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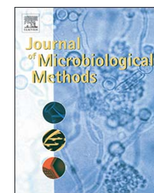

A novel isothermal amplification-based method to detect *Mycobacterium tuberculosis* complex

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ABSTRACT

Mycobacterium tuberculosis (MTB) is the causative agent of pulmonary tuberculosis. Rapid and accurate diagnosis is crucial to tuberculosis control and prevention. A series of diagnostic methods has been available for MTB detection; however, new rapid, simple and affordable methods are needed. In this study, a multiple cross displacement amplification (MCDA)-based assay was developed to detect the IS*6110* gene of the *M. tuberculosis* complex. Hydroxy naphthol blue (HNB), a colorimetric indicator, was used to detect amplification products. Amplification was carried out at a constant temperature (68 °C) for only 40 min, followed by direct determination of amplification products through observation of color variations. The entire detection procedure, from processing of specimens to reading of results, required only 85 min. Moreover, this assay, hereafter designated MTB-MCDA-HNB, was able to detect as little as 1 pg of DNA extracted from the Bacille Calmette-Guerin (BCG) strain of *Mycobacterium bovis*. No cross-reaction with nontuberculous mycobacteria (NTM) species was observed. Moreover, during testing of clinical samples, the sensitivity and specificity of MCDA results were 94.7% and 92.9%, respectively, when compared to results obtained using the Xpert MTB/RIF method. Therefore, the MTB-MCDA-HNB method developed in this study holds promise for application as an effective point-of-care test to detect *M. tuberculosis*.

1. Introduction

Tuberculosis (TB), caused by a group of closely related bacterial species that are members of the *Mycobacterium tuberculosis* complex (MTBC), remains one of the deadliest communicable diseases worldwide. The World Health Organization estimated that there were about 10.4 million TB cases in 2015 globally, resulting in approximately 1.8 million patient deaths from TB that occur mainly in developing countries (World Health Organization, 2016).

Early and correct TB diagnosis is essential to treat and combat this highly contagious disease (Bentaleb et al., 2016). However, traditional bacteriological methods all exhibit drawbacks. For example, the Ziehl-Neelsen (ZN) stain, used for direct routine diagnosis of bacteria in clinical specimens, lacks sensitivity (60%) and often requires multiple patient visits for complete workup (Steingart et al., 2007). Because these pathogens exhibit slow growth, *M. tuberculosis* complex bacterial detection requires one to two months for bacterial growth for conventional culture-based detection, as well as substantial laboratory infrastructure and staff training (Hanna et al., 1999; Otu et al., 2008).

Newer nucleic acid-based molecular diagnostic methods have shown promise for the detection of MTBC in sputum samples, with sensitive, rapid and convenient delivery of results. Xpert MTB/RIF (Cepheid) is an automated, integrated, cartridge-based system that is used with the GeneXpert instrument. This system achieves rapid detection of MTB and rifampin-resistant bacteria. This method has been widely used in tuberculosis detection programs and has contributed much to global tuberculosis control (Xie et al., 2017). However, the expensive infrastructure and high cost of cartridges make this system unworkable in some regions, particularly in less developed areas.

Conversely, accuracy, cost-effectiveness, simplicity and rapid turnaround time are crucial criteria for effective point-of-care and clinical laboratory tests. Subsequently, a novel isothermal amplification technique known as multiple cross displacement amplification (MCDA) (Wang et al., 2017) has been successfully applied to detection of

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Table 1
The primers used in this study.

Primers	Sequence (5′-3′)	Length
F1	GATCGAGCAAGCCATCTG	18
CP1	TCCCCTATCCGTATGGTGGATGACCCGCCAACAAGAAGG	39
C1	TCCCCTATCCGTATGGTGGAT	21
D1	GTCTTTCAGGTCGAGTAC	18
R1	GGATCGATGTGTACTGAGA	19
R2	GTTCAGCGAGCGGCTC	16
D2	TCGGAAGCTCCTATGACAA	19
C2	CATCCAACCGTCGGTCGGAG	20
CP2	CATCCAACCGTCGGTCGGAGATCGTCTCGGCTAGTGCA	38
F2	GGTCTTGTATAGGCCGTTG	19

pathogenic bacteria in food and clinical samples (Wang et al., 2016a, 2016b). MCDA displays unique advantages of simplicity, rapidity, accuracy and cost-effectiveness. Furthermore, hydroxyl naphthol blue (HNB), a colorimetric indicator, permits detection of amplification products through visible color changes. Indeed, an incubator that maintains a constant temperature is the only equipment needed for the MTB-MCDA-HNB assay. In this study, the MTB-MCDA-HNB technique was demonstrated to successfully achieve target analysis. Furthermore, the performance of this test for identifying MTBC from pure cultures and clinical samples was successfully verified.

2. Materials and methods

2.1. Reagents and apparatus

Loop-mediated isothermal amplification (Loopamp or LAMP) kits were purchased from Eiken Chemical Co., Ltd. (Beijing, China). The visual detection reagent (hydroxy naphthol blue, HNB) was purchased from HaiTaiZhengYuan Technology Co., Ltd. (Beijing, China). TE buffer, lysozyme and proteinase K were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Cetyl trimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) were purchased from AMRESCO (USA).

