Original Article | Iran J Pathol.2018; 13(4):429-437
Iranian Journal of Pathology | ISSN: 2345-3656

Investigation of the *rpoB* Mutations Causing Rifampin Resistance by Rapid Screening in *Mycobacterium Tuberculosis* in North-East of Iran

Amir Tajbakhsh[1,2,3], Faezeh Ghasemi[5], Seyedeh Zohre Mirbagheri[6], Mastoureh Momen Heravi[6], Mehdi Rezaee[2,4], Zahra Meshkat[6,7*]

1. *Dept. of New Sciences and Technologies, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
2. *Student Research Committee, Mashhad University of Medical Sciences, Mashhad, Iran*
3. *Young Researchers and Elite Club, Yasooj Branch, Islamic Azad University, Yasooj, Iran*
4. *Dept. of Medical Biotechnology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
5. *Dept. of Biotechnology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran*
6. *Antimicrobial Resistance Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*
7. *Dept. of Microbiology and Virology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

KEYWORDS

Multiplex PCR,
rpoB gene,
Rifampin resistance,
Mycobacterium tuberculosis

Article Info

Received 18 June 2017;
Accepted 13 Sep 2018;
Published Online 25 Sep 2018;

ABSTRACT

Background and Objectives: The incidence of rifampin-resistant strains of *Mycobacterium tuberculosis* has attracted more attention than the tuberculosis infection due to laborious treatment and control. Recognizing the *Mycobacterium tuberculosis* genotypes involving in drug resistance via multiplex PCR, a simple and rapid genotyping method, is an emergency for better treatment and control of tuberculosis. This study was designed to specify the frequency of rifampin-resistant strains of *Mycobacterium tuberculosis* isolated from patients by multiplex allele-specific Polymerase Chain Reaction assay (MAS-PCR).

Methods: In this study, 88 *Mycobacterium tuberculosis* positive samples were included from Qaem Hospital, Mashhad. MAS-PCR was used to detect the rifampin resistance associated mutations in *rpoB* gene.

Results: Mutations in three codons of *rpoB* gene causing rifampin resistance were detected in 51 isolates (58.96%). The detected mutations in codons 531, 526, and 516 were 55.68%, 38.63%, and 13.63%, respectively. The simultaneous mutations were detected in 11 isolates (12.50%) in codons 531, 526 and 516, in 21 isolates (23.86%) in codons 531 and 526, and in one isolate (1.13%) in codons 526 and 516.

Conclusion: According to the results of this study, the frequency of rifampin-resistant strains of *Mycobacterium tuberculosis* isolated from Khorasan province patients (North-East of Iran) was high. The developed MAS-PCR assay can be used for rapid detection in clinical diagnostic laboratories in areas with high prevalence of multidrug-resistant *Mycobacterium tuberculosis* strains. In this respect, MAS-PCR is simple, rapid, and highly sensitive method for drug susceptibility tests for detecting multidrug-resistant *Mycobacterium tuberculosis*.

Corresponding information: Zahra Meshkat, Antimicrobial Resistance Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
Dept. of Microbiology and Virology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran E-mail: meshkatz@mums.ac.ir.

Introduction

Antimicrobial resistance among pathogens has increased recently (1, 2). In this respect, *Mycobacterium tuberculosis* is one of the most resistant and harmful human pathogens in the world which causes tuberculosis (TB) (3). Ss an infectious disease, TB is the second cause of death worldwide (4). According to the World Health Organization (WHO), TB causes mortality and morbidity and becomes a worldwide concern (5). Efficient and rapid detection of TB via molecular techniques have some benefits including detection of multidrug-resistant tuberculosis (MDR-TB), decreasing the mortality of TB and drug resistance through treatment, and decreasing the resistance to the second-line anti-tuberculosis drugs (3). Rifampin (RIF) has been used for the treatment of TB since 1972. RIF binds to the RNA polymerase β-subunit (*rpoB*), coded by *rpoB* gene in M. tubercu-

