Heliyon 10 (2024) e31901

Contents lists available at ScienceDirect

Heliyon

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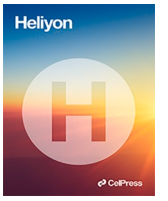

Research article

Development and clinical evaluation of a real-time multiple cross displacement amplification assay for rapid and sensitive detection of *Mycobacterium tuberculosis*

Chunrong Sun [a,1], Chaohong Wang [b,1], Fei Xiao [a], Nan Jia [a], Xiaolan Huang [a], Jin Fu [a], Yu Zhang [a], Juan Zhou [a,**], Guirong Wang [b,***], Yi Wang [a,*]

[a] *Experiment Research Center, Capital Institute of Pediatrics, Beijing, 100020, PR China*
[b] *Department of Clinical Laboratory, Beijing Chest Hospital, Capital Medical University, Beijing Tuberculosis and Thoracic Tumor Institute, Beijing, 101125, PR China*

A R T I C L E I N F O

Keywords:
Mycobacterium tuberculosis
Real-time
Multiple cross displacement amplification
Rapid diagnosis
Lateral flow biosensor

A B S T R A C T

Molecular techniques of nucleic acid testing recommended by the World Health Organization (WHO) for the *Mycobacterium tuberculosis* (MTB) detection were considered to have the potential access to the accurate tuberculosis (TB) notifications. In this study, a new method, which coupled real-time (rt) fluorescence technique with multiple cross displacement amplification (MCDA), was developed for the rapid, sensitive and specific detection of MTB (termed MTB-rt-MCDA). According to the principle of the rt-MCDA test, a set of ten primers were designed for the MCDA reaction, of which one was engineered with a restrictive endonuclease recognition site, a fluorophore and a quencher for achieving the real-time fluorescence detection. MTB-rt-MCDA test was conducted under the optimized conditions (67 °C, 40 min) on the real-time fluorescence platform. The MTB-rt-MCDA assay accurately identified the MTB strains with no cross reaction with other bacteria. The lowest detectable genomic DNA concentration of the MTB-rt-MCDA assay was 25 fg/μl. We employed the genomic DNA templates extracted from sputum of clinical cases for validating the practical applicability of this assay, and the detection power of the MTB-rt-MCDA assay was comparable to that of the Xpert method and MCDA-based biosensor detection and superior to smear microscope method. The complete process of the MTB-rt-MCDA assay, including rapid extraction of DNA and rt-MCDA test, takes less than 1 h. In conclusion, the presented MTB-rt-MCDA assay provided an effective and simple option for the rapid screening of MTB infection.

1. Introduction

Tuberculosis (TB), caused by the infection of *Mycobacterium tuberculosis* (MTB), remains the main cause of infectious mortality worldwide [1]. It is estimated, according to the WHO reports, 10.6 million peoples suffered from tuberculosis in 2021, an increase of

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: zhoujuan2015@126.com (J. Zhou), wangguirong1230@ccmu.edu.cn (G. Wang), wildwolf0101@163.com (Y. Wang).
[1] These authors contributed equally to this article.

https://doi.org/10.1016/j.heliyon.2024.e31901
Received 2 January 2024; Received in revised form 16 May 2024; Accepted 23 May 2024
Available online 24 May 2024

4.5% from 2020, and a total of 1.6 million people died from TB [1].The reported number of people newly diagnosed with TB has partially recovered to 6.4 million in 2021, after the decline due to the COVID-19 pandemic between 2019 and 2020 [2]. However, the number was still well below the pandemic levels. There were many people with TB were not diagnosed and treated for the continued challenges in the access of TB service. The undiagnosed and untreated TB could result in an increase of TB deaths, more community transmission and then, with some lag-time, more people developing TB. Accurate and swift testing of TB is the essential step to address the continued challenges[3].

Despite the molecular tests and other new technologies, culture remains the key method. A positive MTB culture is still the gold standard for the TB diagnosis [4].However, it takes 15 to 60 days for a MTB cultured media to be readily detected [5]. Microscopically detection of acid-fast bacilli (AFB) in stained smears of sputum from suspected patients directly provides a rapid identification of TB, but its sensitivity is 50–60%, or even lower to 30% in children and HIV-positive or immunosuppressed patients [3]. Fluorescence microscopy (FM) could increase the sensitivity of the sputum smears compared to AFB. However, the equipment for FM is expensive, so it is not widely used. In addition, the prepared slides must be read in 24 h, as the fluorescence fades with time [6,7].

Molecular identification is now regarded as a promising approach for the TB diagnostics that must remain simple, rapid and cost-effective [8–12]. GeneXpert MTB/RIF is a kit based on PCR test for the rapid diagnosis of TB, recommended by the WHO early in 2011 [13]. Due to its easy format, this method is now widespread and considered to exhibit higher sensitivity and specificity than that of microbiological and cytological assays [14]. However, the Xpert test remains less sensitive than culture [15,16]. Compared to the based PCR technique (e.g., nested PCR or reverse-transcription PCR), novel isothermal amplification technologies like multiple cross displacement amplification (MCDA) showed more advantages in the detection of MTB infections [10–12]. MCDA could be easily carried out in an isothermal heater or a thermostatic water bath with no requirement of other specialized equipment. The amplification results can be rapidly determined by the color change of pre-added visual indicator or by using lateral flow biosensor (LFB) test with no limitation of instrument or environment. Thus, the MCDA was a strongly recommended molecule tool for the resource-limited areas. However, in MCDA system, the result judgment was not completely impartial as it relied on the color change of indicator pre-added in the reaction tubes. Additionally, the LFB test might potentially introduce product contamination or vulnerability to false positives induced by primer dimerization.

