



AKADÉMIAI KIADÓ

Acta Microbiologica et  
Immunologica Hungarica

69 (2022) 2, 150–157

DOI:


10.1556/030.2022.01730

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## RESEARCH ARTICLE



# Identification of mutations in *rpoB*, *pncA*, *embB*, and *ubiA* genes among drug-resistant *Mycobacterium tuberculosis* clinical isolates from Iran

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Received: February 18, 2022 • Accepted: April 12, 2022

Published online: April 22, 2022

## ABSTRACT

*Mycobacterium tuberculosis* resistant to effective first-line drugs (FLDs) has challenged national and global tuberculosis control programs. This study aimed to identify mutations in 4 genes related to rifampin, pyrazinamide, and ethambutol resistance among clinical isolates of *M. tuberculosis* from southwestern Iran. After drug susceptibility testing of 6620 *M. tuberculosis* clinical isolates by proportional method, a total of 24 FLD-resistant strains were included in the study. Fragments of *rpoB*, *pncA*, *embB*, and *ubiA* genes were amplified and sequenced to mine the mutations by pairwise alignment with the corresponding *M. tuberculosis* H37Rv genes. Phenotypic resistance to rifampin, isoniazid, and ethambutol was detected in 67, 54, and 33% ( $n = 16, 13$ , and  $8$ ) of the isolates, respectively. Of rifampin-resistant isolates, 31% (5/16) were mono-resistant, and 56% (9/16) were multidrug-resistant (MDR). In 100% of rifampin-resistant isolates, mutations were found in the rifampin resistance-determining region (RRDR) of the *rpoB*, with S450L substitution being the most common, especially in MDRs (77.8%, 7/9). Resistance-conferring mutations in *pncA* were present in 12.5% (3/24) of FLD-resistant isolates. The *embB* and *ubiA* mutations were found in 62.5 and 12.5% (5/8 and 1/8) of ethambutol-resistant isolates, respectively, of which the *embB* D354A was the most common substitution (37.5%, 3/8). Sixteen distinct mutations were identified, one of which was novel. The sequence analysis of the RRDR segment was the best way to detect rifampin resistance. The *rpoB* S450L substitution could be a helpful molecular marker to predict MDR. In other genes, no mutation was identified as a reliable marker.

## KEYWORDS

*Mycobacterium tuberculosis*, drug resistance, mutations, *rpoB*, *pncA*, *embB*, *ubiA*

## INTRODUCTION

Tuberculosis (TB) is an ancient disease, yet it is still a significant public health problem, with 10.4 million new cases and 1.4 million deaths annually [1]. There was a rapid decline in the global incidence and mortality of TB after discovering and introducing effective anti-TB drugs, especially isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA) – known as first-line drugs (FLDs) [2]. However, TB returned to the scene, one of the main reasons being the emergence of drug-resistant forms during the last two decades of the 20th century [3].

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In contrast to most other bacteria, horizontal gene transfer by mobile genetic elements has not been reported as a way of acquiring antibiotic resistance in *Mycobacterium tuberculosis*, the causative agent of TB [4]. Instead, the most common principle for antibiotic resistance among *M. tuberculosis* strains is the acquisition of chromosomal mutations under the selective pressure of antibiotic use [5]. Chromosomal mutations mediate drug resistance in *M. tuberculosis* through several distinct mechanisms, including altering drug targets, disabling prodrug activation, and overexpression of drug targets [6].

Drug-resistant TB is a major worldwide health challenge since it needs treating the patients with second-line drugs with less effectiveness, more cost, more toxicity, and low tolerability [7]. Several conventional drug susceptibility testing (DST) methods are available to avoid treatment failure and prevent drug resistance dissemination. However, despite being sensitive and specific, they are time-consuming, which causes a delay in the timely initiation of the treatment regimen. Accordingly, nowadays, rapid and reliable molecular methods for detecting resistance are developed, which require accurate knowledge of genetic mutations related to drug resistance [8].

