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The relationship between *emb*b306 and *emb*b406 mutations and ethambutol resistant in *Mycobacterium tuberculosis* isolated from patiens in west of Iran

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Abstract

Background: Mutations in *emb*B gene have been reported in ethambutol (EMB) resistance *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates. The aim of this study was survey on *emb*B 306 and 406 EMB resistant *M. tuberculosis* isolated from patients in West of Iran (2014-2015).

Methods: Fifty strains of *M. tuberculosis* from patients with pulmonary tuberculosis (TB) were considered. Drug susceptibility using proportional method was performed. Polymerase chain reaction (PCR) -DNA sequencing was applied for mutation in *emb*B 306 and 406 codon detection. Data were analyzed in SPSS 16 software using descriptive statistics and Fisher's exact test ($p<0.05$).

Results: In this study 7 (14%) *M. tuberculosis* isolates were resistant to EMB. 6 (85.71%) and 1 (14.28%) resistant isolates had *emb*B 306 and 304 codon mutations, respectively. Between *emb*B306 mutations and resistance to EMB and MDR isolates had a significant relationship ($p<0.001$).

Conclusion: The data indicated that *emb*B 306 mutations have effect on EMB resistant. Detection of EMB resistant and these mutations prominent for antibiotic prescription.

Keywords: *emb*B gene, Mutations, Ethambutol Resistance, *Mycobacterium tuberculosis*

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Introduction

Ethambutol (EMB) is one of the first-line drugs that used in the treatment of tuberculosis (TB). Mutations in the *emb*B gene codons have effects on the resistance to EMB. Mutations in *emb*B gene, especially those in the EMB resistance-determining region (ERDR), are considered as "hot-spots". These mutations in different *emb*B gene codon have been frequently reported in EMB-resistant *Mycobacterium tuberculosis* (*M. tuberculosis*)isolates (1, 2). In many studies, substitution in different codon of *emb*B gene has been considered as the main position for EMB resistance. BakuBa et al. (2013) in Poland using sequence analysis and polymerase chain reaction (PCR) reported in 17 EMB resistant *M. tuberculosis* isolates, mutations in codon 306 were most common and found in 9 (52.94%) EMB-resistant isolates (2). Bahrami et al. (2013) using multiplex allele-specific (MAS)-PCR from 176 *M. tuberculosis* isolates, 48 isolates were found to be resistant to EMB and in the 14 EMB resistant cases,

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↑What is "already known" in this topic:
There are considerations on relationship between mutations that was occurred in *emb*b306 and *emb*b406 genes that lead to ethambutol and MDR resistant in *Mycobacterium tuberculosis* isolated from patients with TB.

→What this article adds:
This study, proved occurrence of *emb*B306 mutations in EMB-resistant *M. tuberculosis* isolates. A screening using molecular tools in laboratory for EMB-resistant and MDR *M. tuberculosis* that isolated from patients with TB is necessary before drug prescription.

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mutation was found third base of the codon 306 ATG (3). Also Cuevas-Cordoba et al. (2015) in Mexico by sequence analysis and PCR reported that mutation at *emb*B codon 306 in resistance *M. tuberculosis* associated with EMB was related with resistant (4). It is very important to evaluate the prevalence of specific mutations associated with resistance to EMB. The aim of this study was to evaluate the frequency of point mutations (hot spots) in *emb*B gene among *M. tuberculosis* isolates in the West of Iran during 2014 to 2015.

Methods

This cross-sectional survey was done on smear-positive of patients that referred to TB research center in Kermanshah city, Kermanshah Province, west of Iran, during 2014-2015 years.

Sampling

Out of the 1069 strains of *M. tuberculosis*, 50 strains from patients with pulmonary TB (22 men and 28 women with the age range of 23-86 and a median age of 54.5 years) were included in this study and evaluated. At first decontamination method (Modified Petroff) was performed on the samples and then they were stained using the Ziehl-Nelson staining method for observation. In other hand, samples were cultured on Lowenstein-Jensen agar media (Merck, Germany) and incubated for four weeks at 37°C. Colonies were tested with biochemical tests as catalase, nitrate, niacin, pigment production, and colony growth rate.