losis and inhibits the elongation of mRNA. Hereupon, RIF inhibits bacterial growth and metabolism. Currently, RIF is used for the first-line therapy in short-course tuberculosis treatment (3). Resistance to isoniazid (INH) or RIF is known as multi-drug-resistant tuberculosis (MDR-TB), a form of TB infection that are resistant to treatment. Moreover, resistance to RIF is considered as MDR marker of TB. RIF-resistance is caused by mutations generally located in the short 81-bps region between codons 507 to 533 of the *rpoB* gene, also known as the rifampicin resistance determining region (RRDR). Single base insertion, deletion and substitution mutations in RRDR lead to the RIF resistance (6). Mutations in codons 511, 513, 516, 522, 526, 531, and 533 are responsible for the RIF resistance (7). Mutations in three codons 516, 526 and 531 in *rpoB* are conferred with high level (70 to 95%) of the RIF-resistant, particularly in the areas with high prevalence of MDR-TB (8). Determining the frequency of MDR-TB and rapid and effective detection of drug resistance TB infection is important for controlling the spread and the development of further resistant *Mycobacterium tuberculosis* strains. The appropriate anti-TB treatment reduces total cost of therapy and transmission of drug-resistant strains of *Mycobacterium tuberculosis*. Therefore, it is necessary to develop the molecular techniques which can quickly detect TB and MDR-TB (9, 10). According to the reports, Iran is among countries with a high prevalence of drug-resistant TB. However, there is not accurate and comprehensive information of drug resistance in Iran. On the other hand, most studies use a time-consuming technique that increases the time of treatment as well as treatment of resistant strains. In this respect, we have refined a method that provides a rapid and concurrent methodology for target identification that minimizes resource requirements. In the current study, the frequency of *M. tuberculosis* resistance to RIF was investigated using multiplex allele-specific PCR (MAS-PCR). The MAS-PCR method detects main mutations that happen in *rpoB* region in the M. tuberculosis and result in resistance to RIF. The method is simple, rapid (in less than 2.5 hr) with reasonable cost, sensitive and specific based on the PCR and agarose gel electrophoresis. It detects RRDR mutations in DNA extracted from bronchial and sputum. Thus, in our study, point mutations were assessed by MAS-PCR in three codons 516, 526 and 531 of *rpoB* gene in samples isolated from TB patients admitted to Qaem Hospital, Mashhad, Iran.

Materials and Methods

Patient characteristics and bacterial isolates

In this cross-sectional study, all 88 DNA positive samples of patients with tuberculosis (confirmed in the previous study *via* IS6110 amplification (11)) referred to Qaem Hospital, Mashhad, Iran were collected (Table 1). This Hospital is considered as a center for the diagnosis and treatment of tuberculosis in the North-East of Iran. The study was approved by the Ethics Committee of Mashhad University of Medical Sciences. After gathering and decontamination of the samples, the isolates of M. tuberculosis were cultured on Lowenstein-Jensen (LJ) medium and incubated at 37°C for at least three weeks (12). Thus, M. tuberculosis isolates were recognized in consistent with its specific growth rate and colony morphology on LJ medium (11). Finally, the stocks of samples were prepared and bacterial DNAs were extracted via boiling method and then were stored at -20°C. PCR was used to amplify IS6110 and identified the M. tuberculosis positive samples.

Table 1. Frequency of M. tuberculosis positive patients in different groups, categorized based on the sex and type of sampling.

Characteristic		No. (%)of patients (N=88)
Sex	Male	54 (57.95%)
	Female	34 (42.04%)
Type of samples	Sputum	19 (21.59%)
	Bronchial	69 (78.41%)

Multiplex allele-specific PCR (MAS-PCR)

The MAS-PCR assay was performed to determine the mutated and wild-type alleles of *rpoB* gene (13). Multiplex PCR enables amplification of different fragments from limited amount of sample. One reverse primer R (*rpoB*) (5′-TTG ACC CGC GCG TAC AC-3′) was used for MAS-PCR, targeting three different codons of the *rpoB* gene along with three specific forward primers including F1 (*rpoB*) codon 516 (5′-CAG CTG AGC CAA TTC ATG GA-3′), F2 (*rpoB*) codon 526 (5′-CTG TCG GGG TTG ACC CA-3′), and F3 (*rpoB*) codon 531 (5′-CAC AAG CGC CGA CTG TC-3′) (13). In the absence of mutation in these codons of *rpoB* (516 or 526 or 531), wild-type-allele-specific fragments (218, 185, or 170 bp, respectively) were amplified (Figure 1) (14). Thus, when there was a mutation at the targeted codons, no PCR product was generated. Each MAS-PCR reaction was performed in 25 µl volume containing 5-10 ng of purified DNA sample, 1 mM $MgCl_2$, 1 u of *Taq* DNA polymerase (Cinagen, Iran), 2.5 µl PCR Buffer (10X), 100 µM dNTPs, 10 pmol in 1 µl of each primer. The reaction was carried out in an ABI PCR System (Applied Biosystems) under the following thermal conditions: initial denaturation at 95°C for 5 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and final elongation at 72°C for 10 min. The amplified fragments (5 µl) were analyzed using 1.5% agarose gel, stained with DNA Green Viewer, and then visualized *via* UV light (Figure 2).