RIF, a semi-synthetic bactericidal indicated for all forms of TB, reduced the course of treatment from 2 years to 9 months when combined with INH [9]. Slightly less than 5% of global TB cases in 2019 were RIF-resistant (RIF<sup>r</sup>), of which 78% had multidrug-resistance (MDR), characterized by simultaneous resistance to INH and RIF [10]. More than 95% of RIF resistance in *M. tuberculosis* is caused by mutations in an 81-bp fragment of the *rpoB* gene called “rifampin resistance-determining region” or RRDR [11]. The presence of individual mutations in this finite fragment of a particular gene, which is strongly associated with RIF resistance, has paved the way for molecular diagnosis.

PZA, a synthetic analog of nicotinamide, can eradicate semi-dormant *M. tuberculosis* bacilli that are not killed by other anti-TB drugs [11]. This effective sterilizing property allowed the duration of the combined anti-TB treatment regimens to be shortened to 6 months and the recurrence rate to be reduced [12]. The annual prevalence of PZA resistance worldwide is estimated at 16%, amounting to 1.4 million new TB cases [13]. Mutations in three genes have been suggested as the causative agent of PZA resistance in *M. tuberculosis*, but 70–97% of resistant isolates have mutations in the *pncA* gene [11, 14].

EMB is a bacteriostatic agent that acts on replicating bacilli. In the intensive phase of treatment, EMB is used as a companion to other anti-TB drugs to prevent or delay the onset of resistance, especially to RIF [9, 15]. About 4% of *M. tuberculosis* clinical isolates are resistant to EMB [16, 17], and up to 70% of EMB-resistant (EMB<sup>r</sup>) isolates carry mutations in the *embB* gene. The absence of *embB* mutations in other resistants and, on the other hand, its observation in a proportion of susceptible isolates raises the possibility of other mechanisms involved [11]. One of these mechanisms is additional mutations in the *ubiA* gene, which varies in different geographical areas [18].

According to being an endemic region for TB, in the present study, we aimed to investigate the mutations in 4 genes (*rpoB*, *embB*, *ubiA*, and *pncA*) related to resistance to RIF, EMB, and PZA in *M. tuberculosis* isolates obtained from TB patients in Khuzestan province, southwestern Iran.

## MATERIALS AND METHODS

### Ethical approval

The present study was approved by The Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1399.183), and permission was granted for sample collection. The written informed consent was taken from all patients whose samples were used in this study.

### Sample collection and bacterial strains

During seven years from 2014 to 2021, a total of 6,620 detected *M. tuberculosis* isolates from the different counties of Khuzestan province were sent to the Regional TB Reference Laboratory for confirmation and DST. These isolates had been detected by direct microscopy in the sputum sample of patients with suspected pulmonary TB. Isolates were identified as *M. tuberculosis* using acid-fast staining, culture on Löwenstein–Jensen (LJ) medium, and biochemical tests, including niacin accumulation, catalase at 37 °C and 68 °C, and nitrate reduction [19]. The *M. tuberculosis* H37Rv (ATCC<sup>®</sup> 27294) strain was used as the control.

### Drug susceptibility testing (DST)

The DST for FLDs was performed according to the World Health Organization (WHO) recommendations [20]. The critical concentrations of RIF, INH, and EMB (Sigma-Aldrich, Germany), were 40, 0.2, and 2 µg ml<sup>-1</sup>, respectively. If the ratio of colony count on the medium containing an anti-TB drug to the number of colonies on the drug-free control medium was equal to or greater than 1%, the strain was determined as resistant. Resistance to only one FLD was known as mono-resistance. The MDR phenotype was defined as simultaneous resistance to RIF and INH. The isolates resistant to more than one FLD, other than MDRs, were categorized as poly-resistant [21]. All *M. tuberculosis* strains resistant to any FLDs and 4 susceptible isolates were included in the study. The latter strains were selected using the random numbers from the pan-susceptible isolates received in the last 2 months of the study.

### DNA extraction

Genomic DNA was extracted from the isolates using the simple boiling method as described earlier, with slight modification [19]. In brief, a few fresh bacterial colonies were harvested from the LJ culture medium and were dissolved in TE (Tris-EDTA) buffer [pH = 8.0] and boiled twice for 15 min at 100 °C, with a cooling interval of –21 °C



for 5 min. After 5 min of centrifugation at 13,000 rpm, the supernatant was used as a DNA template for polymerase chain reaction (PCR) amplification.

