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RESEARCH ARTICLE

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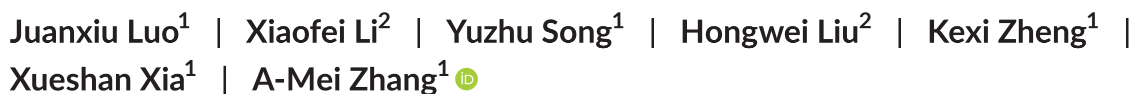
Detection of *Mycobacterium tuberculosis* in clinical sputum by a unique gene in MTB strains called Conserved protein TB18.5 (TB18.5)



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Abstract

A rapid and accurate diagnosis increases the treatment effect and decreases the mortality of tuberculosis (TB) patients. The purpose of this study was to establish an accurate, unique, and rapid molecular diagnostic technique to screen *Mycobacterium tuberculosis* (*MTB*) from clinical sputum. A unique gene in *MTB* strains called *conserved protein TB18.5* (*TB18.5*) was selected by bioinformatics analysis. Two pairs of primers were designed to amplify *TB18.5* using the nested polymerase chain reaction (PCR) or quantitative real-time PCR. Nine pathogens and the *MTB* strain were used to determine the specificity of the *TB18.5* gene. The sensitivity assay was performed after optimizing the PCR conditions. The correct fragment was amplified when a 10 copy number template was used. A total of 232 sputum samples were collected from TB patients (from 2019 to 2020) to evaluate the accuracy of the molecular method in this study. *MTB* was first detected using the BACTEC MGIT-960 culture test and the Gene Xpert MTB/RIF assay. Totals of 195 (84.05%), 182 (78.45%), and 162 (69.83%) sputum samples were determined to be infected with MTB using nested PCR, the Gene Xpert MTB/RIF assay, and the BACTEC MGIT-960 culture test, respectively. In summary, a rapid, unique, and sensitive molecular method was established to diagnose TB infection in clinical sputum samples.

KEYWORDS
conserved protein TB18.5, *Mycobacterium tuberculosis*, sensitivity, specificity, sputum

1 | INTRODUCTION

Tuberculosis (TB) is a common infectious disease caused by *Mycobacterium tuberculosis* (*MTB*). The main organ infected by *MTB* is the lung, but many other tissues or organs can be affected, such as the bone and pleura.[1] *MTB* is mainly transmitted by cough or sneeze droplets,[2] so protecting against TB in the clinic is difficult. Although the Bacillus Calmette-Guérin vaccine and anti-TB drugs are widely used in many countries, approximately ten million people developed TB in 2017, and about 40% of these TB patients lived in South East Asia.[3–5] Two million people die of *MTB* infection each year due to poor quality of life and lack of awareness.[6] China is one of

Juanxiu Luo and Xiaofei Li these two authors contributed equally to this study.

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30 high-burden countries for TB disease and experiences one million new TB cases and 130,000 deaths annually.[5] The number of TB patients has increased recently, and most infections occur in patients with human immunodeficiency virus (HIV) infection. Moreover, TB is the first cause of death from infection with a single agent.

An early and accurate diagnosis of TB is the critical factor for controlling and effectively treating the epidemic. Molecular detection, sputum-smear microscopy, and culture-based methods are widely used to diagnose TB in the clinic.[7] The benefits of molecular diagnosis are rapidity, specificity, and high sensitivity.[8] The Xpert MTB/RIF assay has been recommended and promoted as the molecular criterion for diagnosing TB by the World Health Organization (WHO).[9] However, due to its high cost, the Xpert MTB/RIF assay cannot be widely used in some regions.

In this study, a specific gene called *conserved protein TB18.5* (*TB18.5*) was selected to detect *MTB* using the nested polymerase chain reaction (PCR) or quantitative real-time PCR (qRT-PCR). The specificity and sensitivity of this molecular method were determined to be high after optimizing the PCR conditions.

2 | MATERIALS AND METHODS

2.1 | Collection and DNA extraction of clinical sputum

A total of 232 clinical sputum samples were collected from TB patients by physicians at Kunming Third People Hospital from January 2019 to December 2020. About 1 ml of sputum was obtained from each patient and liquefied using *N*-acetyl-L-cysteine and sodium hydroxide (NZLC-NaOH) (ratio = 2:1). The samples were incubated at 37°C and 200 r/min for 30 min, followed by centrifugation of the liquefied sputum at 10,625 *g* for 30 min, to obtain the precipitate. Genomic DNA of each sample was extracted using the G^+ Bacteria Genomic DNA Kit (ZomanBio, Beijing, China) according to the manufacturer's instructions.

