA, and then the LFB was used for detecting product. DW was regarded as the templates for BC, and the standard strain *M. tuberculosis* (H37Rv, ATCC 27294) genomic templates was used as positive control (PC). All of the strains showed positive results for GeneXpert and MCDA-LFB assay, with 100% detection rate.

Application of MCDA-LFB assay for sputum samples

The 51 sputum samples (provided by pulmonary hospital of Guiyang) were detected by acid-fast staining (AFS) and MCDA-LFB (**Table 3**). All the samples were examined by AFS and microscopy. The results consist of 26 positive samples and 25 negative samples. The positive detection rate was 50.98%. Then MCDA-LFB was applied for the genomic DNA of sputum samples. The results showed that 41 were positive and 10 were negative, the rate of positive detection was 80.39%. DW was used for BC, and the standard strain *M. tuberculosis* (H37Rv, ATCC 27294) genomic templates used as PC.

Discussion

TB, a chronic infectious disease, is caused by *Mycobacterium tuberculosis* complex (MTBC) [1, 5]. TB seriously endangers human health, is a public health and social problem of global concern [5]. Thus, the prevention and treatment of TB need techniques to detect and identify for MTBC, especially a reliable, sensitive and rapid method for detection is important. This method is of great significance for controlling the current global pandemic [6, 8]. A wide variety of detection techniques have been established to meet the diagnostic of TB, such as sputum smear microscopy, mycobacterial cultivation identification, molecular detection and other methods [8, 9]. The molecular detection methods were based on the PCR technique with high reliability and sensitivity [10]. Loop-mediated isothermal amplification (LAMP) technology is anucleic acid amplification technology invented by Notomi T in Japan in 2000 [18]. LAMP has been applied to detect many bacterial pathogens due to its reliability, rapidness and sensitivity [19-21]. In this study, the technique of multiple cross displacement amplification combined with nanoparticles-based lateral flow biosensor (MCDA-LFB) for MTBC detection, was developed from LAMP technology.

In the current study, the *IS6110* and *mpb64* genes were chosen as two specific targets, and 10 MTBC-MCDA primers were designed for each of them. The *IS6110* gene has been widely applied for PCR detections in the *M. tuberculosis* complex genome, which belongs to a family of IS of the IS3 category with high specificity [22]. However, some of *M. tuberculosis* strains absence of *IS6110* gene were found in

several clinical investigations during recent decades [23, 24]. Therefore, in this research, we added another target gene, the *mpb64* gene. The *mpb64* is encoded in the RD2 region of the *M. tuberculosis* genome [25], which creates the MPB64 protein, with a strong specificity for MTBC [25, 26]. While, there is some evidence for absence of the *mpb64* gene in some substrains of *Mycobacterium bovis* BCG [27]. But there are not reports for absence of both *IS6110* gene and the *mpb64* gene. In the study, we chose both the *IS6110* gene and *mpb64* gene as the target genes for MTBC, to ensure its specificity. The MCDA-LFB technique established to assay MTBC was successful by detecting the *IS6110* and *mpb64* genes. In the current study, the detection of 4 MTBC strains, 12 NTM strains and 10 other bacteria strains (**Table 1**) were used to test specificity of MCDA-LFB assay (**Fig. 4**). The specificity of the MCDA-LFB assay was verified by the genomic DNA of the strains, and the MCDA-LFB detection of the *IS6110* and *mpb64* genes tested MTBC with 100% specificity.

In this research, the MCDA products were analyzed through using LFB, colorimetric indicator (Malachite Green, MG) and turbidity method. Comparing the above three assay methods, we deemed the LFB was more visual. And it did no needs special instruments and regents [16, 17]. Besides, LFB can detect both the *IS6110* and *mpb64* genes and make them visualized in a single test at the same time. According to the results, it is found that at the 67°C, both the *IS6110* and *mpb64* genes amplification had the shortest time interval with higher turbidity and faster reaction times at 32 min and 28 min (**Fig. 2**). Then the LFB assay showed that the earliest was observed at the amplification, which lasted 30 min (**Fig. 3**). Hence, the conditions of amplification temperature and time were optimized for MCDA-LFB at 67 °C for 35 min. The all detection process has spent about 70 min, including genomic template preparation (approximately 30 min), MCDA reaction (approximately 35 min) and products assay (approximately 5 min). Base on *IS6110* and *mpb64* genes, the limit of detection of MCDA assay was 100 fg of genomic DNA template per reaction (**Fig. 4**).

The semi-nested automatic real-time PCR (Xpert MTB/RIF) was a sensitive detection of MTB with rifampin (RIF) resistance [28], and the detection time was about 2.5 h. In this research, the application of MCDA-LFB for MTB was equal to the Xpert MTB/RIF (**Table 2**). Usually, Xpert MTB/RIF have four-channel detection system, which means this machine can test 4 samples at the time. But it needs to use the matching kit, which is a high cost. The Xpert MTB/RIF is suitable as a rapid screening technology for MTB with RIF resistance, not suitable for the large detection of MTB samples. While the MCDA-LFB technique was a better choice for detecting MTBC with large specimen size. Importantly, it low cost. Comparing with the acid-fast staining (AFS) combined with microscopy detected MTB of sputum specimens, an additional 15 sputum samples were detected by MCDA-LFB technique, and the detection rate was 29.41%, which is higher than the sputum smear microscopy, with a 100% coverage rate of the positives. The sputum smear microscopy lacks sensitivity [10, 11], takes approximately 2 h, including fixing, dyeing, drying naturally and microscopy. And the microscopy takes more time as the samples increasing. However, MCDA-LFB technique can elegantly solve this problem. Since it does not cost much time, at the meantime the maximum detection quantity of it was 96 samples.