In this study, we leveraged the high amplification efficiency of MCDA technique and the specific cleavage activity of restriction endonuclease to develop a swift, sensitive and accurate detection platform for MTB (termed MTB-rt-MCDA). Results of MTB-rt-MCDA assay was transformed into fluorescence signals, which could be reported by a fluorescent equipment in a real-time manner. The MTB-rt-MCDA assay was carried out in a one-step reaction, eliminating risk of aerosol contaminations. Via testing the clinical samples, we validated feasibility of the MTB-rt-MCDA assay by comparing its results with those obtained using the MCDA-based biosensor detection, Xpert, and sputum smear microscopy.

2. Materials and methods

2.1. Reagents and instruments

The DNA extraction kits were purchased from Bejing Transgen Biotech Co., Ltd (Beijing, China). The Isothermal® Amplification Kit, disposable lateral flow biosensor (LFB), color indicator and biotin-14-dCTP were purchased from Huidexin Biotech Co., Ltd (Tianjin, China). Restriction endonuclease (Nb.*Bsr*DI) was marketed from New England Biolabs (America). All the primers were synthesized by TianYiHuiYuan Biotech Co., Ltd (Beijing, China). The Real Time Turbidimeter LA-320C was purchased from Eiken Chemical Co., Ltd (Japan). The Applied Biosystems 7500 Fast Real-Time PCR System was purchased from Applied Biosystem Inc. (USA).

Table 1
Primers used in the study.

Primer name	Sequence and modifications	length
MTB-F1	GTTCTTGGGCCGACACG	17
MTB -F2	TTCCGGTCCCGCTGAC	16
MTB -CP1	AGGGTTCATCGTCACGGAACAG-GGAAGGGCAACTGAGCAT	42
MTB -CP2	CGGCAGTGAACGTCATTCCGTATCGACCTGCATCCCGAC	38
MTB -C1	AGGGTTCATCGTCACGGAACAG	22
MTB -C2	CGGCAGTGAACGTCATTCCGT	21
MTB -D1	CAACAGCGGCCAACAT	16
MTB -D2	TGCGTTGGCAGAACGAT	17
MTB -R1	CTTGGCCACCGAGACTT	17
MTB -R2	ACGCGGCCGTGTGG	14
MTB-CP1[a]	5'-FAM-TGCAATG-AGGGT(BHQ1)TCATCGTCACGGAACAG-GGAAGGGCAACTGAGCAT-3'	
MTB-D1[b]	5'-FAM-CAACAGCGGCCAACAT-3'	

[a] The modified primer for the MTB-rt-MCDA assay.
[b] The modified primer for the MCDA-LFB assay.

2.2. Primer design

According to principle of MCDA technique, primer set containing ten primers were designed based on the sequence of the sdaA gene (GenBank No. NC_00962.3) of MTB, using Primer Premier 5.0 software. The primer set included six amplification primers (C1, C2, D1, D2, R1 and R2), two displacement primers (F1 and F2) and two cross primers (CP1 and CP2). For real-time fluorescence detection, the primer CP1 was extended with a short sequence (Ss, TGCAATG) and a fluorophore FAM at the 5′ end and the Black Hole Quencher 1 (BHQ1) at the middle of the sequence (termed CP1*). In addition, for LFB detection, primer D1 was also labelled with FAM at the 5' end (termed D1$^{\#}$). All the primers, including the sequences, location, and modifications are listed in Table 1 and Fig. 1.