The presence of the allele-specific PCR product bands (218, 185, or 170 bp) indicated the absence of mutations in the codons (516 or 526 or 531), thus, the lack of these specific bands demonstrated the mutation in the specific codons and the isolated sample was considered as resistant to rifampin (MDR-TB).

In this study, another PCR which amplified *rpoB* gene and validated the presence of this gene, was conducted to check all DNA samples and confirmed the results especially for the samples with three simultaneous mutations. This PCR was performed with another primer set including forward primer F (5′-TACGGTCGGCGAGCTGATCC-3′) and reverse primer R (5′- TACGGCGTTTCGATGAACC-3′) (15). The PCR reaction was carried out in 25 µl volume with the same situation as stated above, except for 1 mM $MgCl_2$ and 20 pmol in 1 µl of each primers. The reaction was carried out in an ABI PCR System (Applied Biosystems) under the following thermal conditions: initial denaturation at 95°C for 6 min; 36 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min; and final elongation at 72°C for 7 min. Electrophoresis was done in the same condition as MAS-PCR. The presence of PCR product band (411 bp) confirmed the presence of genomic DNA of *M. tuberculosis*.

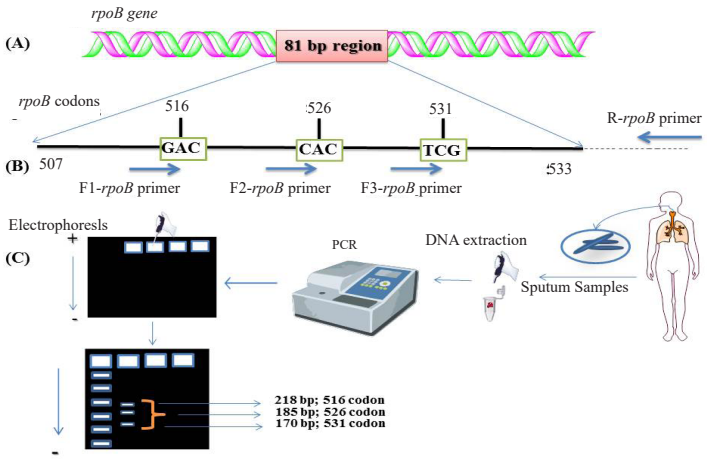

Figure 1. A schematic overview of *rpoB* gene and the way to identify hotspots for mutations by MAS-PCR. A: *rpoB* gene and 81-bp in the region; B: position of codons 516, 526 and 531 in the region of 81-bp and primers used to detect them; C: the steps of mutation detection including collecting patient sputum samples, extracting DNA from the sputum, doing MAS-PCR and then electrophoresis of the products of MAS-PCR; The bands of 170, 185 and 218 bp represent the absence of the mutation at the codons 531, 526 and 516.

rpoB: RNA polymerase subunit β gene; MAS-PCR: multiplex-allele-specific Polymerase Chain Reaction; bp: base pair