Conclusions

In the study, a reliable, rapid, visual, inexpensive and simple MCDA-LFB assay based on the *IS6110* and *mpb64* genes have been successfully established for detection of MTBC. This technique could identify *M. tuberculosis* complex with high specificity, sensitivity, and rapidness, and it was visual and simple assay the MCDA products. Thus, the MTBC-MCDA-LFB method could be regarded as a useful technique for rapid and reliable identification of *M. tuberculosis* complex in clinical samples. Moreover the technique did not require complicated instrument and expensive reagents, and could be use widespreadly, especially, in resource-limited areas of developing countries with a high TB burden.

Methods

Reagents and Apparatus

DNA isothermal amplification kits, Colorimetric indicator (Malachite Green, MG), biotin-14-dCTP and nanoparticles-based lateral flow biosensor (Disposable Lateral Flow Biosensor, LFB#2) were purchased from Bei-Jing HaiTaiZhengYuan. Co., Ltd. (Beijing, China). The DNA extraction kits (QIAamp DNA minikits; Qiagen, Hilden, Germany) were purchased from Qiagen (Beijing, China).

Design of MCDA primer

There are two specific MTC-MCDA targets gene of the *IS6110* (GenBank, Sequence ID: CP053903.1) and *mpb64* (GenBank, Sequence ID: CP053903.1) were chosen. The 10 specific primers were designed by primer software PRIMER PREMIER 5.0 and Primer Explorer V4 [13] for each of them. In addition, FITC (fluorescein isothiocyanate) was labeled at 5ʹ end of the *IS6110*-C1* primer, digoxigenin (Dig) was labeled at 5ʹ end of the *mpb64*-C1* and biotin labeled at 5ʹ end of the *mpb64*-C1 *mpb64*-D1* primers. The details, including primer design, sequences and modifications, locations in the expression site of the *IS6110* and *mpb64* genes were listed in **Table 4** and **Fig. 6**. All of the primer sequences were synthesized and purified through Tian-Yi Biotech (Beijing, China) at HPLC purification grade.

Bacterial Strains and Genomic DNA Preparation

There were 60 strains among MTBC 38 strains, *Non-tuberculous mycobacterium* (NTM) 12 strains and other bacteria 10 strains, the detail in **Table 1**. The bacterial strains were stored in 10% (w/v) glycerol broth at −80 °C, then were refreshed and cultured. According to the instruction book, the genomic templates were extracted from the cultured strains by the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA). Sputum samples were treated with 4% NaOH solution and genomic templates were extracted by the Kit. Then the genomic templates were tested by ultraviolet spectrophotometer at A260/280, and

stored at -20°C before the templates were used. The above experiments are required to be carried out in the biosafety Level II laboratory.

MCDA Reactions and Detection

Three detection methods, including real-time turbidimeter (LA-320C), disposable lateral flow biosensor and colorimetric indicator (MG), were applied to detecting the MCDA amplification products. The suitability of two target genes of MCDA primers were examined through MCDA reaction of the single *IS6110* gene either for *mpb64* gene. Then both of the two target genes were detected at the same time. The standard strain *M. tuberculosis* (H37Rv, ATCC 27294) genomic templates were used for MCDA assay according to the DNA isothermal amplification kits. Briefly, 12.5 μl 2 ×Buffer, 1 μl Bst 2.0 DNA polymerase (10 U), 1 μl biotin-14-dCTP, 1 μl malachite green, 2 μl genomic templates, 5.3 μl DW and 2.2 μl amplification primers or mix primers containing 0.4 μM each of displacement primers F1 and F2, 0.4 μM each of amplification primers C1* and C2, 1.2 μM each of amplification primers R1, R2, D1 (D1*) and D2, 2.4 μM each of cross primers CP1 and CP2, the reaction mixtures of MCDA were in a final volume of 25 μl. Then they were reacted 35 min on the 67 °C by PCR, and 1 μl reaction products were used for LFB detection (**Fig. 1**). Double distilled water was used as the template for the blank control, the *M. avium* genomic DNA was used as the templates in the negative control.

Optimizing the reaction temperature and time of MCDA assay

Both *IS6110* gene and *mpb64* gene were detected at different temperatures by real-time turbidimeter (LA-320C). We examined the effect of different temperatures, from 63 °C to 70 °C with 1 °C intervals for MCDA amplification. The *M. avium* genomic templates were used as negative control. Subsequently, the two target genes were confirmed at different amplification reaction times, 20 min, 30 min, 40 min and 50 min, detected by LFB and MG.

Analytical Sensitivity and Specificity of MCDA Assay

The genomic DNA templates of MTB (H37Rv, ATCC 27294) were serially diluted (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg) for sensitivity analysis by MCDA-LFB detection for single gene and double genes. And double distilled water was the template for the blank control. MTBC 38 strains, *Non-tuberculous mycobacterium* (NTM) 12 strains and other bacteria 10 strains (**Table 1**) were used for specificity assay. The genomic DNA from the strains was amplification by MCDA reactions, then assayed through LFB. The experiments were repeated at least two times.

Culture strains detecting by MCDA-LFB

In this study, 34 MTBC strains were isolated and cultured by Guizhou Provincial Center for Disease Control and Prevention (GZCDC). The semi-nested automatic real-time fluorescence quantitative PCR (Xpert MTB/RIF) and MCDA-LFB method were applied for the culture strains. In short, in the biological safety cabinet, a small amount of bacteria were scraped and placed in the sample reagent following instructions of GeneXpert MTB/RIF kit. Then they were mixed well, reacted and stand for 15 min. The 2 ml reacting solution was added into the Cartridge reaction box, and was tested by GeneXpert system. All the experiments needed the biosafety Level II laboratory.