Written informed consent was obtained from all patients before the study, which conformed to the tenets of the Declaration of Helsinki. This study was approved by the Institutional Review Board of Kunming University of Science and Technology (Approval No. 2014SK027). All experiments were performed following relevant guidelines and regulations.

2.2 | BACTEC MGIT-960 CULTURE test and the Gene Xpert MTB/RIF assay

Sputum from all patients was subjected to the BACT MGIT-960 test and Gene Xpert MTB/RIF assay at Kunming Third People's Hospital. The sputum was liquefied using NZLC-NaOH (2:1) before the BACT MGIT-960 test (Becton-Dickinson, Brea, CA, USA). Fluorescence intensity was tested to evaluate *MTB* positivity. A 192-bp region of the *rpoB* gene was amplified and detected using GeneXpert (Cepheid, Sunnyvale, CA, USA).

2.3 | Selection of specific genes and primer design

A total of 173 genome sequences of *Mycobacterium* were downloaded and analyzed. A specific *MTB* gene was selected using the method from our previous study.[10] Briefly, 173 genomes of *Mycobacterium* (including 10 *MTB* sequences) were downloaded from the NCBI to a local database and blasted using BLAST software. The potential specific genes were further blasted using online BLAST at the NCBI. Finally, the *TB18.5* gene was considered a specific gene of the *MTB* strains. *TB18.5* primers were designed for further study.

2.4 | Nested PCR amplification

One-step PCR was performed in a 20 μl reaction volume, including 3 μL of DNA extracted from sputum, 10 μl of 2× TSINGKE Master Mix, and 0.3 μM each of the myco-F1 and myco-R1 primers. The PCR conditions for the first step were one cycle of 94°C for 5 min, 25 cycles of 94°C for 30 s, 57.5°C for 30 s, 72°C for 10 s, and one extension cycle of 72°C for 5 min. Nested PCR was also performed in a 20 μl reaction volume, including 3 μl of the amplicon from the first-step PCR, 10 μl of 2× TSINGKETM Master Mix (or 2× ChamQ SYBR qPCR Master Mix [Vazyme, Beijing, China] for qRT-PCR), and 0.3 μM of each of the myco-F2 and myco-R2 primers. The PCR conditions were one cycle of 94°C for 5 min, 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 10 s, and one extension cycle of 72°C for 5 min.

2.5 | Specificity and sensitivity testing

Six common clinical pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus heamolyticus*, and *Acinetobacter baumannii*) and three other *Mycobacterium* species (*Mycobacterium avium*, *Mycobacterium chelonei*, and *Mycobacterium intracellulare*) were used as negative controls to investigate the specificity of the *TB18.5* gene and the primers. The *TB18.5* gene was constructed using the pMD 18-T vector (Takara, Shiga Japan) to test the sensitivity of the primers. Then, the plasmids were diluted to 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copy numbers to achieve a sensitive assay.

2.6 | Statistical analysis

The Pearson chi-square test with Yates' correction was used to distinguish the statistical difference among results of three methods. The *p*-value <.05 was statistical significance. Statistical analysis was performed by using GraphPad Prism 5 software (GraphPad Software Inc. USA).

3 | RESULTS

The bioinformatics analysis revealed that the *TB18.5* gene was considered a specific *MTB* gene. Two primer pairs were designed for nested PCR to amplify this gene in clinical samples (Table 1). To

test the specificity of the *TB18.5* gene and the primers, nine common clinical pathogens and the *MTB* strain were amplified using the myco-F1/myco-R1 and myco-F2/myco-R2 primers. As shown in Figure 1, the *MTB* strain was successfully amplified by the two pairs of primers, but the other nine pathogens could not be amplified. Thus, the *TB18.5* gene was highly specific to the *MTB* strain and could be used to clinically diagnose TB patients.

The sensitivity of the molecular testing method was evaluated in this study using plasmids with 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copy numbers. The results showed that the amplicon band was clear when the plasmid with 10 copy numbers was used as the template (Figure 2). We tested primer sensitivity using qRT-PCR to directly observe the results. The melting curve was detected when the template was a plasmid with 10 copy numbers (Figure 3). All 232 sputum samples from TB patients were tested using the BACTEC MGIT-960 CULTURE test, the Gene Xpert MTB/RIF assay, and nested PCR in this study. The results showed that 195 (84.05%), 182 (78.45%), and 162 (69.83%) samples were identified as *MTB* using nested PCR, the Gene Xpert MTB/RIF assay, and the BACTEC MGIT-960 CULTURE test, respectively. Although the nested PCR-positive ratio was the highest among the three methods, no statistical difference was identified between results of nested PCR and Gene Xpert MTB/RIF assay. However, the *P*-value showed significant difference between results of BACTEC MGIT-960 CULTURE test and nested PCR ($p = 0.0004$)/ or Gene Xpert MTB/RIF assay ($p = 0.044$).