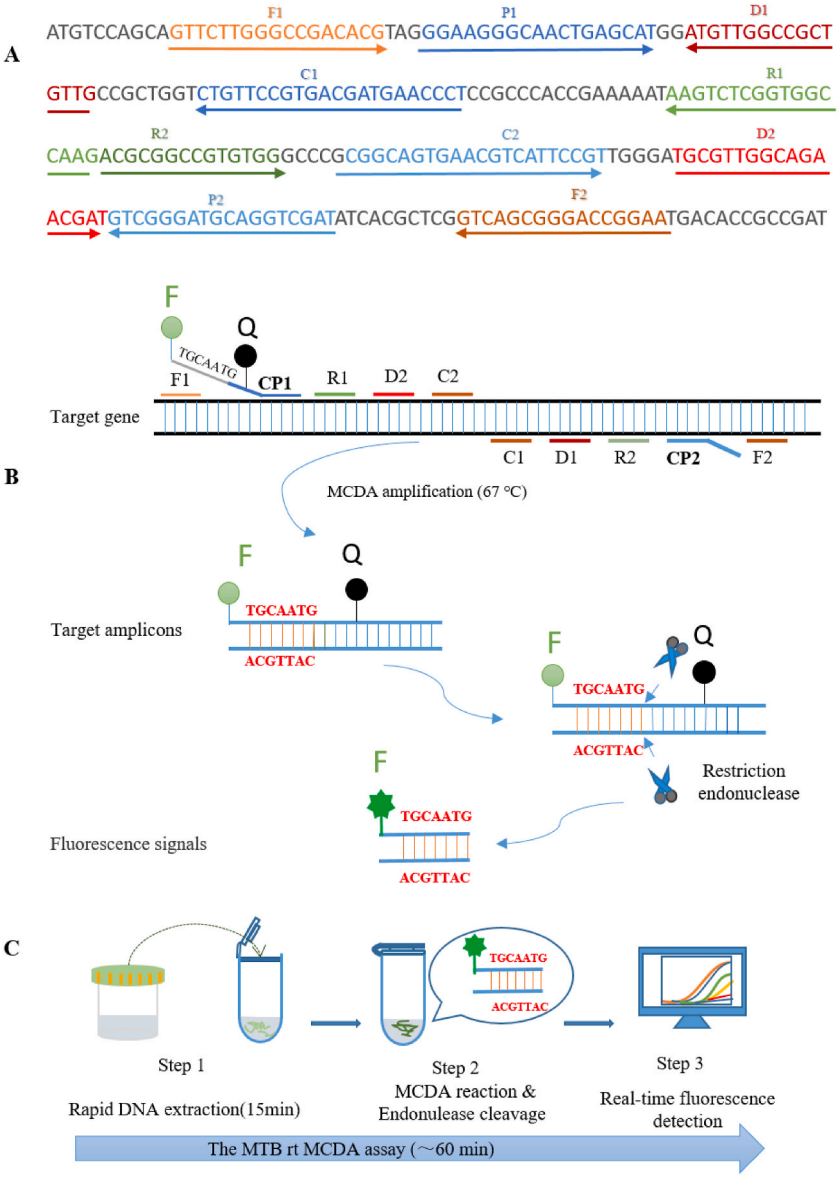

Fig. 1. Schematic illustration of the principle of the MTB-rt-MCDA assay. A, Location and sequences of the primers used in this study targeting the sdaA gene of MTB. Right and left arrows show sense and complementary sequences, respectively. The colored text indicates the position of primers, including two displacement primers (F1 and F2), two cross primers (CP1 and CP2), and six amplification primers (C1, C2, D1, D2, R1 and R2). **B**, Schematic diagram of the MCDA reactions with the modified primers. Primer CP1 extended with an endonuclease recognition site at the 5′ end was modified with a fluorophore (FAM, F) at the 5′ end and a quencher (BHQ1, Q) in the middle. After amplification, double stranded target amplicons with the restriction endonuclease recognition site were generated. After recognized and cleaved by restriction endonuclease, the fluorescence signal was released. **C**, The entire process of the MTB-rt-MCDA detection system. The whole process, including rapid DNA extraction, MCDA reaction and endonuclease cleavage, and real-time fluorescence detection, could be completed within 1 h.

2.3. The standard MTB-MCDA reaction

The standard MTB-MCDA reaction was executed in a 25 μl reaction mixture, which contained 0.2 μl each of the amplification primers (D1was replaced by D1$^{\#}$) (100 μM), 0.1 μl each of the displacement primers (100 μM), 0.4 μl each of the cross primers (100 μM), 0.5 μl of biotin-14-dCTP, 8U of Bst 2.0 DNA polymerase, 1.2 μl of color indicator, 1 μl of template (or 4 μl of clinical templates), 12.5 μl of 2 × isothermal reaction buffer and according distilled water. The amplification reaction was performed using the conventional PCR instrument or a real-time turbidimeter at 67 °C for 40 min. The results could be real-time monitored by tubidimeter according to the accumulation of the products, directly interpreted according to the color change of the visual indicator, or interpreted with LFB. In the case of LFB, 5 μl of MCDA amplicons and 100 μl running buffer (containing 10 mM PBS, PH 7.4 with 1 % Tween 20) were subsequently dropped onto the sample pad. Then, in 2 min, the positive result displayed two red bands respectively at the testing line (TL) and the control line (CL) and the negative with one red band at CL.

2.4. Optimal reaction temperature of the MTB-rt-MCDA assay

To optimize the operation of MTB-rt-MCDA assay, MTB-MCDA reactions were firstly performed at temperatures from 64 to 69 °C with 1 °C interval to determine the optimal amplification temperature. The reactions were conducted and real-time monitored by the turbidimeter, using extracted DNA of MTB as the positive control and double-distilled water (DW) as blank. The processes were operated at the corresponding temperatures for 40 min followed with a termination of the reaction at 90 °C for 5 min. The temperature at which the best performance was obtained was considered as the optimum one. The achieved optimal reaction temperature was utilized in the following MTB-rt-MCDA assay.

2.5. The standard MTB-rt-MCDA reaction

The reaction mixture of the MTB-rt-MCDA assay was 25 μl as well, which was similar to the above mentioned MTB-MCDA reaction with some adjustment: replacing CP1 and D1$^{\#}$ to CP1* and D1, respectively; extra addition of 1.0 μl of restriction endonuclease Nb.*Bsr*DI and disposal of biotin-14-dCTP and visual indicator. The MTB-rt-MCDA assay was proceeded at 67 °C for 40 min with the real-time PCR system. Genomic DNA of MTB was prepared as positive control, and DW as blank control. The performance of MTB-rt-MCDA reaction was accessed on the basis of fluorescence generation.