Sputum samples detecting by MCDA-LFB

The research had 51 sputum samples (provided by pulmonary hospital of Guiyang), which were detected by acid-fast staining (AFS) and MCDA-LFB for MTBC. AFS and microscopy examination, briefly, a small amount of sticky phlegm were selected and spread evenly on the glassslides to form a film. Then the flame was fixed for 30 seconds, and acid-fast staining was carried out. Glass slides were dried naturally and microscopy. The above experiments are required to be carried out in the biosafety Level II laboratory.

Abbreviations

MTBC, *M. tuberculosis* complex; TB, tuberculosis; MCDA, multiple cross displacement amplification; LFB, nanoparticles-based lateral flow biosensor; AFS, acid-fast staining; WHO, World Health Organization; FITC, fluorescein isothiocyanate; Dig, digoxigenin. mer, monomeric unit; nt, nucleotide; ATCC, American type culture collection;LoD, limit of detection; MG, malachite green; GZCDC, Guizhou Provincial Center for Disease Control and Prevention; NTM, *Non-tuberculous mycobacterium*; ATCC, American type culture collection; GZCDC, Guizhou Provincial Center for Disease Control and Prevention; P, positive; N, negative; TL1, test line 1; TL2, test line 2; CL, control line; MG, malachite green; DW, double-distilled water; BC, blank control; PC, positive control.

Declarations

Acknowledgments

Authors' contributions

JH and SL conceived and designed the study. JH, ZX and SL participated in primer design. JH, ZX, XY, and XC contributed to all the laboratory work. WX and YC contributed to the data collection. WZ, WC and

HC performed the statistical analysis. JH and SL wrote the initial draft of the manuscript. JH and SL revised the manuscript. All the authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The raw sequence data reported in this paper were came from GenBank, Sequence ID: CP053903.1, Mycobacterium tuberculosis strain H37Rv_IC1 chromosome (https://www.ncbi.nlm.nih.gov/nuccore/ CP053903.1?report=fasta)

Ethics approval and consent to participate

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.

Consent for publication

Not applicable

Competing interests

All of the authors declare that there are no competing interests in this article.

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Tables

Table 1. The detail of strains

Bacteria	Strain no. (source of strains)	No. of strains	MCDA-LFB result
MTC			
M. tuberculosis	H37Rv ATCC27294	1	P
M. tuberculosis	Isolated strains (GZCDC)	34	P
M. bovis	ATCC19210	1	P
M. africanum	ATCC25420	1	P
Bacillus Calmette-Guerin	Vaccine strain (GZCDC-BCG)	1	P
NTM			
M. aureus	ATCC23366	1	N
M. flavum	ATCC43999	1	N
M. avium	ATCC25291	1	N
M. marinum	ATACC927	1	N
M. abscess	ATCC19977	1	N
M. chelonae	ATCC14472	1	N
M. gordon	ATCC14470	1	N
M. phlei	ATCC11758	1	N
M. nonchromogenic	ATCC19530	1	N
M. xenopi	ATCC19250	1	N
M. aichiense	ATCC27280	1	N
M.microti	ATCC19422	1	N
Other bacteria species			
Enterococcus faecalis	Isolate strains (GZCDC)	1	N
Salmonella	Isolate strains (GZCDC)	1	N
Klebsiella pneumoniae	Isolate strains (GZCDC)	1	N
Pseudomonas aeruginosa	Isolate strains (GZCDC)	1	N
Staphylococcus aureus	Isolate strains (GZCDC)	1	N
Escherichia coli	Isolate strains (GZCDC)	1	N
Bacillus cereus	Isolate strains (GZCDC)	1	N
Listeria monocytogenes	Isolate strains (GZCDC)	1	N

Streptococcus pneumoniae	Isolate strains (GZCDC)	1	**N**
Campylobacter jejuni	Isolate strains (GZCDC)	1	**N**

Abbreviations: MTC, *Mycobacterium tuberculosis complex*; NTM, *Non-tuberculous mycobacterium*; ATCC, American type culture collection; GZCDC, Guizhou Provincial Center for Disease Control and Prevention; P, positive; N, negative.

Table 2. Culture strains Detecting by MCDA-LFB

Culture strains (n=34)	Gene-Xpert	MCDA-LFB
Positive	34	34
Negative	0	0
Positive rate (%)	100%	100%

Abbreviations: MCDA-LFB, multiple cross displacement amplification with lateral flow biosensor.

Table 3. Sputum samples Detecting by MCDA-LFB

Clinical Sputum samples (n=51)	Acid-fast staining	MCDA-LFB
Positive	26	41
Negative	25	10
Positive rate (%)	50.98%	80.39%

Abbreviations: MCDA-LFB, multiple cross displacement amplification with lateral flow biosensor.