### PCR amplification and sequencing

The *M. tuberculosis* isolates were molecularly confirmed using PCR based on the *IS6110* gene. Fragments of four genes (*rpoB*, *embB*, *ubiA*, and *pncA*) were amplified to investigate resistance to RIF, EMB, and PZA. The oligonucleotide primers used for PCR amplification have been described in previous studies and are shown in Table 1.

The PCR reaction mixtures were prepared with the components as follows: 12.5 µl of *Taq* DNA Polymerase 2x Master Mix (AMPLIQON, Denmark), 1 µl of the DNA template (65 ng µl<sup>-1</sup>) except for *IS6110* and *pncA*, which were 5 µl, 10 pmol of each primer except *pncA* and *embB* which were 5 pmol, and double-distilled water up to a final volume of 25 µl. The final concentration of MgCl<sub>2</sub> in each mixture was 1.5 mM.

DNA amplification was done in a Bio-Rad thermal cycler (USA) with the following cycling program: initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at variable temperatures for 30 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The optimal annealing temperatures for each primer set are listed in Table 1.

The PCR products were sent for sequencing after electrophoresis on 1.5% agarose gel for 60 min. Bidirectional sequencing was performed in the Cardiogenetic Research Center (Tehran, Iran) laboratory using the amplification primers.

### Genotyping

The genotypes of resistant *M. tuberculosis* isolates were determined by performing a manual 24-locus MIRU-VNTR typing as described earlier [27] and applying the MIRU-VNTRplus web application [28].

### Data analysis

The BLAST algorithm (available at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was employed to analyze the sequences: blastn and blastx for nucleotide and translated nucleotide

sequences, respectively [29, 30]. The sequences were aligned with the corresponding wild gene sequences in the *M. tuberculosis* H37Rv genome, available in the GenBank (RefSeq sequence: NC\_000962.3). Any nucleotide change compared to the reference wild-type sequence was defined as a mutation. To determine whether the mutations found were resistance-conferring, a search in the Comprehensive Antibiotic Resistance Database, available at <https://card.mcmaster.ca> [31], and the TB mutations catalog [32] was performed. Chi-square (or Fisher's exact) test was used to check the relationship or independence of the two qualitative variables. In statistical tests, the *P*-value less than 0.05 was considered significant.

## RESULTS

In the present study, 24 *M. tuberculosis* isolates with resistance to FLDs were detected using conventional DST and molecular techniques. In Table 2, the resistance profile of the isolates according to their genotypic sub-lineages is presented. Single or combined resistance to INH, RIF, and EMB was detected in 54.2% (*n* = 13), 66.7% (*n* = 16), and 33.3% (*n* = 8) of isolates, respectively. MDR isolates accounted for

Table 2. Resistance profile and genotypic sub-lineage of 24 drug-resistant *M. tuberculosis* clinical isolates

Resistance profiles	Sub-lineages				Total
	Delhi/CAS	NEW-1	EAI	Others <sup>a</sup>	
INH	1	1	0	1	3
RIF	2	3	0	0	5
EMB	2	2	0	0	4
INH + EMB	0	0	0	1	1
RIF + EMB	1	0	1	0	2
INH + RIF	4	0	1	3	8
INH + RIF + EMB	0	0	0	1	1
Total	10	6	2	6	24

Abbreviations: INH, isoniazid; RIF, rifampin; EMB, ethambutol; Delhi/CAS, Delhi/Central Asian; EAI, East-African-Indian.

<sup>a</sup> Included one LAM (Latin American-Mediterranean) and five unknown isolates.