2.6. Sensitivity and specificity evaluation of the MTB-rt-MCDA assay

To determine the detection limit of the MTB-rt-MCDA assay, DNA templates extracted from pure culture of MTB (strain:H37Rv) were continuously diluted to 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 25 fg and 10 fg per microliter. Accordingly, three replicates were conducted for each of the serial dilutions, as well as the blank control of distilled water. For the specificity analysis, the assay were

Table 2
Pathogens used in this study.

Pathogen	Strain no. (source of strain)[a]	No. of strains	MTB-rt-MCDA[b]
Mycobacterium tuberculosis	Isolated strains (BCH)	4	P
Mycobacterium gordonae	Isolated strains (BCH)	1	N
Mycobacterium gadium	Isolated strains (BCH)	1	N
Mycobacterium fortuitum	Isolated strains (BCH)	1	N
Mycobacterium cosmeticum	Isolated strains (BCH)	1	N
Nocardia spp.	Isolated strains (BCH)	1	N
Bordetella pertussis	Isolated strains (CDC)	1	N
Haemophilus influenzae	Isolated strains (CDC)	1	N
*Staphylococcus am*ber	Isolated strains(CDC)	1	N
*Shigella son*nei	Isolated strains (CDC)	1	N
*Staphylococcus epidermi*dis	Isolated strains (CDC)	2	N
*Salmone*lla spp.	Isolated strains (CDC)	1	N
*Citrobat*er spp.	Isolated strains (CDC)	1	N
*Enteroinvasive Escherichia C*oli	Isolated strains (CDC)	1	N
Enterotoxic Escherichia coli	Isolated strains(CDC)	1	N
*Staphylococcus haemolytic*us	Isolated strains (CDC)	1	N
*Stenotrophomonas maltophi*lia	Isolated strain s(CDC)	1	N
Streptococcus suis	Isolated strains (CDC)	1	N
*Moraxella Catarrhal*is	Isolated strains (CDC)	1	N
*Corynebacterium strial*um	Isolated strains (CDC)	1	N
*Streptococcus salivar*ius	Isolated strains (CDC)	1	N
*Candida albic*ans	Isolated strains (CDC)	1	N
*Pseudomonas aerugino*sa	Isolated strains (CDC)	1	N
*Klebsiella pneumoni*ae	Isolated strains (CDC)	1	N

[a] BCH, Beijing Chest Hospital Affiliated to Capital Medical University. CDC, Chinese Center for Disease Control and prevention.
[b] P, positive; N, negative.

performed with DNA templates of 24 non-MTB strains (Table 2) and 4 MTB strains. The results were displayed with real-time fluorescence detection platform, as well as the LFB platform for comparison.

2.7. Clinical feasibility evaluation of the MTB-rt-MCDA assay

In order to evaluate the feasibility of the MTB-rt-MCDA assay in clinical settings, nucleic acid extractions from 32 sputum specimens were retrospectively analyzed. Each specimen used in this study received the informed consent from the participants. The collected sputum specimens (1–2 ml) were transferred and stored at 4 °C within 2 h before the following process within 24 h. All the specimens have been detected by smear microscopy and Xpert MTB/RIF assay for MTB infection diagnosis at Beijing Chest Hospital Affiliated to Capital Medical University (BCH). The routinely extracted DNA of these sputum specimens were kept at −20 °C for the