Table 4. The details of primers for the *IS6110* and *mpb64* genes

Genes	Primers name [a]	Sequences and modifications	Length
IS6110	IS6110-F1	5'-GGATGGTCGCAGAGATCC-3'	18 nt
	IS6110-F2	5'-ATCGCGTTCGCCCTT-3'	15 nt
	IS6110-CP1	5'-CGCGCAGCCAACACCAAGTAGCAGCACGATTCGGAGTG-3'	38 mer
	IS6110-CP2	5'-CCGGGACCACGACCGAAGACGCAATTCGGCGTTGTC-3'	36 mer
	IS6110-C1*	5'-FITC-CGCGCAGCCAACACCAAGTAG-3'	21 nt
	IS6110-C2	5'-CCGGGACCACGACCGAAGA-3'	19 nt
	IS6110-D1	5'-ACCTCACTGATCGCTG-3'	16 nt
	IS6110-D2	5'-ATCCGCTGAGCTGAAGC-3'	17 nt
	IS6110-R1	5'-ACTTACGCACCGTCTC-3'	16 nt
	IS6110-R2	5'-CAGGCGCAGGTCGATG-3'	16 nt
mpb64	mpb64-F1	5'-CCCCGGGTTGAAGAAGA-3'	17 nt
	mpb64-F2	5'-GCTCAAGGTCTACCAGAAC-3'	19 nt
	mpb64-CP1	5'-ACAGGTATCGATAGCGCCGAATGCCCCGTCGTTCGTGACT-3'	40 mer
	mpb64-CP2	5'-TGCCACAGCGTGTCATAGGTACGACCACGTACAAGGC-3'	37 mer
	mpb64-C1*	5'-Dig-ACAGGTATCGATAGCGCCGAATG-3'	23 nt
	mpb64-C2	5'-TGCCACAGCGTGTCATAGGT-3'	20 nt
	mpb64-D1*	5'-Biotin-CGGTGAATTATCAGAACTTC-3'	20 nt
	mpb64-D2	5'-GCCTGGTCCCAATCGAA-3'	17 nt
	mpb64-R1	5'-GTGAACTGAGCAAGCAGA-3'	18 nt
	mpb64-R2	5'-ACAATGGGGAAGACGACT-3'	18 nt

Notes: [a] IS6110-C1*, 5⃞-labeled with FITC when used in MCDA-LFB assay; mpb64-C1*, 5⃞-labeled with Dig when used in MCDA-LFB assay; mpb64-D1*, 5⃞-labeled with biotin when used in MCDA-LFB assay.

Abbreviations: FITC, fluorescein isothiocyanate; Dig, digoxigenin. mer, monomeric unit; nt, nucleotide.
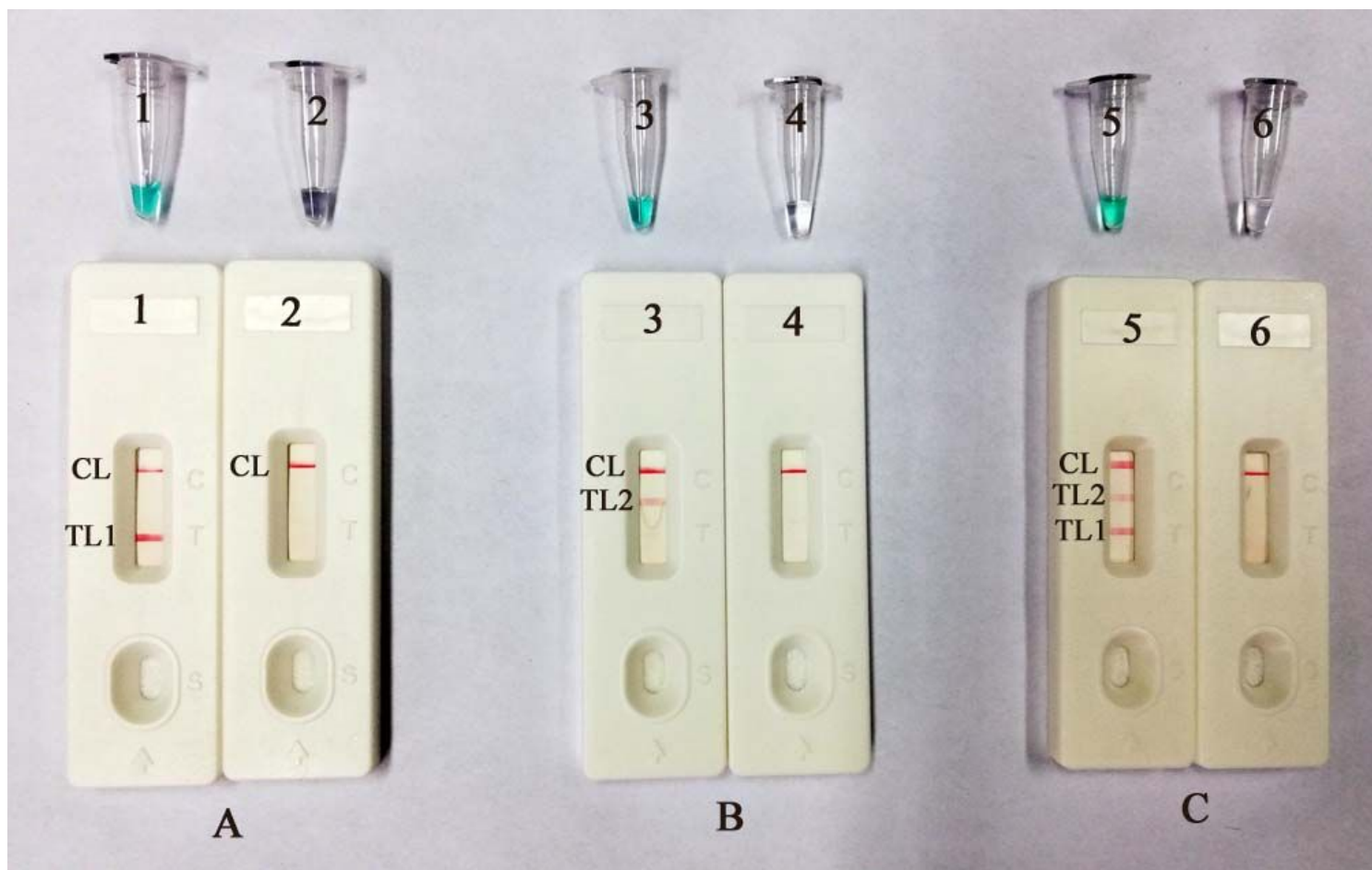
Figures