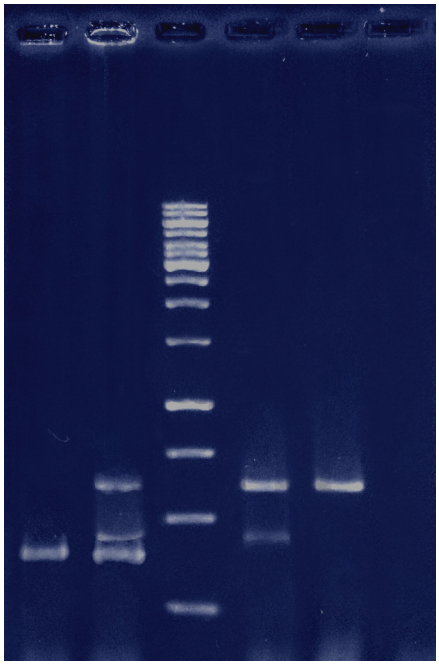

Figure 2. MAS-PCR products from clinical *M. tuberculosis* strains targeting three *rpoB* codons (lanes are presented left to right): Lane 1: mutaion in codon 526 (185 bp), and codon 516 (218 bp) ; Lane 2: no mutaion in codon 531 (170 bp), codon 526 (185 bp), and codon 516 (218 bp); Lane 3: DNA marker; Lane 4: mutaion in codon 531 (170 bp); Lane 5: mutaion in codon 531 (170 bp) and codon 526 (185 bp); and lane 6: mutaion in codon 531 (170 bp), codon 526 (185 bp), and codon 516 (218 bp). Products were analyzed using 1.5% agarose gel electrophoresis and visualized via UV light.

Results

Mutations in *rpoB* 516, *rpoB* 526, and *rpoB* 531 in DNA positive samples (88 samples) were recognized using MAS-PCR method. Using MAS-PCR, 51 rifampin-resistant (57.96%) and 37 susceptible (40.04%) isolates were detected. The mutation in codons 516, 526, and 531 were detected in 12 isolates (13.63%), 34 isolates (38.63%), and 49 isolates (55.68%), respectively (Table 2). The MAS-PCR results demonstrated that the mutation with high frequency at codon 531 (55.68%) was followed by codons 526 (38.63%) and 516 (13.63%). Amongst the isolates, 21 isolates showed simultaneous mutation in codons 526 and 531 (23.86%), one isolate in codons 526 and 516 (1.13%), and none isolate in codons 531 and 516. Moreover, 11 isolates (12.5%) showed simultaneous mutations in all 3 foregoing codons. The PCR products were analyzed on 1.5% agarose gel electrophoresis and visualized *via* UV light. The 218, 185 and 170 bp bands represented the wild-type codons 516, 526, and 531, respectively (Figure 2). Furthermore, the second PCR results confirmed that the genomic DNA of *M. tuberculosis* was presented in all of the samples.

Table 2. The mutation frequency at 3 different loci of the rpoB gene in rifampin-resistant *M. tuberculosis* isolated from North-East of Iran.

	Mutations		Samples		Total Percent	
No mutation	None		37		42.046%	
With Mutation	3 mutations	516+526+531	11	12.50%	12.50%	57.954%
	2 mutations	526+531	21	23.86%	24.996%	
		516+531	0	0.00%		
		516+526	1	1.136%		
	1 mutation	531	17	19.31%	20.446%	
		526	1	1.136%		
		516	0	0.00%		
Total			88		100%	

Discussion

Based on the previous reports, rifampin resistance (more than 95%) was caused by at least a mutation in the RRDR of β-subunit of RNA polymerase (*rpoB*) gene. This achievement showed that the mutations in codon 531 followed by codon 526 and codon 516 were the most frequent mutations in RRDR of the *rpoB* gene (15, 16)

Also, previous studies reported the codon 526 as the most common site of mutation in rifampin resistant cases (17). The results of the current study, conducted on 88 samples isolated from the TB patients of North-East of Iran, demonstrated that 57.96% of samples had *rpoB* gene mutations in the short 81-bps region with codons mutations 531, 526 and 516 which 20.44%, 24.99%, and 12.50% of these mutations were single-, double-, and triple-codon mutations, respectively. The most abundant mutation was related to mutation in codon 531 (55.68%). According to WHO statistics, 140 per 100,000 of people in the world annually, and also, based on the Ministry of Health Tuberculosis and Leprosy Centre Management of Iran, 14.4 per hundred thousand of people in Iran were infected with TB (18, 19). Based on the results of this study, there is a high prevalence of multidrug-resistant *Mycobacterium tuberculosis* in North-East of Iran. Recognizing MDR-TB will be helpful in determining drugs and timely treatment regimens as anti-tuberculous therapy. In particular, the rapid detection of resistance to rifampin leads to choose an effective treatment with appropriate antibiotics to inhibit further outbreaks of resistance infection and create MDR and extensively drug-resistant (XDR) strains. Thus, it needs simple, rapid, inexpensive, and genetics-based assays targeting *rpoB* mutations to detect RIF-resistant *M. tuberculosis*. Particular methods described for the detection of *rpoB* mutations associated with RIF-resistance were PCR-single-strand conformation polymorphism, direct DNA sequencing, real-time PCR with fluorimetry, heteroduplex mobility, dot spot, RNA-RNA mismatch, PCR-based denaturing gradient gel electrophoresis (DGGE), MAS-PCR and some other methods (19, 20).