Table 1. Oligonucleotide primers used in gene amplification in present study

Gene	Primer sequence	Product size	Reference	Annealing temperature
<i>rpoB</i>	F: 5'-TACGGTCGGCGAGCTGATCC-3' R: 5'-TACGGCGTTTCGATGAACC-3'	411 bp	[22]	62 °C
<i>embB</i>	F: 5'-CGACGCCGTGGTGATATTCG-3' R: 5'-CCACGCTGGGAATTCGCTTG-3'	863 bp	[23]	61.5 °C
<i>ubiA</i>	F: 5'-ACGTTGAGCTTGAGGCTAGC-3' R: 5'-CGCTGTCGCGAATACTGCT-3'	909 bp	[24]	61.6 °C
<i>pncA</i>	F: 5'-CGGATTTGTCGCTCACTACA-3' R: 5'-TCCGCCGCCGAACAGTTTCATCCCGGT-3'	756 bp	[25]	62 °C
<i>IS6110</i>	F: 5'-CTCGTCCAGCGCCGCTTCGG-3' R: 5'-CCTGCGAGCGTAGGCGTTCGG-3'	130 bp	[26]	63 °C



37.5% (9/24) of all resistant and 56% (9/16) of RIF<sup>r</sup> isolates. Among them, one isolate was resistant to EMB in addition to INH and RIF. Mono-resistance and poly-resistance were observed in 50% (12/24) and 12.5% (3/24) of isolates, respectively. RIF mono-resistance was revealed as 31.3% (5/16) of all RIF<sup>r</sup> cases. Statistically significant, all isolates belonging to the NEW-1 sub-lineage were mono-resistant ( $P$ -value = 0.0137). There was no significant relationship between MDR isolates and Delhi/CAS sub-lineage ( $P$ -value = 0.5582).

### Mutations in the *rpoB* gene

Mutations in this gene were observed exclusively in 16 RIF<sup>r</sup> isolates and only within RRDR. Thus, the association between RRDR mutation and RIF resistance was significant ( $P$ -value < 0.00001). Six non-synonymous substitutions (in 4 codons) were identified (Table 3). The most common substitution, S450L, was found in 50% (8/16) of RIF<sup>r</sup> isolates, including 77.8% (7/9) of MDR isolates. There was a significant relationship between this mutation and the MDR pattern ( $P$ -value = 0.0406). Three different nucleotide changes caused the amino acids tyrosine, proline, and asparagine to replace histidine at codon 445. These 3 mutations were detected separately in 25% (4/16) of isolates, and there was no significant relationship between them and resistance patterns. Each L452P and L430P substitution was

detected in 12.5% (2/16) of isolates. These 2 mutations did not occur in any MDR isolates; Nevertheless, this difference was insignificant ( $P$ -value = 0.175).

### Mutations in the *pncA* gene

Of drug-resistant isolates, 54.2% (13/24) had mutations in this gene related to PZA resistance. One point mutation (367 C>T), which led to the synonymous substitution of S65S, was observed in 41.7% (10/24) of drug-resistant and 75% (3/4) of susceptible isolates. Four non-synonymous substitutions were detected in 12.5% (3/24) of FLD-resistant isolates but not susceptible isolates. These 3 isolates were 2 RIF mono-resistant strains and one MDR, but there was no significant relationship between a mutation in *pncA* and RIF resistance ( $P$ -value = 0.5257). Non-synonymous substitutions of V7L and T135P occurred in one isolate, and D8N and T100P were separately in the other 2 isolates. The last 3 isolates did not have S65S mutation.

### Mutations in the *embB* gene

Among EMB<sup>r</sup> and ethambutol-sensitive (EMB<sup>s</sup>) isolates, 62.5% (5/8) and 5% (1/20) had mutations, respectively, a significant difference ( $P$ -value = 0.0008). Four non-synonymous substitutions were identified (Table 4). The most common was D345A in 37.5% (3/8) of EMB<sup>r</sup> isolates, one

Table 3. Detected mutations in the *rpoB* gene of RIF-resistant *M. tuberculosis* clinical isolates ( $n = 16$ )

Mutations			No. of Mono-resistant	No. of Multidrug-resistant	No. of Poly-resistant	Total
Nucleotide	Amino acid <sup>a</sup>	<i>E.coli</i> equivalent <sup>b</sup>				
1209 T>C	L430P	L511P	1	0	1	2
1333 C>T	H445Y	H526Y	1	0	0	1
1334 A>C	H445P	H526P	1	1	0	2
1333 C>A	H445N	H526N	0	1	0	1
1349 C>T	S450L	S531L	0	7*	1	8
1355 T>C	L452P	L533P	2	0	0	2

<sup>a</sup> Mutations are shown as single-letter designations for amino acids.