Figure 1

Confirmation and detection of M. tuberculosis-MCDA products. (A) The LFB and MG method, were applied for IS6110 gene amplification. The products of the M. tuberculosis-MCDA assay were visually analyzed by observation of the TL1 and color change. Tube 1/Biosensor 1: positive amplification of M. tuberculosis; Tube 2/Biosensor 2: black control of DW. (B) The LFB and MG method, were applied for mpb64 gene amplification. The products of the M. tuberculosis-MCDA assay were visually analyzed by observation of the TL2 and color change. Tube 3/Biosensor 3: positive amplification of M. tuberculosis; Tube 4/Biosensor 4: black control of DW. (C) The LFB and MG method, were applied for both IS6110 and mpb64 genes amplification. The products of the M. tuberculosis-MCDA assay were visually analyzed by observation of the TL1, TL2 and color change. Tube 5/Biosensor 5: positive amplification of M. tuberculosis; Tube 6/Biosensor 6: black control of DW.
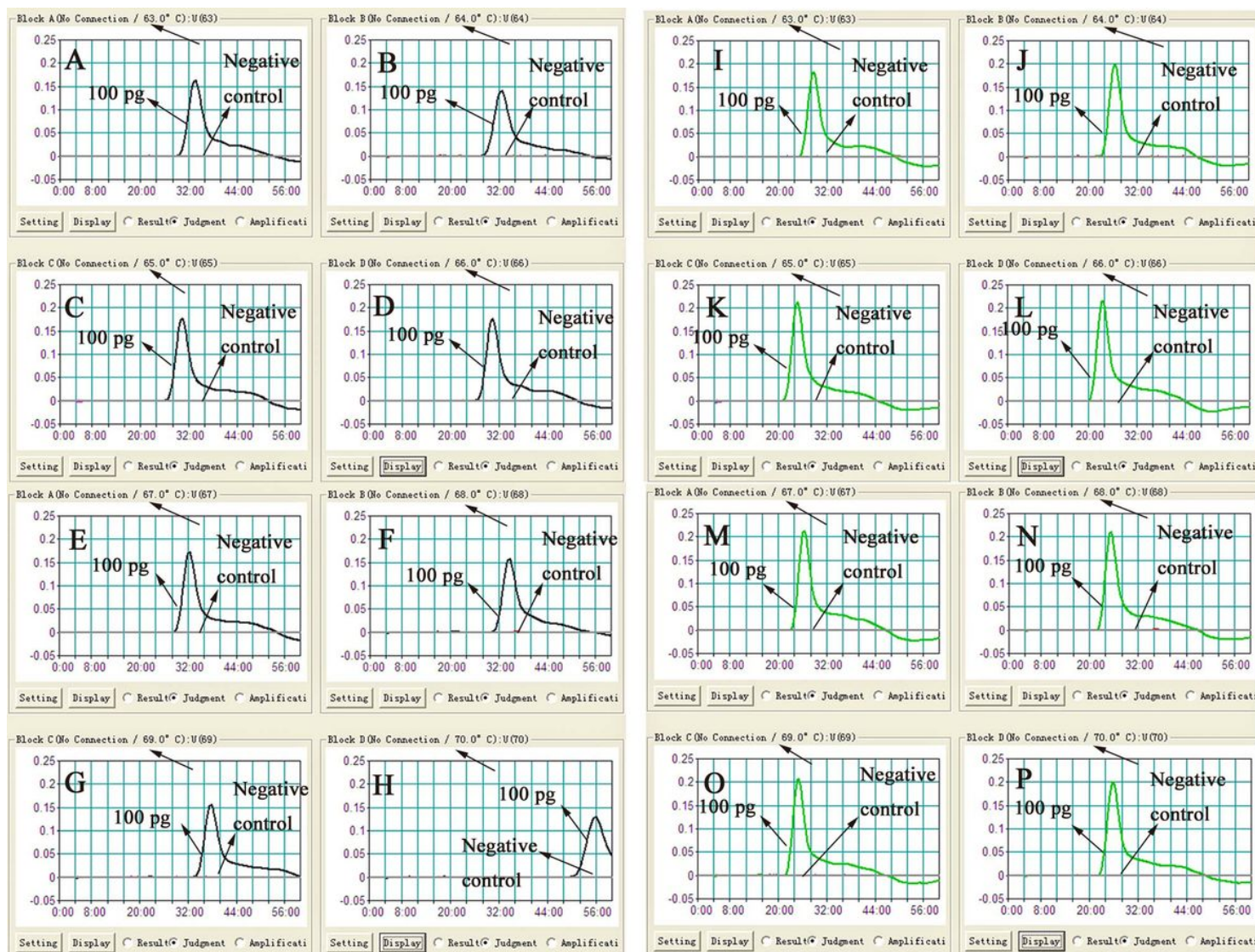
Figure 2

Reaction temperature optimization for M. tuberculosis-MCDA primers, both IS6110 gene and mpb64 gene were detected for different temperature. The genes were detected for different temperatures (63-70 °C, 1 °C intervals) with target DNA at the level of 100 pg per reaction. The standard MCDA reactions for detection of M. tuberculosis were monitored by the determination of real-time turbidity, and the threshold value was 0.1 and a turbidity >0.1 was set as positive. The M. avium genomic templates were used as negative control. (A-H) The gene of IS6110 was detected by real-time turbidity shown 65-67 °C had higher turbidity (>0.15) and more fast reaction times (30-32 min). (I-P) The gene of mpb64 was detected by real-time turbidity shown 65-67 °C had higher turbidity (>0.20) and more fast reaction times (24-26 min). According to the generated typical kinetics graphs, both the IS6110 and mpb64 genes amplification at the 67 °C had shortest time interval with higher turbidity and more fast reaction times. Thus, the amplification temperature of 67 °C was applied to perform the remaining experiments performed in the study.