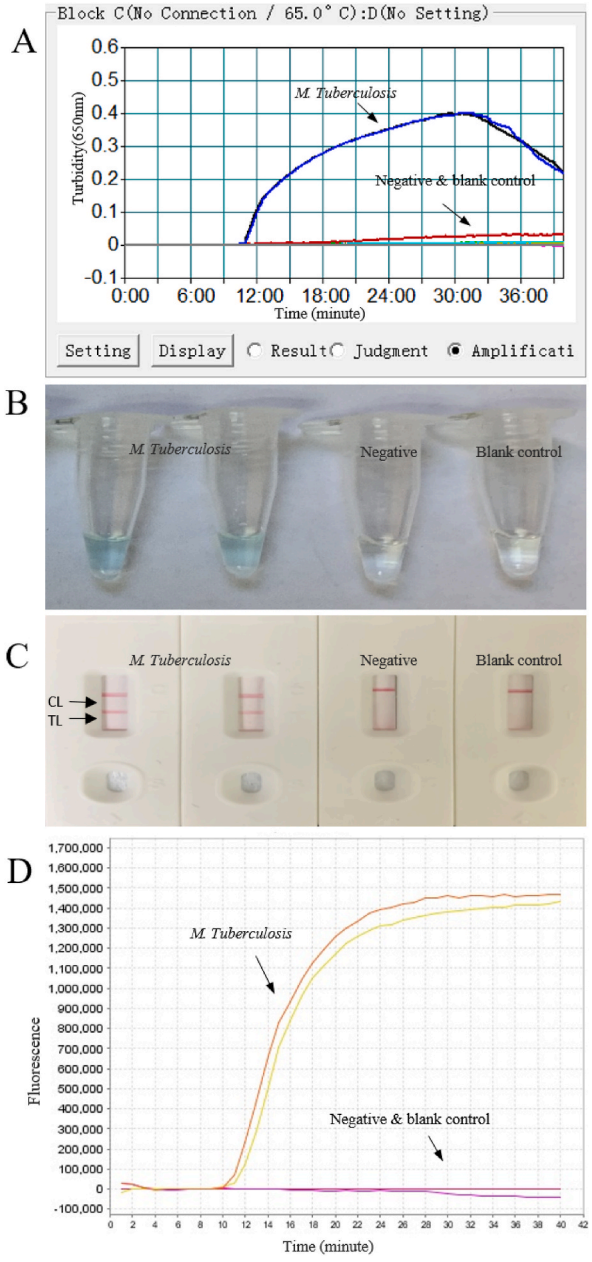

Fig. 2. Confirmation of the MTB-rt-MCDA assay. For primer verification, MTB-MCDA assay was carried out at 65 °C for 40 min and the results were reported using real-time turbidimeter (**A**), VDR (**B**), and LFB (**C**). The MTB-rt-MCDA assay was performed at 65 °C for 40 min using real-time fluorescence detector monitoring the results (**D**). DNA templates extracted from MTB strains and *Mycoplasma pneumoni*ae were used as positive and negative controls, respectively, and the DW as blank control. DW, distilled water; TL, testing line; CL, control line.

process of testing with MTB-rt-MCDA assay and MTB-MCDA-LFB test.

3. Results

3.1. Establishment of the MTB-rt-MCDA assay

The MTB-rt-MCDA assay was designed on the basis of MCDA amplification and restriction endonuclease (Nb.*Bsr*DI) cleavage, and real-time fluorescence quantification PCR technique. The MCDA reaction was performed with the selected primer set targeting sdaA gene of MTB (*Fig.* 1A). With the primer CP1* that labelled a restriction endonuclease recognition sequence and FAM at the 5' end and BHQ1 in the middle, the MCDA reaction produced plenty of amplicons containing restriction endonuclease recognition sequence, which will be recognized and cleaved by the restriction endonuclease Nb.*Bsr*DI, resulting in the release of the fluorescence group (FAM) from the quenching of BHQ1 and emission of fluorescence signal for detection (Fig. 1B). Thus, the MTB-rt-MCDA assay was able to achieve MTB detection by real-time fluorescence detection in one tube. The detection process of the MTB-rt-MCDA assay, totally takes less than 1 h (Fig. 1C), including rapid extraction of DNA and rt-MCDA test.

3.2. Performance confirmation of the MTB-rt-MCDA assay

In order to confirm the performance of the MTB-rt-MCDA assay, primer effectiveness was firstly examined by performing MTB-MCDA assay with the selected primer set. After amplification at 65 °C for 40 min, the reactions with genomic DNA of MTB (positive controls) exhibited obvious turbidity increase using the real-time turbidimeter (Fig. 2A), displayed green color with visual indicator (Fig. 2B), and produced two red lines (TL and CL) in LFB (Fig. 2C); whereas the negative and blank controls displayed as no turbidity increase, colorless and only one red band in the CL of LFB. These results indicated the usefulness of the selected primer set for MTB detection with MCDA technique. Then, the confirmed primer set was utilized in the MTB-rt-MCDA assay. As shown in Fig. 2D, fluorescence signal was observed from the reactions with MTB rather than the negative and blank controls. The performance of the MTB-rt-MCDA assay demonstrated its feasibility for MTB diagnosis with the selected primer set.

3.3. The optimal reaction temperature for the MTB-rt-MCDA assay

We examined six temperatures (from 64 to 69 °C with 1 °C increment) for the optimal reaction temperature of the MTB-rt-MCDA assay by implementing the MCDA reaction on the real-time turbidimeter. The results were recorded through the real-time produced corresponding kinetics graphs. As displayed in Fig. 3(A-F), the optimal result was obtained at 67 °C. Thus, 67 °C was employed in the following tests.

3.4. Sensitivity and specificity of the MTB-rt-MCDA assay

In order to assess the detection limit of the MTB-rt-MCDA assay, prepared dilutions of genomic DNA templates of MTB were tested for three replicates. As shown in Fig. 4A (I~III), the MTB-rt-MCDA reaction can reach the minimum detectable level at 25 fg of DNA templates of MTB per test, which displayed as generation of obvious fluorescence signals. The results of MTB-rt-MCDA detection were completely consistent with that of MTB-MCDA-LFB assay (Fig. 4B). Thus, the sensitivity of the MTB-rt-MCDA assay was as low as 25 fg