Drug susceptibility test

Drug susceptibility testing of *M. tuberculosis* (the proportional method) was done according to the standards of mycobacteriological procedures for isoniazid (INH) (0.2 µg/ml), rifampin (RIF) (40 µg/ml), streptomycin (STR) (4 µg/ml), and EMB (2 µg/ml). The H37RV reference strain of *M. tuberculosis* was served as quality control for EMB susceptibility testing (5).

Polymerase Chain Reaction (PCR) technique

Bacteria that were cultured on Lowenstein-Jensen media were deactivated in the temperature 80°C for one hour. Then DNAs were extracted with the cetyl-trimethyl ammonium bromide (CTAB) method. For EMB-resistant and susceptible isolates, an 863-bp fragment from the ERDR of the *embB* gene was PCR-amplified. The oligonucleotide primers *emb*B F (5′-CGACGCCGTGGTGA TATTCG-3′) and *emb*B R (5′-CCACGCTGGGAATTC GCTTG-3′) were used as described previously (2).

Sequencing

For all the samples (50; 100%) that had 863bp band in the electrophoresis, nucleotide sequencing was performed for *emb*B 306 and 406 codon mutations detection automatically (by Macro Gene, a Korean company). The sequence data were analyzed with the Chromas Pro ver. 1.7.1 software program. The nucleotide sequences were translated to amino acids using the European Bioinformatics Institute (EMBL-EBI) website (http://www.ebi.ac.uk/Tools/psa/emboss_needle/index.html). The existence of mutations was determined by comparing the achieved sequences with the *M. tuberculosis* reference strain H37Rv (ATCC 27294) sequence of *emb*B from the GenBank database (www.ncbi.nlm. nih.gov/genbank/) using the BLASTn algorithm (blast.ncbi.nlm.nih.gov/).

Statically method

Data were analyzed using SPSS 16 software. Descriptive statistics to determine the frequency and percentage and also Fisher's exact test to compare qualitative findings were used ($p<0.05$).

Results

Drug susceptibility test result

Drug susceptibility proportional method results showed that of 50 *M. tuberculosis*isolates, only 17 (86%) strains were sensitive to all drugs, 8 (16%) of strains were multidrug resistant (MDR) and 25 (50%) were non-MDR. Also 7 (14%) and 43 (86%) *M. tuberculosis*isolates were resistant and suspect to EMB, respectively. 5 (71.42%) EMB-resistant (100%) *M. tuberculosis* isolates were in MDR and 2 (28.57%) were in non-MDR group. From 43 nonresistant EMB isolates, 17 (39.53%) were found in sensitive to all drugs group and 23 (53.48%) were in non-MDR group and 3 (6.97%) samples in MDR group.

Polymerase Chain Reaction (PCR) technique test

PCR results confirmed *emb*B gene for all 50 samples (Fig. 1).

Sequencing results

Also PCR-DNA sequencing for*emb*B 306 and 406 codon showed that in EMB susceptible strains, mutations at the *emb*B 306 codon were not detected. But *emb*B 306 codon point mutation in 6 (85.71%) and *emb*B 406 codon point mutation in 1 (14.28%) of EMB resistant isolates were found (Table 1, Fig. 2).

Statistical results showed that *emb*B306 mutations and resistance to EMB and MDR had a direct relationship with each other ($p<0.001$).