<sup>b</sup> Based on the *Escherichia coli* sequence annotation.

\* Statistically significant.

Table 4. Mutations in *embB* and *ubiA* genes in ethambutol-resistant ( $n = 8$ ) and ethambutol-susceptible ( $n = 20$ ) isolates of *M. tuberculosis*

Resistance phenotype	No. of isolates	<i>embB</i>		<i>ubiA</i>	
		Nucleotide	Amino acid <sup>a</sup>	Nucleotide	Amino acid
Ethambutol-resistant	1	916 A>G	M306V	WT	WT
	1	931 G>C	D311H	463 A>C	E149D
	3	1061 A>C	D354A*	682 T>C	R76R
	3	WT	WT	WT	WT
Ethambutol-sensitive	1	WT	WT	463 A>C	E149D
	1	1036 G>C	A346P	682 T>C	R76R
	18	WT	WT	WT	WT

Abbreviation: WT, wild-type.

<sup>a</sup> Mutations are shown as single-letter designations for amino acids.

\* Statistically significant.





mono-resistant, and two poly-resistants. The association of this mutation with EMB resistance was significant ( $P$ -value = 0.0171). M306V and D311H substitutions were detected in one MDR and one poly-resistant, respectively. Non-synonymous substitution A346P was identified only in a pan-susceptible isolate.

### Mutations in the *ubiA* gene

The mutation was detected in 12.5% (1/8) of EMB<sup>r</sup> isolates and 5% (1/20) of EMB<sup>s</sup> isolates (Table 4). There was no significant association between mutations in *ubiA* and EMB resistance ( $P$ -value = 0.4863). A non-synonymous (E149D) and a synonymous (R76R) substitution coexisted in an EMB<sup>r</sup> and an EMB<sup>s</sup> isolate; both belonged to the EAI sub-lineage. The mentioned mutations had a significant relationship with the sub-lineage ( $P$ -value = 0.0152). The EMB<sup>r</sup> isolates with D354A and M306V substitutions in *embB* had no mutations in *ubiA*.

## DISCUSSION

In 2020, the incidence rate of TB reported in Iran was 6.74 cases per 100,000 population. With an incidence of 9.72 cases per 100,000 people, Khuzestan ranks fifth among the provinces of Iran [33]. This border province, which has a population of more than 4.5 million, is faced with a considerable volume of domestic and foreign traffic for commercial, tourism, and pilgrimage purposes annually [34]. Therefore, TB control is essential for local health officials. This study sought to find and describe mutations in 24 clinical isolates of drug-resistant *M. tuberculosis* in Khuzestan, Iran, by exploring sequences of 4 genes associated with resistance to the 3 FLDs. Sixteen distinct mutations were identified, one of which was novel.

RIF mono-resistance was considered a rare phenomenon until about two decades ago, and RIF resistance was practically accepted as a proxy for MDR [35]. However, some evidence suggests an increasing prevalence of RIF mono-resistance in recent years [36]. According to the WHO report in 2020, of the 470,000 RIF-resistant TB cases worldwide, 23.4% were not MDR [10]. In a systematic review study, 33.3% of RIF<sup>r</sup> isolates from new TB cases in Iran were not resistant to INH [37], which is very close to the finding in our study (31.3%). This significant proportion of mono-resistance among RIF<sup>r</sup> isolates suggests the need to develop guidelines and treatment recommendations separate from MDR for this type of drug-resistant TB [36]. RIF exerts its bactericidal effect by binding to the  $\beta$ -subunit of DNA-dependent RNA polymerase – encoded by *rpoB* – and inhibiting mRNA elongation. For this reason, 95% of RIF<sup>r</sup> isolates carry mutations in this gene [11]. The 81-base pair RRDR segment, located between codons 426 and 452 of the *rpoB* gene of *M. tuberculosis* (equivalent to 507 to 533 in *Escherichia coli*), encodes the enzyme's active site. Its mutations confer resistance by a mechanism of drug target alteration [6]. All mutations found in RIF<sup>r</sup> isolates were within the RRDR

segment. Therefore, analysis of this fragment could predict RIF resistance with 100% sensitivity, specificity, and accuracy. Although the proportion of RRDR mutations in RIF<sup>r</sup> isolates is significantly higher in most studies, some have reported mutations outside the segment, including a previous study from Iran [38].

