Iranian Journal of Microbiology
Volume 15 Number 3 (June 2023) 401-407

ORIGINAL ARTICLE

Simultaneous detection and differentiation of *Mycobacterium tuberculosis* and nontuberculous mycobacteria coexisting in patients with pulmonary tuberculosis by single-tube multiplex PCR

Leila Heidari[1], Gholamreza Rafiei Dehbidi[1], Ali Farhadi[1], Golnar Sami Kashkooli[2], Farzaneh Zarghampoor[1], Sepide Namdari[1], Noorossadat Seyyedi[1], Saeid Amirzadh Fard[1], Abbas Behzad-Behbahani[1*]

[1]Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Shiraz Referral Mycobacteriology Laboratory, Shiraz University of Medical Sciences, Shiraz, Iran

Received: January 2023**, Accepted:** April 2023

ABSTRACT

Background and Objectives: In clinical diagnostics, molecular methods are used to detect *Mycobacterium tuberculosis* bacilli (MTB) and to distinguish them from non-tuberculous mycobacteria (NTM). They are also used to make the right treatment decision for the patient as soon as possible. The aim of this study was to establish a rapid and novel multiplex PCR (mPCR) assay for the detection and differentiation of MTB and NTM in a single tube.

Materials and Methods: 100 sputum samples positive for acid-fast bacilli (AFB) were included in this study. Mycobacterial culture, biochemical tests, and antibiotic susceptibility testing were performed on samples. After alkaline decontamination, total DNA was extracted from the samples. A primer pair targeting the *rpoB* gene, encoding the beta-subunit of RNA polymerase, was used to detect MTB and NTM, amplifying a 235-bp fragment of MTB and a 136-bp sequence of NTM. A pair of primers targeting a 190-bp fragment of the IS6110 region of MTB was also used to confirm the results. The sensitivity and specificity of the mPCR assay were evaluated using DNA extracted from standard strains. The amplified products were then analyzed by conventional agarose gel electrophoresis.

Results: Of 100 AFB smear-positive sputum samples, 92 MTB DNA, 7 NTM DNA, and one mixed-infection sample were identified in a single tube using mPCR assay. There was no correlation between the AFB degree of smear positivity and PCR results. Of seven NTM isolates, 6 (86%) were resistant to rifampin, isoniazid, and ethambutol, the three first-line anti-tuberculosis drugs.

Conclusion: A single-tube mPCR assay based on the *rpoB* gene provides a rapid and reliable means of detecting and differentiating MTB and NTM in sputum specimens.

Keywords: Isoniazid; Multiplex polymerase chain reaction; *Mycobacterium tuberculosis*; Nontuberculous mycobacteria; Rifampin; Sputum

*Corresponding author: Abbas Behzad-Behbahani, PhD, Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran. Tel: +98-71-32270301 Fax: +98-71-32270301 Email: behzadba@gmail.com; behzadba@sums.ac.ir

INTRODUCTION

The World Health Organization (WHO) estimates that almost a quarter of the world's population are infected with *Mycobacterium tuberculosis* (MTB) (1). Even after the coronavirus (COVID-19) became a pandemic, tuberculosis (TB) remains the leading cause of deaths from infectious diseases worldwide (2-4). Although MTB infections have declined worldwide, pulmonary tuberculosis caused by non-tuberculous mycobacteria (NTM) as opportunistic pathogens has increased. This syndrome accounts for more than 90% of clinical cases of NTM disease (5-7). Due to the unique progression pattern of this infection and the disease characteristics, NTM is usually resistant to primary anti-tuberculosis drugs (8). Moreover, the identification of infectious NTM species is crucial to determining treatment outcomes and resistance to treatment (9).

On the other hand, there is increasing evidence that the prevalence of NTM-MTB co-infections varies widely across geographic regions (10, 11). Therefore, it makes sense to consider NTM-MTB coinfections in samples from patients with pulmonary tuberculosis, as failure to detect NTM-MTB coinfections could lead to incorrect treatment of patients.

In many developing countries, TB diagnosis is based on clinical findings, chest X-rays, and acid-fast bacilli (AFB) detection in sputum samples. Although tuberculosis is always detected and diagnosed using AFB microscopy, mycobacterial cultures remain the gold standard for the laboratory confirmation of TB disease (12, 13).