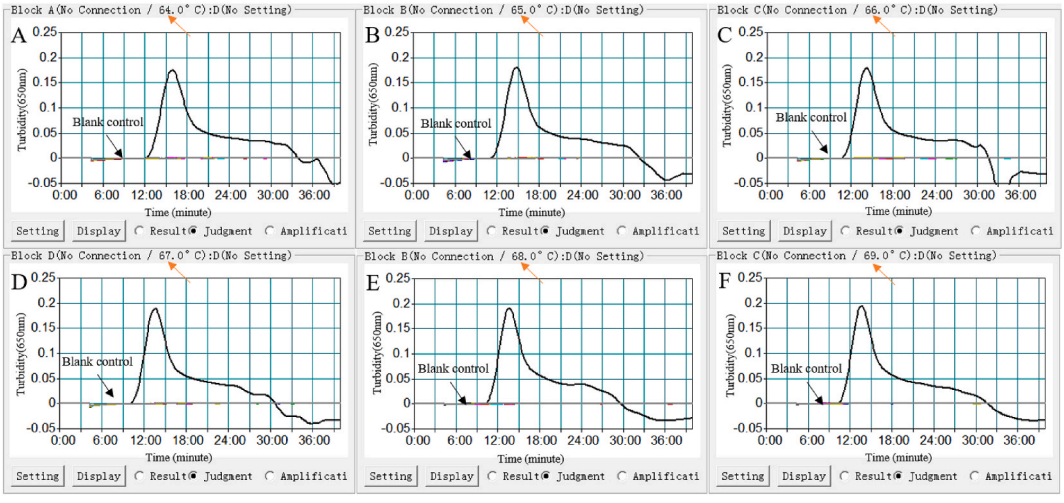

Fig. 3. Temperature optimization for MTB-rt-MCDA assay. The MTB-MCDA reactions were conducted at temperatures ranging from 64 to 69 °C and the kinetic curves at different temperatures (**A~F**) were acquired from real-time tubidimeter.

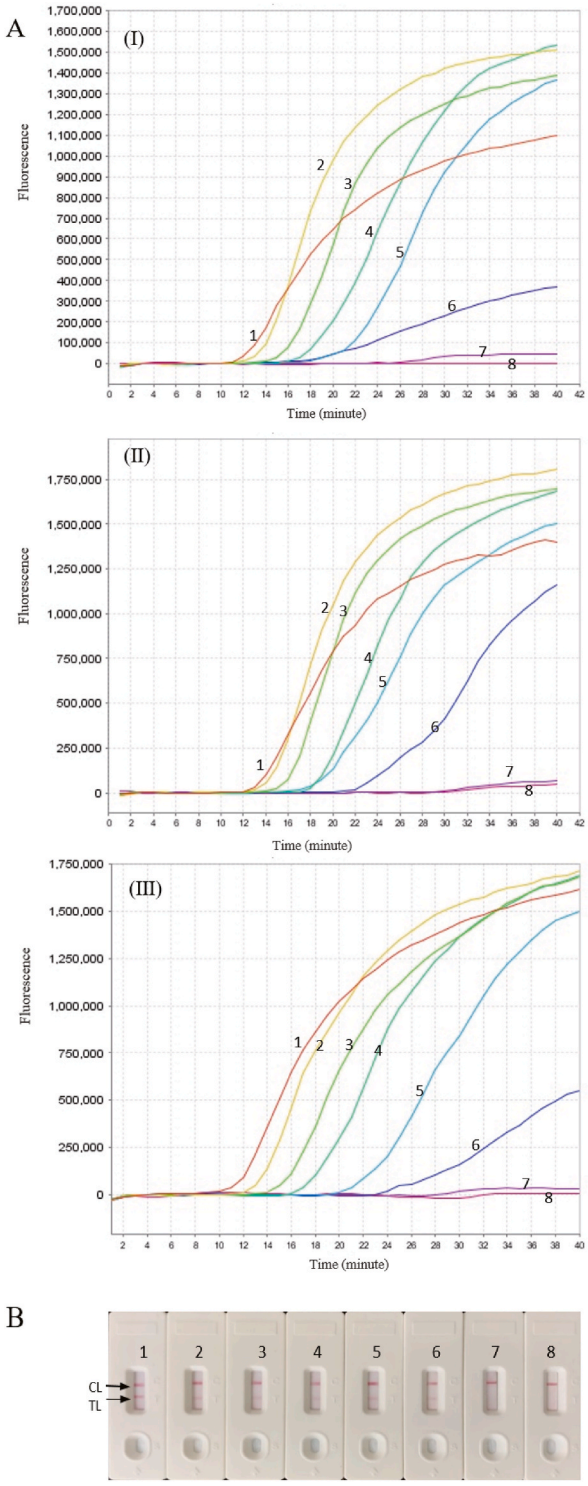

Fig. 4. Sensitivity confirmation of the MTB-rt-MCDA assay. Sensitivity of the MTB-rt-MCDA assay was analyzed using serial dilutions of DNA templates extracted from pure MTB strains. The MTB-rt-MCDA reactions were repeatedly tested three times (**A I∼III**). In addition, the MTB-MCDA-LFB assay was performed in parallel for comparison and confirmation (**B**). Signals/strips1: 1 ng/ml, 2: 100 pg/ml, 3: 10 pg/ml, 4: 1 pg/ml, 5: 100 fg/ml, 6: 25 fg/ml, 7: 10 fg/ml, 8: blank control. TL, testing line; CL, control line.

per microliter.

For the determination of the specificity of the MTB-rt-MCDA assay, we examined 4 MTB strains and 24 non-MTB strains (Table 2). As expected, only the 4 MTB strains obtained positive results, meanwhile the non-MTB strains produced negative (Fig. 5A), which was identical to that of MTB-MCDA-LFB (Fig. 5). These data demonstrated a specificity of 100 % of MTB-rt-MCDA assay for MTB detection.