Figure 3

The optimal duration of time required for M. tuberculosis-MCDA-LFB method. Four distinct reaction times (20 min; 30 min; 40 min; 50 min) were examined and compared at 67 °C. Biosensors represent DNA levels of 100 pg of M. tuberculosis templates. The earliest was observed when the amplification lasted for 30 min.
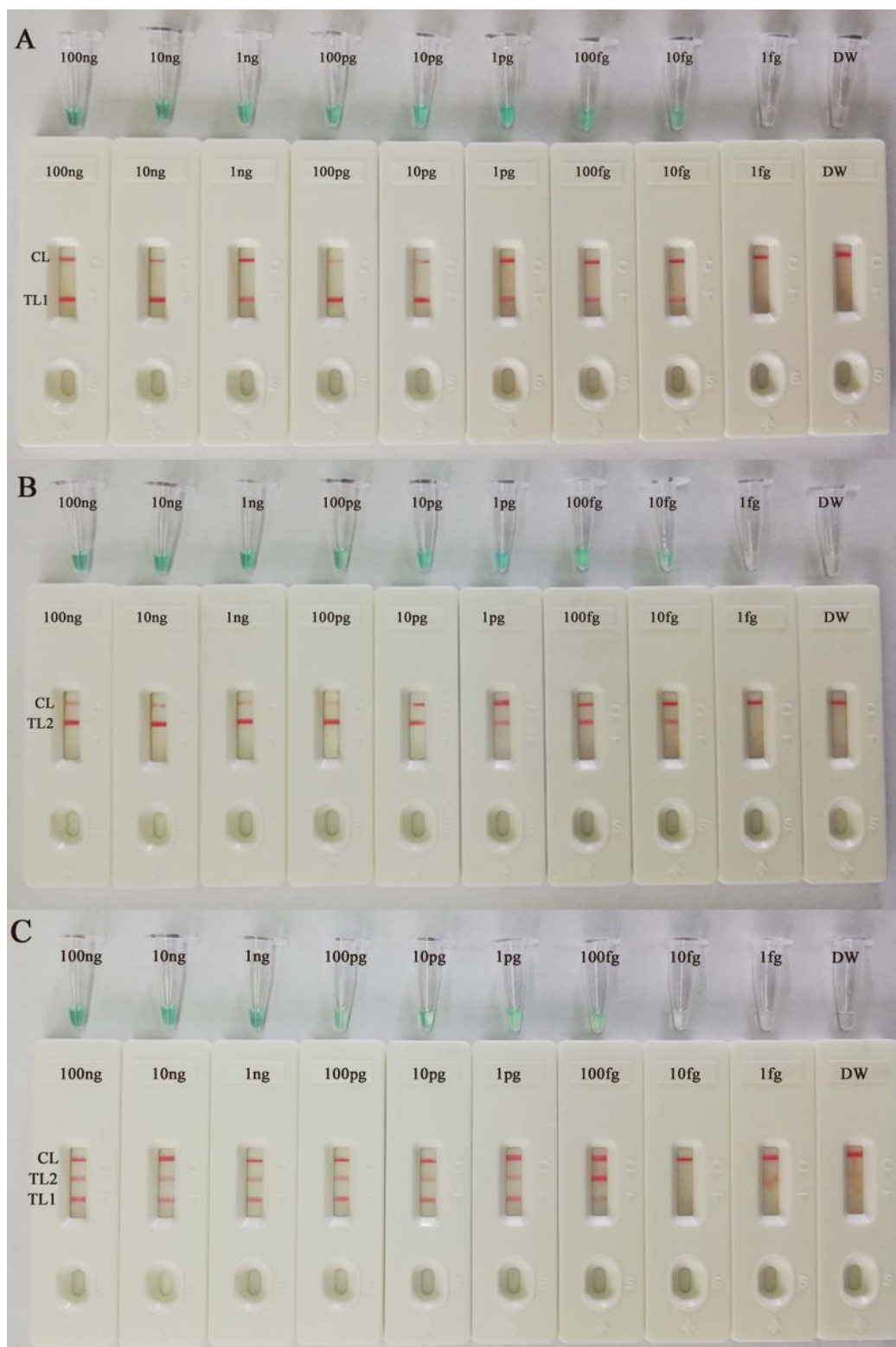
Figure 4

Sensitivity of MCDA method using serially diluted genomic DNA extracted from M. tuberculosis (H37Rv, ATCC27294). A total of two detecting techniques, including the lateral flow biosensor (LFB) and colorimetric indicator (MG) methods, were applied to analyze the amplification products. Serial dilutions of target templates were subjected to standard MCDA reactions. The DNA levels of 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg per reaction. And double distilled water was the template for