There have been advances in molecular diagnostic methods that are superior to culture-based TB tests. For example, the PCR-based Xpert MTB/RIF test can accelerate the diagnosis and increase the detection sensitivity in multibacillary TB infections (12, 14, 15). Although Xpert MTB/RIF technology provides important information on rifampin resistance, it is less sensitive than TB sputum culture and cannot distinguish NTM infections.

The multiplex polymerase chain reaction (mPCR) method is one of the cost-effective and easy-to-use molecular methods for the detection of MTB and NTM alone or in coexistence in a single tube. In addition, depending on the results, patients can be treated to prevent disease progression until the results of bacterial culture and antibiotic susceptibility tests are available.

In this study, we evaluated the performance of an mPCR assay to detect MTB and NTM in AFB-positive and clinically suspicious pulmonary tuberculosis patients visiting the Shiraz Mycobacteriology Regional Reference Laboratory.

MATERIALS AND METHODS

Patients and samples. In this cross-sectional study, 100 new cases of AFB smear-positive patients who attended Shiraz University of Medical Sciences Regional Reference Laboratory for Mycobacteriology between March 2019 and December 2021 were included. All cases of pulmonary tuberculosis with at least two positive sputum AFB and without prior treatment with anti-tuberculosis drugs were considered as new cases. The Medical Ethics Committee of Shiraz University of Medical Sciences, Iran, approved the study (No. IR.SUMS.REC.1392.3567). All experiments were performed according to the appropriate guidelines and regulations (16). Participants provided informed consent prior to sample collection.

The patients included 34 women between the ages of 22 and 79 (mean=48 years), and 66 men between the ages of 22 and 86 (mean=52 years). Sputum samples were collected in sterile Bijou bottles, and a portion of the samples were immediately aliquoted into sterile tubes and stored at -20°C for further molecular analysis.

Acid-fast staining and bacterial culture. Sputum samples were first decontaminated by sodium hydroxide digestion and subjected to conventional mycobacterial culture and bacteriological examination. For conventional mycobacterial identification, 200 µl of each decontaminated sputum sample was inoculated in duplicate onto two Lowenstein-Jensen (L-J) medium slant cultures. The slant cultures were incubated at 37°C and monitored weekly for eight weeks. Acid-fast staining and biochemical tests such as nitrate reduction and niacin assay were used to identify MTB and NTM (17).

Drug susceptibility testing. Susceptibility to conventional anti-tuberculosis drugs was assessed based on the World Health Organization guideline (17) using isoniazid, rifampicin, and ethambutol.

DNA extraction from sputum samples. As pre-

viously reported, all samples were pretreated with NaOH, and total DNA was extracted from sputum samples using the classical phenol-chloroform method (18). Genomic DNA from *M. tuberculosis* strain H37Rv (ATCC 25618) and NTM *M. gordonii* (ATCC 14470) were used as reference strains and positive controls for establishing mPCR assays. In addition, genomic DNA from several common bacteria, such as *Staphylococcus aureus* (ATCC 2523), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumoniae* (ATCC 25920) were also used to assess the specificity of the designed mPCR.

Each independent PCR assay included positive and negative controls. Positive controls were *M. tuberculosis* H37Rv strain DNA and *M. gordonii*. The negative control was PCR-grade water.

Development of multiplex PCR. Two pairs of primers were used to simultaneously detect and amplify MBT and NTM in one sample, specifically targeting the *rpoB* gene of MTB and NTM, but with two different PCR product sizes. In addition, the assay was validated using a set of PCR primers that specifically targeted the IS6110 region of *M. tuberculosis* (Table 1).

The multiplex PCR assay was performed using an in-house reaction mix. After the optimal conditions were reached, the amplification reactions were performed in a final volume of 20 µL containing 0.5 µM of each specific primer set for the MTB *rpoB* region and 0.2 µM of the *rpoB*-specific primer set for NTM, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit Taq DNA polymerase (Fermetas, Latonia), and 2 µL (about 30 ng/reaction) of extracted DNA. Amplification was performed as follows: first denaturation step at 95°C for 5 min, followed by 35 cycles of 30-sec denaturation at 95°C and one-min annealing and extension at 72°C. Final extension was carried out at 72°C for 10 min. Amplified DNA fragments were then electrophoresed in 2% agarose gel and 1× TBE buffer and stained with 1x GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA).

Positive amplification of IS6110 confirmed MTB positivity irrespective of *rpoB* amplification result, and the amplification of a 136-bp sequence of the *rpoB* region along with a negative IS6110-specific reaction was considered NTM positivity.