The most common mutated codons in *rpoB* were 450 and 445, comparable to most studies from other countries [39] and consistent with other parts of Iran [38]. Mutation at codon 450 has the lowest fitness cost, making it the most common mutation in RIF<sup>r</sup> isolates worldwide, especially MDR isolates [5]. In this study, S450L substitution was dominant among all *rpoB* mutations (50%) and had absolute superiority in MDR isolates (77.8%). The frequency of this mutation was higher than the results of studies in Taiwan (66.7%) [40] and Pakistan (58%) [41], and its relationship with the MDR resistance pattern was statistically significant. Thus, it may be suggested as a molecular marker of MDR isolates with 77.8% sensitivity, 93.3% specificity, and 87.5% accuracy. Mutations in codons 452 and 430 have also been reported in previous domestic and foreign studies – albeit at a lower frequency [38, 40–43]. The mutations identified in codons 450, 445, 430, and 452 are all resistance-conferring [31, 32], and substitutions S450L and H445Y are associated with high-level resistance [44]. Unlike most studies, we did not find any mutations in codon 435.

PZA is a prodrug that, after entering the bacterial cell, requires the enzyme pyrazinamidase, encoded by *pncA*, to convert it to the active form – pyrazinoic acid [5]. Therefore, mutations in this gene may lead to PZA resistance by disrupting prodrug activation [6]. More than 600 unique mutations have been reported in approximately 400 positions scattered throughout the length of the *pncA* gene and its flanking regions [13]. This incredible variety of mutations indicates that the enzyme pyrazinamidase is not essential for the growth and survival of *M. tuberculosis* [6]. In this study, the proportion of FLD-resistant isolates with *pncA* mutation was higher than in Vietnam with a similar setting (54.2 vs. 46.8%) [12]. The difference in sample size and the presence of a relatively high frequent mutation common between resistant and susceptible isolates may explain the discrepancy.

PZA resistance is not assayed in routine DST because it is technically challenging and inaccurate [45]. Newly recommended methods, such as MGIT 960, have had high rates of false-positive results, and many countries lack the resources to apply them [12, 45]. For this reason, the molecular diagnosis of resistance to PZA has been proposed as the reference method [20]. Synonymous substitution of S65S, recently reported in PZA-resistant and -sensitive isolates [46, 47], is a single-nucleotide polymorphism that, as a rule, does not confer resistance. Regardless of this silent mutation, only 12.5% of FLD-resistant isolates had distinct mutations in the *pncA* gene. The 4 non-synonymous substitutions found in the 3 isolates were previously reported and accepted as resistance-conferring [32, 48, 49]. Considering the 84% frequency of *pncA* mutations in PZA-resistant isolates, reported in a recent study in Iran [47], the maximum number



of PZA-resistant isolates among the isolates under study was 4. Thus, the prevalence of PZA resistance in FLD-resistant isolates was calculated to be 16.7% (4/24). Contrary to reports of a high prevalence of PZA resistance, especially among MDR isolates [47–49], the relatively low rate in the studied region can be promising for the favorable effect of PZA-containing regimens on resistant isolates, including MDR.