the blank control. (A) Singly IS6110 gene at the genomic DNA levels of 100 ng per reaction, 10 ng per reaction, 1 ng per reaction, 100 pg per reaction, 10 pg per reaction, 1 pg per reaction, 100 fg per reaction and 10 fg per reaction produced positive reactions. (B) The mpb64 gene at the genomic DNA levels of 100 ng per reaction, 10 ng per reaction, 1 ng per reaction, 100 pg per reaction, 10 pg per reaction, 1 pg per reaction, 100 fg per reaction and 10 fg per reaction produced positive reactions. (C) Both IS6110 gene and mpb64 gene at the genomic DNA levels of 100 ng per reaction, 10 ng per reaction, 1 ng per reaction, 100 pg per reaction, 10 pg per reaction, 1 pg per reaction and100 fg per reaction produced positive reactions.
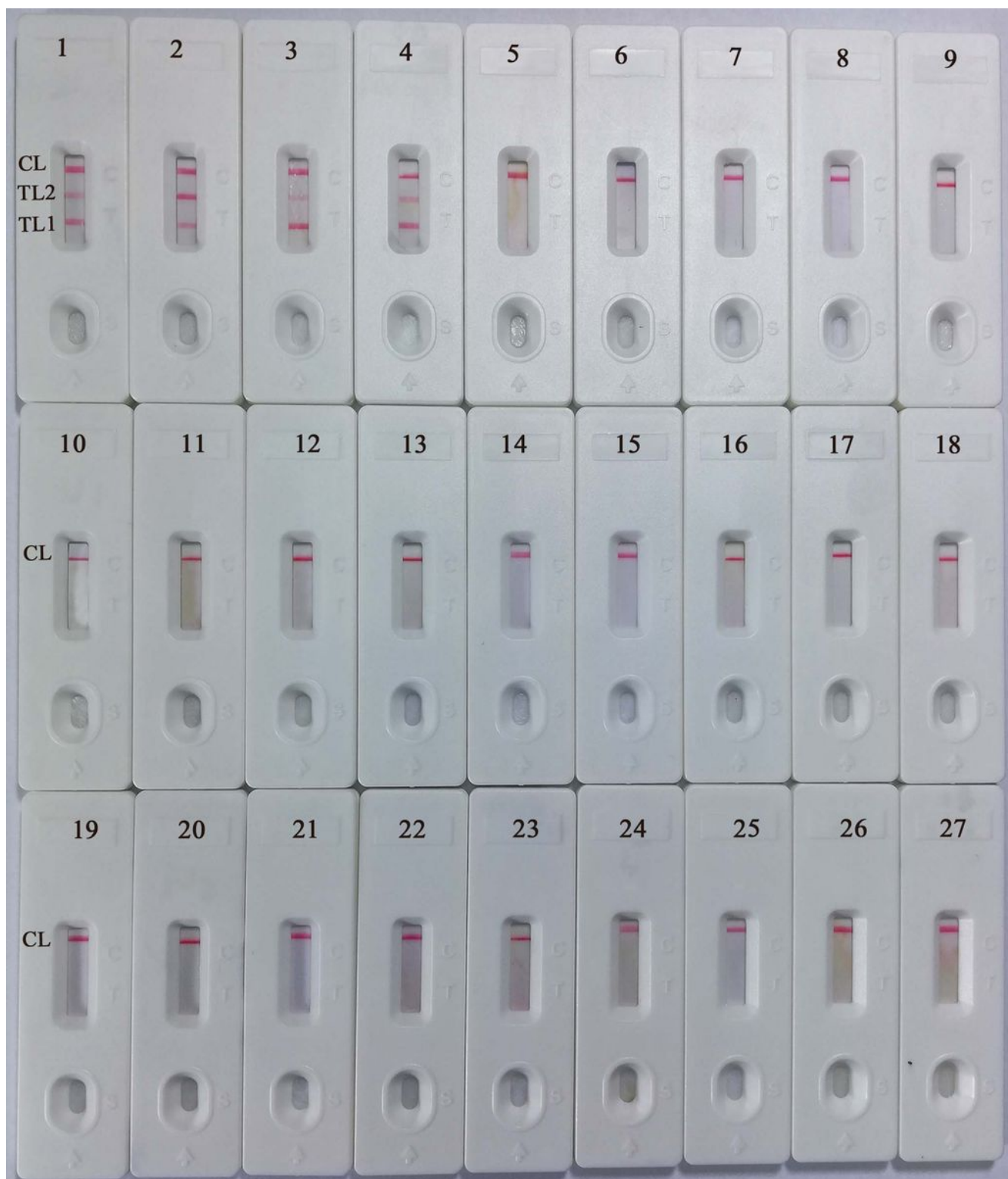
Figure 5

Specificity analysis of the MTBC-MCDA-LFB assay for different bacterial strains. The genomic DNA from the strains was analyzed visually by MCDA amplification and LFB detected. Biosensors 1-4 used MTBC strains including M. tuberculosis (H37Rv, ATCC 27294), M. bovis (ATCC 19210), M. africanum (ATCC 25420) and Bacillus Calmette-Guerin (Vaccine strain store at GZCDC); Biosensors 5-16 used Non-tuberculous mycobacterium (NTM) strains including M. aureus (ATCC 23366), M. flavum (ATCC 43999),