The reaction mixture was the same as above, except that the final concentration of the specific primer set for the IS6110 region was 8 pmol/reaction. Amplification was performed as follows: initial denaturation step at 95°C for 5 min, followed by 30 cycles of 20-sec denaturation at 95°C, one-min at 65°C and 20-sec extension at 72°C. The final extension was performed at 72°C for 10 min. Amplified DNA fragments were then electrophoresed in 2% agarose gel and 1 x TBE buffer and stained with 1×GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA , United States).

Sensitivity analysis with limit of detection. To determine the limit of detection (LOD) of the current multiplex PCR assay, 10-fold serial dilutions of purified genomic DNA (1×10^6 copies/µl) from *M. tuberculosis* and NTM cultures were used. To check the specificity of primers, the Primer-Blast software tool was used (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). In addition, *ropB* and IS6110 primer sets were used to amplify other bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* in the samples.

RESULTS

Culture positivity rate by Löwenstein–Jensen (L-J) medium. Based on phenotypic characteristics, including growth rate (fast or slow) and pigment production, and biochemical identification of bacteria

Table 1. Primer nucleotide sequences used in the study

Primer name	Sequences (5 to 3') for the *rpoB* gene	Product size (bp)
MTBc1-F	CGT ACG GTC GGC GAG CTG ATC CAA	235
MTBc5-R	CCA CCA GTC GGC GCT TGT GGG TCA	
NTM5-F	GGA GCG GAT GAC CAC CCA GGA CGT	136
NTMR3-R	CAG CGG GTT GTT CTG GTC CAT GAA	
	Sequences (5' to 3') for IS6110 region	
MTB1 F	ATC CTG CGA GCG TAG GCG TCG	190
MTB2 R	CAG GAC CAC GAT CGC TGA TCC GG	

isolated from L-J medium, 92 out of 100 (92%) AFB smear-positive specimens were diagnosed as MTB. Of these, 55 samples (59.8%) were associated with the fast-growing MTB with a growth rate of approximately 16-25 days, 34 samples (34%) had an average growth rate of 26-35 days, and three samples (3.2%) were associated with slow-growing MTB.

Fast-growing NTM with a growth rate of 16-25 days were isolated from 7 out of 100 (7%) specimens. In addition, NTM with the coexistence of MTB was isolated in one out of 100 (1%) samples 26-35 days after bacterial culture.

Susceptibility to antituberculosis drug. In the present study, 49 cases were sensitive to both rifampin and isoniazid and 35 cases were resistant to these two drugs. We found 12 cases sensitive to isoniazid but resistant to rifampin and four cases sensitive to rifampin and resistant to isoniazid. Of seven NTM isolates, 6 (86%) were resistant to rifampin, isoniazid, and ethambutol, the three first-line antituberculosis drugs.

Validation of the PCR method. The detection limit of the multiplex PCR assay using the *rpoB* plasmid for detecting MTB and/or NTM DNA was 100 copies/L. The same limit of detection was achieved when using IS6110 plasmid DNA to specifically detect and confirm MTB in sputum samples. No cross-reaction was observed when the *rpoB* and IS6110 primer sets were used to amplify other bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* in the samples.

PCR conditions were optimized with three pairs of primers, using *M. tuberculosis* H37Rv and *M. gordonii* as representative strains for MTB and NTM, respectively. When primers were used to amplify the *rpoB* gene, a 235-bp PCR product was obtained for the MTB strain and a 136-bp PCR product for NTM (Fig. 1). When primers were used to specifically amplify the sequence in the IS6110 region, a single 190-bp product was obtained that confirmed the *M. tuberculosis* strain. However, when primers for bacterial genomes not belonging to the genus *Mycobacterium* were used, no PCR products were obtained.

Detection of MTB and NTM by multiplex PCR. Mycobacterial DNA was detected in all AFB swab-positive specimens by multiplex PCR. Using *rpoB* primers and PCR with primers recognizing the IS6110 region, MTB was detected in 53 AFB