The *embCAB* operon encodes membrane-associated arabinosyl transferase enzymes that polymerize arabinose into arabinan [24]. EMB, an analog of arabinose, binds to these enzymes to inhibit the biosynthesis of arabinogalactan and arabinomannan, essential components of the mycobacterial cell wall [50]. Mutations in the *embB* gene can cause EMB resistance by a mechanism of drug target alteration [6]. In previous studies, the *embB* mutation rates in EMB<sup>r</sup> isolates varied widely, from 10 [50] to 100% [23]. In this study, 62.5% of the EMB<sup>r</sup> isolates mutated in *embB*, closer to Bakula et al.'s finding, which focused only on MDR isolates [17]. The most common *embB* mutation led to the D354A substitution, which was also significantly associated with EMB resistance. This substitution was previously reported as a unique mutation in EMB<sup>r</sup> isolates [8] and is now known as resistance-conferring [31, 32]. In most earlier studies, mutations in codon 306, known as the EMB resistance-determining region (ERDR), were overwhelming [8, 17, 23, 51, 52] and suggested as markers of EMB resistance. The frequency of M306V substitution in EMB<sup>r</sup> isolates was equal to that of the D311H substitution reported only in a relatively old study [53]. The absence of *embB* mutations in some EMB<sup>r</sup> isolates has more or less been reported in previous studies [17]. Such isolates may have resistance-conferring mutations in other loci of the operon. Nevertheless, other studies also found discord between phenotypic resistance assays and genotypic changes in the *embCAB* operon [50]. *EmbB* mutations were also detected in 5% of phenotypically EMB<sup>s</sup> isolates which varied from 14.3 [54] to 45% [8] in previous studies. Earlier studies did not report the A346P substitution in EMB<sup>s</sup> isolates.

The enzyme encoded by the *ubiA* (Rv3806c) gene is involved in the biosynthesis pathway of DPA (decaprenylphosphoryl- $\beta$ -D-arabinose), which donates arabinose to the enzyme arabinosyl transferase. This enzyme, vital for the growth and survival of *M. tuberculosis*, is not an EMB target; however, mutations in *ubiA* may increase DPA levels that compete with EMB for binding to arabinosyl transferase [24]. It was shown that *ubiA* mutations multiply the effect of *embB* mutations on EMB MIC values and increase resistance levels [55]. The prevalence of *ubiA* mutations in EMB<sup>r</sup> isolates varied in different geographical areas, and they were more common in African isolates than in Asian ones (52 vs. 11%) [18]. In a recent study from Thailand, about 9% of EMB<sup>r</sup> isolates mutated in the *ubiA* [24]. The mutation rate of this gene in EMB<sup>r</sup> isolates under the present study was 12.5%, closer to Asian isolates. R76R and E149D substitutions, in an EMB<sup>r</sup> and an EMB<sup>s</sup> isolate belonging to the EAI sub-lineage, are phylogenetic markers and have no role in resistance [24, 52].

An important limitation of the present study was the small sample size. Nevertheless, from another perspective,

enrolment of the resistant isolates identified in the last few years in a region with a population of 4.5 million may be a strength.

In conclusion, This study characterized mutations in the *rpoB*, *pncA*, *embB*, and *ubiA* genes – related to RIF, PZA, and EMB resistance, respectively – in 24 clinical isolates of *M. tuberculosis* with resistance to FLDs in southwestern Iran. RIF mono-resistance was not uncommon, and in the region studied, RIF resistance can not be considered a surrogate marker for MDR. Sequencing the RRDR fragment of *rpoB* was the best way to detect RIF resistance. The *rpoB* S450L mutation was a predictive molecular marker for the MDR pattern. Given the relatively low prevalence of PZA resistance in FLD-resistant isolates in this region, it can be more confidently incorporated into treatment regimens for drug-resistant TB. Due to the discordance between phenotype and genotype, neither of the mutations of *embB* and *ubiA* genes would be considered as a reliable molecular marker to predict EMB resistance.

**Conflict of interest:** The authors declare that there is no conflict of interest.

## ACKNOWLEDGMENT

This work was financially supported by the Ahvaz Jundishapur University of Medical Sciences (AJUMS), Ahvaz, Iran (grant No: OG-9903). It was conducted at the Infectious and Tropical Diseases Research Center of AJUMS. We sincerely appreciate the staff of the Regional Tuberculosis Reference Laboratory of Khuzestan province for providing the isolates, the staff of the Infectious and Tropical Diseases Research Center, and the Department of Microbiology to provide the necessary equipment and an appropriate atmosphere for experiments.

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