M.avium (ATCC 25291), M.marinum (ATACC 927), M. abscess (ATCC 19977), M. Chelonae (ATCC 14472), M. Gordon (ATCC 14470), M. Phlei (ATCC 11758), M. Nonchromogenic (ATCC 19530), M. xenopi (ATCC 19250) and M. aichiense (ATCC 27280), M. microti (ATCC 19422); Biosensors 17-26 used other bacteria strains which isolated by GZCDC including Enterococcus faecalis, Salmonella, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Escherichia coli, acillus cereus, Listeria monocytogenes, Streptococcus pneumoniae and Campylobacter jejuni; Biosensors 27 used double distilled water as the blank control.
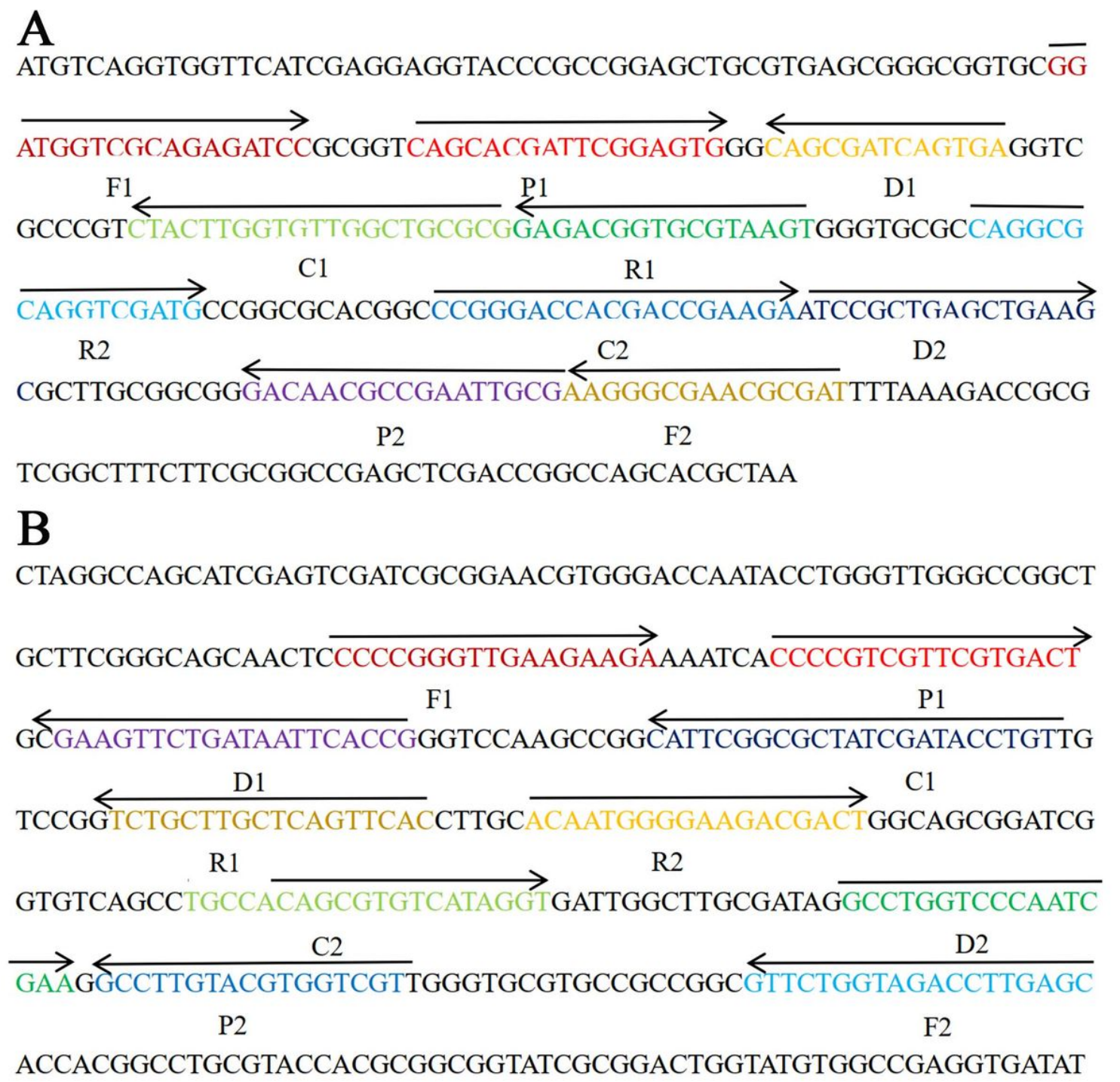
A

ATGTCAGGTGGTTCATCGAGGAGGTACCCGCCGGAGCTGCGTGAGCGGGCGGTGCGG

ATGGTCGCAGAGATCCGCGGTCAGCACGATTCGGAGTGGGCAGCGATCAGTGAGGTC

F1   P1   D1
GCCCGTCTACTTGGTGTTGGCTGCGCGGAGACGGTGCGTAAGTGGGTGCGCCAGGCG

C1   R1
CAGGTCGATGCCGGCGCACGGCCCGGGACCACGACCGAAGAATCGCTGAGCTGAAG

R2   C2   D2
CGCTTGCGGCGGGACAACGCCGAATTGCGAAGGGCGAACGCGATTTTAAAGACCGCG

P2   F2
TCGGCTTTCTTCGCGGCCGAGCTCGACCGGCCAGCACGCTAA

B

CTAGGCCAGCATCGAGTCGATCGCGGAACGTGGGACCAATACCTGGGTTGGGCCGGCT

GCTTCGGGCAGCAACTCCCCCGGGTTGAAGAAGAAAATCACCCCGTCGTTCGTGACT

F1   P1
GCGAAGTTCTGATAATTCACCGGGTCCAAGCCGGCATTCGGCGCTATCGATACCTGTTG

D1   C1
TCCGGTCTGCTTGCTCAGTTCACCTTGCACAATGGGGAAGACGACTGGCAGCGGATCG

R1   R2
GTGTCAGCCTGCCACAGCGTGTCATAGGTGATTGGCTTGCGATAGGCCTGGTCCCAATC

C2   D2
GAAGGCCTTGTACGTGGTCGTTGGGTGCGTGCCGCCGGCGTTCTGGTAGACCTTGAGC

P2   F2
ACCACGGCCTGCGTACCACGCGGCGGTATCGCGGACTGGTATGTGGCCGAGGTGATAT

Figure 6

The sequences and modifications, locations in the expression site of the IS6110 and mpb64 genes. (A) Location and nucleotide sequence of M. tuberculosisIS6110 gene used to design the MCDA primers. (B) Location and nucleotide sequence of M. tuberculosismpb64 gene used to design the MCDA primers. All the sequences of the primer sites are underlined. Right and left arrows indicate sense and complementary sequences that are used.