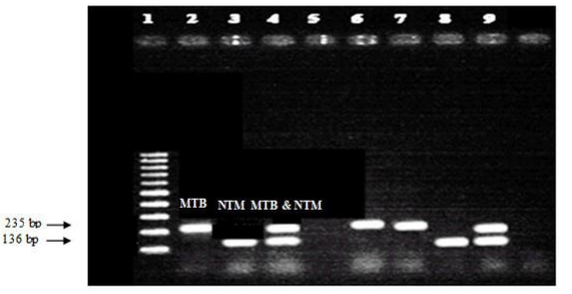

Fig. 1. Optimized multiplex PCR to detect MTB (235 bp) and NTM (136 bp) in one tube in different combinations. Lane 1. 100-bp DNA ladder (Fermentas, Lithuania); Lane 2: *M. tuberculosis* positive control; lane 3: NTM positive control; Lane 4 both *M. tuberculosis* and NTM genome in one tube; Lane 5: negative control; Lane 6-9: clinical samples.

smear-positive grade 1+, 21 grade 2+, and 18 grade 3+ specimens. Using the same primers, on the other hand, multiplex PCR was able to detect NTM in 3% of the AFB smear-positive grade 1+, 3% of the AFB smear-positive grade 2+, and 1% of AFB smear-positive grade 1+ specimens. *M. tuberculosis* bacilli were detected in 91/100 (91%) sputum samples, while 7/100 (7%) were detected as NTM, and one sample had a mixed infection of MTB and NTM detected by multiplex PCR (Fig. 2).

When the frequency of MTB and NTM infections in different age groups was determined, the highest rate of infection with both MTB (24.1%) and NTM (2.5%) was found in patients between 41 and 50 years of age (Fig. 3).

Antibiogram susceptibility testing in positive *M. tuberculosis* cultures showed that 49% of the isolates were susceptible to both rifampin and isoniazid, while 35% of them were resistant to these two antibiotics. In addition, 12% of the isolates were sensitive to isoniazid and resistant to rifampin. On the other hand, 4% of the isolates were sensitive to rifampin and resistant to isoniazid. None of the NTM isolated from sputum cultures were sensitive to both rifampin and isoniazid.

DISCUSSION

Early identification of the infecting microorganisms is necessary for patient management. In the case of MTB infection, rapid diagnosis and identification is an important factor in initiating anti-TB drug therapy and controlling the person-to-person spread of MTB bacilli. In addition, it is necessary

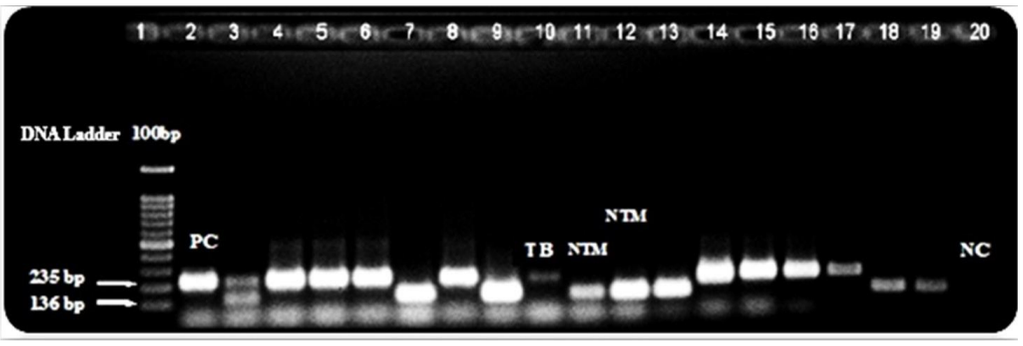

Fig. 2. Detection of MTB and NTM DNA in sputum samples from patients with AFB smear positive
Lane 1: 100 bp DNA ladder; Lane 2: MTB positive control (PC); Lane 3: MTB and NTM mixed infection; Lane 4-6: MTB; Lane 7: NTM; Lane 8: MTB; Lane 9: NTM; Lane 10: MTB; Lane 11-13: NTM; Lane 14-17: MTB; Lane 18-19: NTM; Lane 20 Negative control (NC).