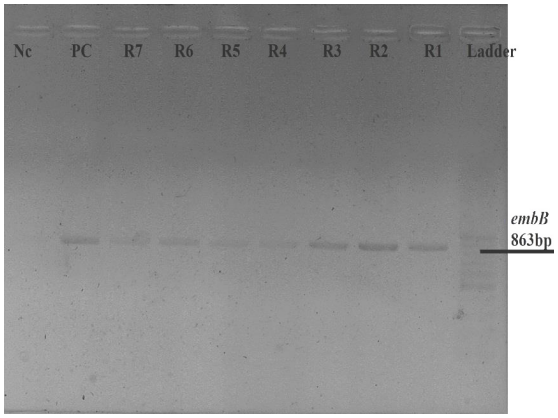


Fig. 1. Amplified Fragments of *emb*B Gene with Electrophoresis of PCR products in *M. tuberculosis* isolates;Line 1, Ladder (100-1500bp); Line 2-8, *emb*B gene in EMB resistance samples (863bp); Line 9, Positive control; Line 10, Negative control.

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*Table 1.*Point mutation in EMB-resistant and EMB-susceptible M. tuberculosis isolates

Nucleotide	Mutation* Aminoacid	No. (%) of isolates mutation EMB-resistant (n=7)	EMB-susceptible (n=43)
ATG → ATA	Met→IL (306)	3 (42.85)	0 (0)
ATG → GTG	Met→Val (306)	3 (42.85)	0 (0)
GGC → AGC	Gly→Ser (406)	1 (14.28)	0 (0)



*Fig. 2.*Amino acid sequence compared to H37RV in (EMBL-EBI) with nBLAST algorithms, mutation amino acid isoleucine (I) instead of methionine (M) at codon 306

Discussion

EMB resistance is a serious problem in many countries, so early detection of EMB resistance is very important to avoid of resistant strains spread (6). In our study, 86% of strains were sensitive to all antibiotics that were applied. But 16% of strains were MDR, also 50% were non-MDR. In a study in 2015 in Iran, Tavanaee Sani et al. using standard proportional method showed MDR-TB in 56 new cases (1.78%) and 26 (11.53%) patients with relapse during 2012-2013 that it was less than our study. Different rate of resistant can be due to obtaining sample from restricted regions with low or high rate of resistance. For example high rate of resistance can be observed in neighboring of region such as Afghanistan and Pakistan as the most prevalent area for TB (7). In our results, 14% of *M. tuberculosis* isolates in our results were related with EMB resistant. In other hand PCR-DNA sequencing proved that in all EMB resistant isolates point mutation were occurred. *emb*B 306 codon mutations in 85.71% and *emb*B 406 codon mutation in 14.28% of EMB resistant isolates were found. Nasr Esfahani et al. in Iran in 2016 using standard proportional method reported from 32 *M. tuberculosis* isolates, 6.25% were resistant to EMB. PCR- Single-strand conformation polymorphism (SSCP) and direct sequencing in Nasr Esfahani Esfahani study detected 2 EMB resistant isolates had mutation in codons 309 and 299 (8). Prevalence of EMB resistant isolates with mutation in our work is higher than Nasr Esfahani study. *M. tuberculosis* has different mechanisms to ovoid killed by drugs. One of these mechanisms is mutations in *emb*B gene. This gene encodes an arabinosyltransferase that is involved in cell wall arabinan biosynthesis and it is the target for the EMB. Mutation in this gene causes EMB resistant (9). Mour et al. in 2014 in Spain reported that from 53 strains resistant to EMB, 77.4% of strains had mutation substitutions in the *emb*B gene. 53.7% of them were related to codon 306 and 26.8% strains showed mutation in codon406. Mutations in *emb*B406 were related to EMB resistance and MDR (10). Also *emb*B306 mutations in our study had a significant relationship with EMB resistant and MDR strains. *M. tuberculosis* is a human pathogen that causes TB and this disease damages the lungs, central nervous system, lymphatic system and circulatory system. So a prevention and appropriate management in treatment for this infection is necessary (11, 12).

Conclusion

In conclusion, the results presented in this study suggest that the frequency of *emb*B306 mutations in EMB-resistant *M. tuberculosis* isolates, is much higher than its occurrence in EMB-susceptible isolates. These results suggest that the sequencing of this region of *emb*B gene is sufficiently sensitive to be used as a fast screening tool for finding high-level resistance to EMB, specifically in the population served by our research laboratory.

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Conflict of Interests

The authors declare that they have no competing interests.

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