3.5. Clinical feasibility confirmation of MTB-rt-MCDA assay

The clinical feasibility of the MTB-rt-MCDA assay was validated by retrospectively testing 32 sputum samples with this method. After examination, 24 samples displayed positive results, and the remaining 8 was negative ones (Fig. 6A), in accordance with that produced by the MTB-MCDA-LFB test (Fig. 6B). Of note, by comparing with the previous results by smear microscopy and Xpert MTB/RIF assay, it was found that the MTB-rt-MCDA assay detected all the Xpert MTB/RIF-positive samples, and characterized another 16 smear microscopy-negative samples (Fig. 7), indicating that the clinical feasibility of the MTB-rt-MCDA assay was comparable to the Xpert MTB/RIF assay but superior to smear microscopy. All the data above demonstrated that the MTB-rt-MCDA assay could be a hopeful tool for MTB diagnosis in clinical settings.

4. Discussion

TB is still a global concern of public health problem, and remains one of the major infectious cause of death. Although the mortality rate and incidence of TB had declined in the past decades with the whole world's effort, the COVID-19 pandemic had abruptly reversed this trend due to substantial reduction of TB testing and case notifications, resulting in increase in TB incidence and mortality and deleterious effects on TB control [17]. Thus, strategies to mitigate the situation, including more easily access to rapid and accurate testing MTB, were highly appreciated. Under this context, we verified the developed MTB-rt-MCDA assay for the immediate and accurate diagnosis of MTB infection and further to prompt TB control.

The MTB-rt-MCDA assay developed here was a MCDA-based detection platform using MCDA technique for target amplification and real-time fluorescence detector for result reporting. As a rapid, simple and economical amplification method, MCDA technique has more and more applications to diagnose bacteria, viruses and fungi infections [18–21]. Due to its simplicity, convenience and low cost, MCDA reactions could be easily conducted under an isothermal condition provided by a thermostatic heater or a water bath. And the amplification results were visually inspected by a LFB test or the color change of the chromogenic indicator pre added in the reaction mixture [22–25]. The whole process is easy-to-perform, timely, and cost-effective. However, these methods had some shortcomings, including the subjectivity when judging the outcomes by color change and the risk of contamination when using biosensors. These shortcomings could be overcome by the MTB-rt-MCDA method devised here, which monitored the results by a real-time fluorescence detection format. By modifying the MCDA primer with the short sequence 5′-TGCAATG-3′, the newly synthetic products were cleaved

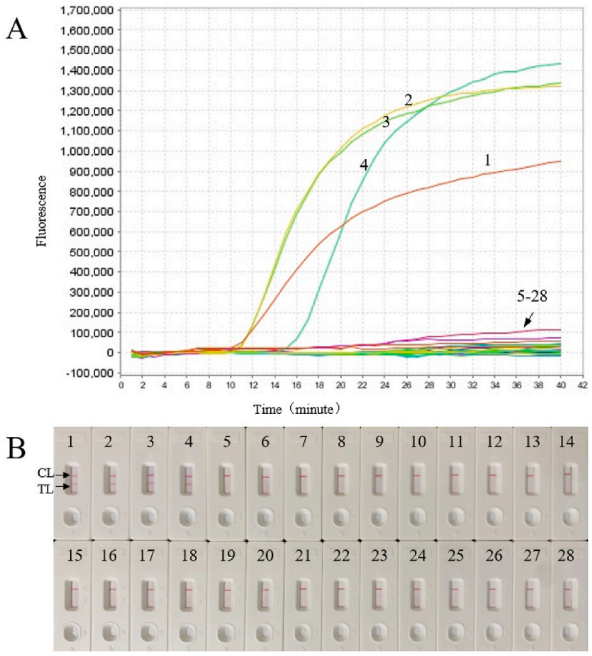

Fig. 5. Specificity conformation for MTB-rt-MCDA assay. DNA templates from 4 MTB strains and 24 non-MTB strains were tested by the MTB-rt-MCDA assay (**A**) to confirm its specificity. In addition, the MTB-MCDA-LFB assay was performed in parallel for comparison and confirmation (**B**). Signals/tubes/strips 1 to 4 represented four MTB strains, and the other ones represented non- MTB strains. TL, testing line; CL, control line.

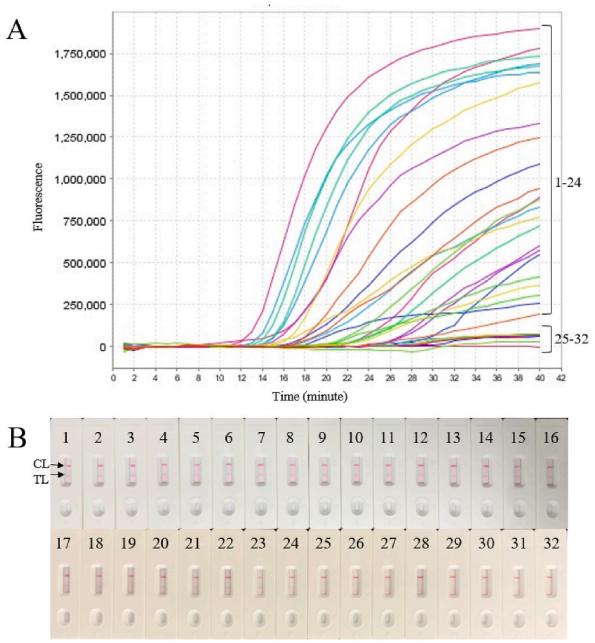

Fig. 6. Application of MTB -rt-MCDA assay in clinical specimens. DNA templates from 32 sputum samples were detected by the MTB-rt-MCDA assay (**A**) and the MCDA-LFB test (**B**). TL, testing line; CL, control line. Samples 1–24 displayed positive results, while the others were tested negative.