TABLE 1 Information of nested PCR primers

primer	Sequence (5′–3′)	Amplicon size (bp)
Myco-F1	ATGACGGCAATCTCGTGCTCA	481
Myco-R1	TTAGCTGGCCGCCAGCTGCTCG	
Myco-F2	GCAAGACCGTCGAGGTCACC	375
Myco-R2	CCAGGACGTTGTTGAGCAGCA	

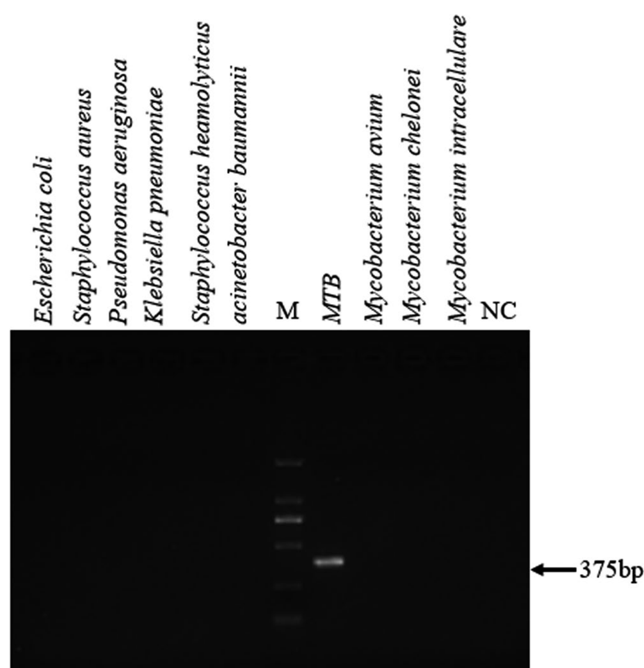

FIGURE 1 Evaluating specificity of the *conserved protein TB18.5* gene in *MTB*. Bands 1–6 are amplification with template *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus heamolyticus*, and *acinetobacter baumannii*, respectively. Bands 8–11 are amplification with template *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium chelonei*, and *Mycobacterium intracellulare*. M means DNA marker DL2000; NC means negative control

4 | DISCUSSION

TB is one of the top ten causes of death in the world,[11] and also causes high morbidity in poor regions or HIV-positive patients.[12,13] Although TB vaccines and drugs have been widely used to control *MTB* infections, many patients develop TB each year. Although the WHO has proposed to eliminate TB by 2035,[14] it will be difficult to accomplish this task. An early and rapid diagnosis of TB patients could increase the treatment effect and decrease the chances for drug-resistant TB.[14,15]

Molecular detection, sputum-smear microscopy, and culture-based methods are generally used to diagnose TB.[16] Sputum-smear microscopy is currently recommended for regular detection and is performed using an acid-fast staining microscopic examination. Although this method was invented more than 100 years ago, it is still widely used in clinics because of its high speed and low price.[15] However, the lower specificity and sensitivity of sputum-smear microscopy can lead to false-positive or false-negative results.[17] Fluorescence microscopy increases the sensitivity but not the specificity of sputum-smear microscopy[18]; therefore, it is used as a preliminary evaluation of *MTB* infection. *MTB* culture is the golden standard to diagnose TB, but it is time-consuming.[19] Although the BACTEC MGIT-960 CULTURE test shortened the *MTB* culture time, 1 week is required to obtain the results. Thus, molecular detection of *MTB* is an accurate and rapid method compared with phenotypic methods. The Xpert MTB/RIF assay was first recommended by the WHO in 2010 to diagnose pulmonary TB disease in adults[20] and was considered to complement proportional methods,[21] but this method requires expensive equipment. A simple and lower-cost molecular method, similar to the LCD-array used in testing drug-resistant mutations,[22] should be established to apply in developing countries.

To establish a rapid, sensitive, and specific molecular method to detect *MTB*, a unique *MTB* gene (*TB18.5*) was selected and primers were designed for nested PCR (or nested RT-PCR). Then, the specificity and sensitivity of this method were determined. After analyzing the results of the Xpert MTB/RIF assay, the BACTEC MGIT-960 CULTURE test, and the molecular method in this study, the positive rate was higher using the established molecular method than the other two methods. Moreover, the low cost, high accuracy, specificity, and sensitivity suggested that the molecular method could be used in the clinic or laboratory in the future.