2.2. Primer design for MTB-MCDA-HNB assay

In order to design MTB-specific MCDA primers, the nucleotide sequence of gene IS*6110* (GenBank accession number: Y17220.1) was downloaded from the NCBI GenBank database and a set of MCDA primers was designed using primer software PRIMER PREMIER 5.0 and Primer Explorer V4 according to the MCDA method. Blast analysis was used to confirm that the MCDA primers were specific for MTB. The details of primer design, premier sequences and locations of MCDA primers are shown in Table 1 and Fig. 1. All oligomers were synthesized and purified by TsingKe Biological Technology (Beijing, China) and were of high-performance liquid chromatography purification grade.

2.3. Bacterial strains and genomic template preparation

A total of 165 strains of bacteria were used in this study (Table 2). All mycobacterial strains were stored in 20% (w/v) glycerol in 7H9 broth at − 70 °C and then revived on Löwenstein–Jensen (LJ) medium at 37 °C. The genomic DNA templates of mycobacterial strains were extracted using a CTAB-phenol-chloroform extraction method (Lekhak et al., 2016) and quantified using a NanoDrop ND-1000 instrument (Calibre, Beijing, China). The BCG DNA template was serially diluted to provide various amounts of template per reaction (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg) for limit of detection analysis of MTB-MCDA-HNB results.

2.4. The standard MCDA assay

Multiple cross displacement amplification reactions were carried out in amplification mixtures in 25 μL per reaction as described by Wang et al. (2017). Briefly, each reaction contained 2.4 μM cross primer CP1 and 2.4 μM cross primer CP2, 0.8 μM each of amplification primers C1 and C2, 1.2 μM each of amplification primers R1, R2, D1 and D2, 0.4 μM each of displacement primers F1 and F2, 12.5 μL of 2 × reaction mix (Loopamp kit), 1.25 μL of *Bst* DNA polymerase (10 U), 1 μL HNB and 1 μL DNA template. Next, the isothermal amplification was performed using a real-time turbidimeter (Loopamp, LA-320c). MCDA products were verified by means of a colorimetric indictor (HNB), turbidimeter and gel electrophoresis.

The optimal reaction temperature was determined within the range of 65 °C to 70 °C for 60 min. A mixture containing 1 μL DNA extraction of *Mycobacterium kansasii* (*M. kansasii*, ATCC 12478) strain was selected as a negative control and a mixture containing 1 μL double distilled water without added DNA template was used as a blank control.

2.5. Specificity and limit of detection of the MTB-MCDA-HNB assay

To verify the specificity of the MTB-MCDA-HNB assay, DNA templates from 165 mycobacterial strains were used (Table 2); all strains were identified using 16S-23S rDNA internal transcribed spacer sequence analysis and the LJ culture method described above. A turbidimeter (LA-320C) and colorimetric indicator (HNB) were utilized to analyze MCDA products and each assay was repeated twice.

The BCG strain contains just one copy of the IS*6110* element. The limit of detection (LoD) was determined using serial dilutions of BCG genomic DNA template to deliver 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg to the reaction. Three replicates of each dilution were tested.

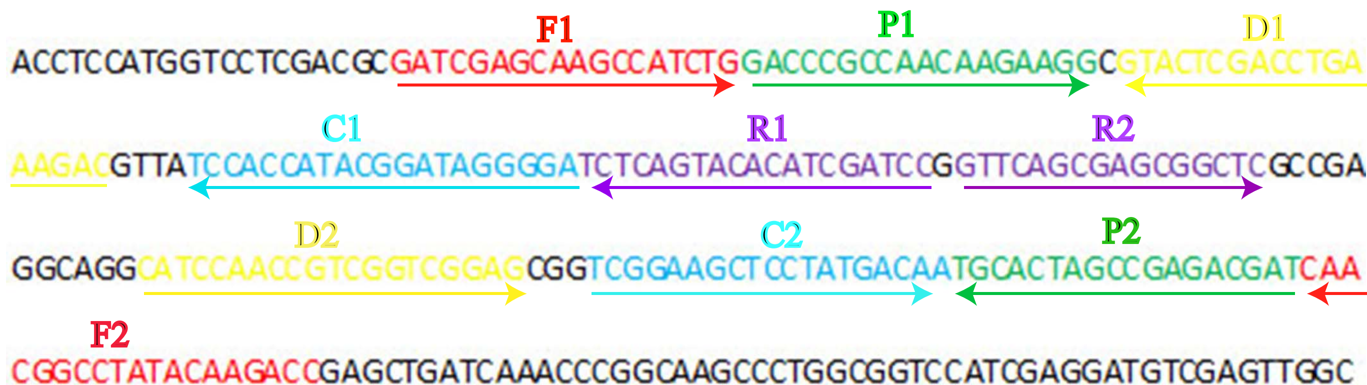

Fig. 1. Location and sequence of selected gene (part of IS*6110*) used to design multiple cross displacement amplification primers. The sites of primer sequences were underlined. Left arrows and right arrows showed complementary and sense sequences that are used.

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