Sample ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
MTB-rt-MCDA assay	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xpert MTB/RIF assay	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sputum smear microscopy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Sample ID	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
MTB-rt-MCDA assay	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Xpert MTB/RIF assay	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Sputum smear microscopy	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-

Fig. 7. Comparison of the performance of the MTB-rt-MCDA assay, MTB- MCDA-LFB assay, the Xpert MTB/RIF assay and sputum smear microscopy in detection of the 32 clinical specimens. 1–32 represented 32 sputum samples. +, positive; -, negative.

Table 3
Overview of the most used methods for the detection of *M. tuberculosis* and its effectiveness.

Assay	Turnaround time	Sensitivity[a]/Dynamic range	Resource
MTB-rt-MCDA	~60 min	5 copies/μl	This study
MTB-MCDA-LFB	~60 min	5 copies/μl	This study
Xpert-RIF	~90 min	15.6 CFU/ml	Chakravorty S et al. [28]
RT-PCR	~60–180 min	32 CFU/ml, 5000 CFU/ml	Kostera J et al. [32], Yu G et al. [33]
TB-LAMP	~60 min	20 copies/μl	Wu D et al. [30], Chen X et al. [31]
Sputum smear microscopy	~30 min	5000–10000 CFU/ml	WHO
Culture	15–60 days	10–100 CFU/ml	WHO

[a] The detection limit of the assay.

by restriction endonuclease Nb.*Bsr*DI, producing fluorecence and results reporting by real-time fluorescence detector. The process of MTB detection by MTB-rt-MCDA assay was fluorescently monitored and could obtained positive results within low to 12 min without production of contamination because this assay did not need to open the reaction tube.

Here for the MTB-MCDA reaction, *s*daA gene of MTB was targeted to design primers. The *s*daA region performed excellent specificity and sensitivity as the target region for a PCR assay in the previous study [26]. The ten primers for MCDA reaction targeted different parts of the *s*daA gene, enhancing the specificity of amplification. As demonstrated in this study, the primers we used enabled effective and correct identification of genomic DNA of MTB strains with the optimum condition, and no cross-reaction was observed from non-MTB pathogens or blank control, demonstrating the excellent specificity of the presented MTB-rt-MCDA method in detecting MTB. The lowest detection level of MTB-rt-MCDA assay was 25 fg/μl (~5 copies), which was comparable to that of the MTB-MCDA-LFB test (this study), the Xpert MTB/RIF Ultra assay (15.6 colony forming unit (CFU)/ml) [27] and the CRISPR-Cas12a-based detection platform (~8 copies) [19]. Moreover, we compared the detection limit of the most used methods for the MTB diagnosis according to the previous studies (Table 3) [5,28–34], further implying the feasibility of MTB-rt-MCDA in MTB diagnosis.

In the clinical evaluation the MTB-rt-MCDA assay correctly identified MTB from all the Xpert MTB/RIF-positive samples and characterized MTB from 16 smear microscopy-negative samples besides the 8 smear microscopy-positive samples, validating that the presented assay has an excellent performance in the clinical identification of MTB infection.

Above all, the MTB-rt-MCDA assay described here, combining the fluorescence detection technique and MCDA strategy, can excellently detect MTB nucleic acid with the minimum limit of 25 fg, without cross-reaction with other pathogens. The MTB-rt-MCDA assay proves to be promising for clinical application, and the entire process can be accomplished within 1 h. The results can be obtained in real-time form using the fluorescence quantitative PCR platform, negating the need for expensive apparatus and professional technicians. However, the limitation of this study is the manual steps in the process. The manual DNA extraction and transfer to the reaction vessel could possibly lead to cross contamination. Meanwhile, the application of the assay could be limited for its requirement of operator training and the lack of the equipment and standardized laboratory. In summary, the MTB-rt-MCDA assay projected in this article offered a good option for detecting MTB infection in accuracy and rapidity, but still needs much improvement.

Funding

This study was funded by Beijing Nova Program (Grant Nos. Z211100002121042) and National Natural Science Foundation of China (82200115).

Ethics approval

The studies involving human participants were reviewed and approved by Beijing Chest Hospital Ethics Committee (Ethical approval number: KY-2018-020).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Chunrong Sun: Writing – original draft, Visualization, Validation, Data curation. **Chaohong Wang:** Writing – original draft, Validation. **Fei Xiao:** Writing – original draft, Validation, Data curation. **Nan Jia:** Writing – original draft, Formal analysis, Data curation. **Xiaolan Huang:** Writing – original draft, Visualization, Validation. **Jin Fu:** Writing – original draft, Resources. **Yu Zhang:** Writing – original draft, Resources. **Juan Zhou:** Writing – review & editing, Supervision, Methodology. **Guirong Wang:** Writing – review & editing, Supervision. **Yi Wang:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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