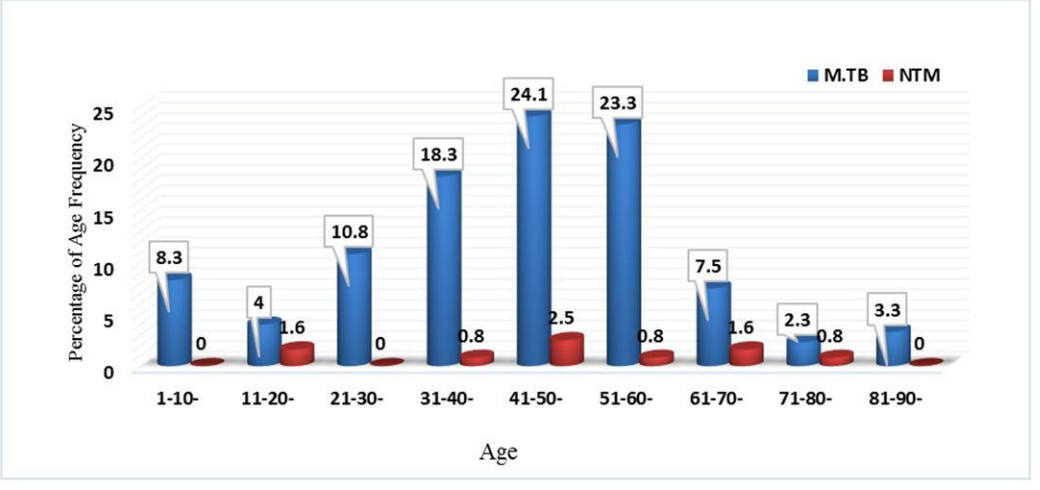

Fig. 3. Frequency of MTB and NTM infections in different age groups of patients.

to distinguish MTB from NTM in order to properly treat tuberculosis. NTM represents over 190 species and subspecies, some of which can cause disease in people of all ages and affect both pulmonary and extrapulmonary sites.

Although Xpert MTB/RIF Ultra technology has facilitated the detection of *M. tuberculosis* and resistance to rifampin in a single test, it is less sensitive than TB sputum culture and cannot distinguish NTM infections. In addition, the technology is difficult to access in low-income countries. On the other hand, multiplex PCR assays are technically more demanding than conventional monoplex PCR protocols. Using an in-house multiplex PCR assay, we were able to distinguish between *M. tuberculosis* at the species level and *Mycobacterium* spp.

Our results showed that the multiplex PCR assay was specific for *M. tuberculosis* and non-tuberculous Mycobacteria. Primers in the conserved region of the *Mycobacterium rpoB* gene were designed for two reasons. Firstly, the selected region does not exist in any bacterial genus or species other than mycobacteria. Secondly, with minor changes in the sequence of the primers, *Mycobacterium tuberculosis* was distinguishable from NTM in a single test (19). Using this assay, we were able to detect MTB and NTM in the sputum samples of patients with positive AFB smears much more quickly than culture.

Although AFB smears were classified differently, multiplex PCR was able to amplify DNA from all smear-positive specimens regardless of classification, suggesting that there was no relationship between PCR positivity and the number of AFB in smear-positive specimens.

The coexistence of pleuropulmonary TB and NTM infection was diagnosed in one patient by multiplex

PCR testing. In addition, NTM was detected in 7 of 100 AFB smear-positive specimens. The coexistence of MTB and NTM has been reported in several studies (20-22), but most of them were case reports. This shows the importance of diagnosing the co-infection of MTB and NTM in patients with active tuberculosis. Individuals with a history of lung damage are susceptible to NTM. The coexistence of TB and NTM has led to inaccurate diagnoses and inappropriate treatments (23).

Recently, a quantitative multiplex PCR assay has been developed for the simultaneous detection of MTB and NTM in clinical samples (24). They showed that the multiplex assay had better specificity than Xpert, and they suggested that the assay could complement the widely used Xpert assay and enhance the discrimination of TB and NTM infections.

NTM used to be considered commensal organisms. However, infection with NTM in some patients such as those with cystic fibrosis is severe (25). Furthermore, different therapy regimens are recommended based on the species of NTM isolated from patients with cystic fibrosis. We did not set up multiplex PCR to distinguish different NTM species. However, it is noteworthy that this multiplex PCR allows the simultaneous detection of MTB and NTM, if present, in a single tube.

Although diagnosing *M. tuberculosis* and other *Mycobacterium* species in a patient sample is very important, determining drug resistance is ultimately just as important as its diagnosis (26, 27).

CONCLUSION

Overall, compared to bacterial culture, our in-house multiplex PCR assay based on the *rpoB* gene is a fast, sensitive, and inexpensive molecular method capable of detecting and distinguishing MTB and NTM in less than four hours in a single tube. The method can help physicians make the right treatment decision for the patient as quickly as possible.

ACKNOWLEDGEMENTS

We would like to thank the staff at the Shiraz Mycobacteriology Regional Reference Laboratory, Shiraz University of Medical Sciences for their technical assistance.

This work is part of a project funded by Shiraz University of Medical Sciences, Shiraz, Iran under Agreement No. 1392/3567.

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