MAS-PCR assay uses several pairs of primers, which are specific for different DNA fragments. It is performed in one assay to amplify multiple specific target sequences. MAS-PCR has several applications related to the clinical diagnosis including pathogen identification, pathogen differentiation without extra analysis, recognizing mixed infections, SNP genotyping, mutation analysis, and identifying the existence of pathogens (minor) which cover the lack of conventional sequencing methods (21). Furthermore, the main advantages of MAS-PCR (one tube) as compared to the conventional PCR (several tubes) are cost, preparation, and analysis time, which are reduced in MAS-PCR. On the other hand, MAS-PCR approximately eliminates the limitations of microbiological culture such as time, cost, contamination and identifying one pathogen (22). Thus, MAS-PCR assay is a rapid and inexpensive molecular technique for detection of *M. tuberculosis* resistance to anti-tuberculous drugs, especially RIF22 and 38 (20, 23). Resistance to RIF serves as a surrogate marker for MDR-TB of *M. tuberculosis*. Also, *rpoB* gene with a mutation in the 81-bps hot spot region (codons 516, 526, and 531) is mainly correlated with the RIF resistance (13, 24).

Some studies conducted in Iran, showed high levels of resistance to rifampin (25, 26). Investigating the prevalence of resistance to rifampin in Iran was studied *via* MAS-PCR technique (27). In our study, the prevalence of two and three point mutations was 37.49% (33.88) samples. This prevalence for other studies was different: two studies in India showed 19.66% (23.117) and 48.57% (28). A study in Thailand (29) observed 5.2% and another study in China (30) showed 12.4% prevalence.

In the present study, 55.68 % of isolates had mutation in codon 531 of *rpoB* gene which was similar to other studies including 53% (Brazil) (30), 59.83% (Northern India) (31), 58% (Thailand) (29), 60% (India) (32), 53% (Greece) (33), 59% (Italy) (34) and 59% (India) (35), and was higher than other studies including 41% (36) and two studies conducted in Iran including 40% (37) and 26% (38). Furthermore, some studies reported a higher mutation ratio including the

studies conducted in South Africa (70.5%), northern Lima (68%), and Iran 63.3% (39). The mutation in codon 526 was observed among 38.63% of isolates which was similar to the results of the studies conducted in Italy (30%) (34), Japan (33%) (40) and USA 36% (41). Also, the frequency of mutation at codon 526 was much higher than the studies conducted in Iran (16.6%) (42), China (4%) (40), Greece (19%) (33), Brazil (19%) (43) and India (23%, 26%) (32, 36). The frequency of mutation in the codon 516 was higher (22.22%) than that of reported in other studies such as South Africa and East Asian countries (32, 40).

Moreover, the results of the current study were in accordance with the studies conducted in East of Asia (29). The worldwide frequencies of mutations in codons 531 and 526 ranged from 29% to 74% and 0% to 43%, respectively (44). Thus, our reported frequencies were in accordance with those reported from other locations. Also, the results of this study showed that the most frequent mutations in RRDR of the *rpoB* gene were in accordance with codon 531 followed by codon 526 and codon 516, whereas, the study of Velayati *et* al. in Iran showed that the second frequent mutation was observed in codon 516 (10.8%) (39).

In conclusion, we showed that the frequency of the drug resistance to rifampin in North-East of Iran was high and besides, we illustrated the role of MAS-PCR procedure that speeds up the implementation of resistance assays that is inexpensive, easy to carry out and interpret, and needs routine standard PCR and agarose standard gel electrophoresis in healthcare centers. Therefore, quickly genotyping with this technique is suggested to be considered as an effective treatment for tuberculosis patients to consequently prevent the development of drug-resistant strains of Mycobacterium in this region.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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How to Cite This Article

Tajbakhsh A, Ghasemi F, Mirbagheri S, Momen Heravi M, Rezaee M, Meshkat Z. Investigation of the rpoB Mutations Causing Rifampin Resistance by Rapid Screening in Mycobacterium Tuberculosis in North-East of Iran. Iranian Journal of Pathology, 2018; 13(4): 429-437.