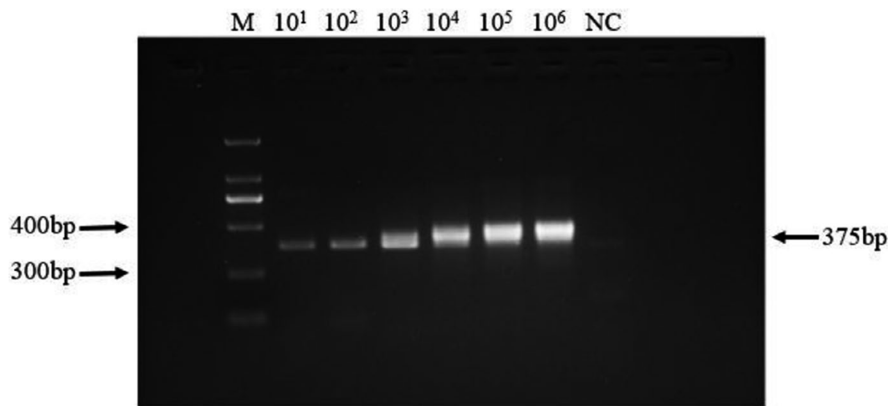

FIGURE 2 Sensitivity of the primers for the *conserved protein TB18.5* gene by nested PCR. The copy numbers of template are 10, 10^2, 10^3, 10^4, 10^5, and 10^6 in bands 2–7, respectively. M means DNA marker DL2000; NC means negative control

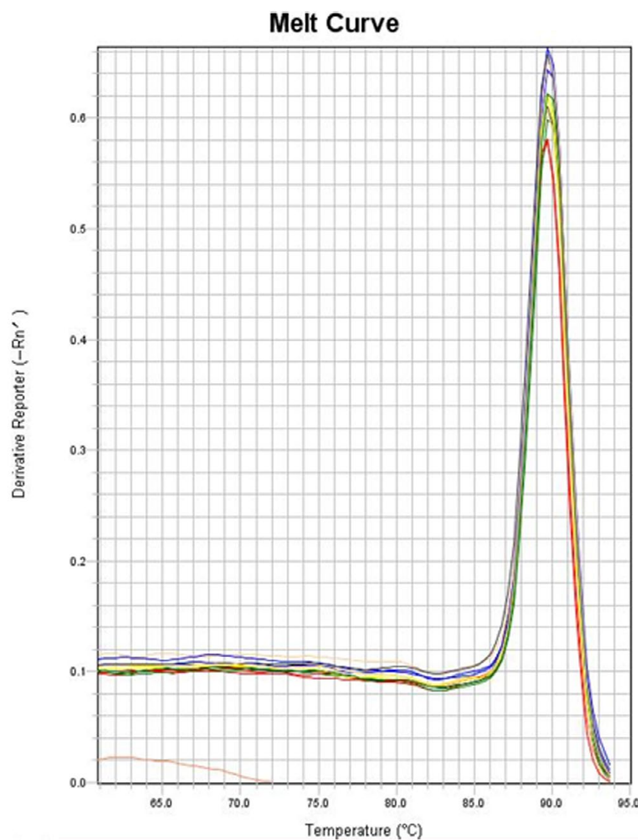

FIGURE 3 Sensitivity of the primers for the *conserved protein TB18.5* gene by nested qRT-PCR. Orange curve is negative control (NC); red curve is template with 10 copy numbers; yellow curve is template with 10^2 copy numbers; blue curve is template with 10^3 copy numbers; green curve is template with 10^4 copy numbers; black curve is template with 10^5 copy numbers; and pink curve is template with 10^6 copy numbers

Although high detection ratio of nested PCR was identified, there were still a few limitations. Firstly, the numbers of bacteria used for testing the specificity of nested PCR were less, so we should collect more kinds of bacteria to further identify its specificity. Secondly, whether this nested PCR method could refer to the other kind of TB samples (such as ascites and synovial fluid) was not clear. Finally, we should improve protocol of sample preparation to increase the positive detection ratio of the nest-PCR method.

In summary, the *TB18.5* gene, which was identified as a unique gene in *MTB* strains, was used to evaluate *MTB* infections. The optimized nested PCR/nested qRT-PCR method was established to detect *MTB* in clinical sputum samples, which showed higher positive ratio than Xpert MTB/RIF assay and the BACTEC MGIT-960 CULTURE test. Thus, it is benefit for TB patients to obtain early and sensitively diagnose and treatment by using this nest-PCR method.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

AMZ, YZS, and XSX designed the experiments. JXL and KXZ performed the experiments. AMZ, JXL, and YZS analyzed the data and prepared for the article. XFL and HWL collected the sputa samples and performed the clinical evaluation.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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