JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Frequency of mutational changes in the *embB* among the ethambutol-resistant strains of *Mycobacterium tuberculosis* in Iran

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Abstract

Introduction: Early detection of drug resistant tuberculosis is one of the main priorities of TB control program. Ethambutol (EMB) is a first-line anti-TB drug that is effective for preventing treatment failures caused by *Mycobacterium tuberculosis* strains that are resistant to other drugs. The aim of this study was to sequence the *embB* gene to characterize the mutations causing resistance to EMB and to analyze the relationship between bacterial genotype and EMB resistance among *M. tuberculosis* isolates in Iran.

Methodology: A total of 20 *M. tuberculosis* isolates comprising 10 multidrug-resistant (MDR) and 10 non-MDR isolates, recovered from TB patients in four regions: Tehran, Isfahan, Zahedan, Khorasan, were analyzed. Mutational profiling was performed by amplifying and sequencing the *embB* gene. Spoligotyping was carried out to characterize the bacterial genotype.

Results: Phenotypic EMB resistance was found in 13 strains. Mutations affecting ethambutol resistance-determining region (ERDR) of the *embB* were identified in 6 of 13 EMB-resistant isolates. The majority of these mutations resulted in amino acid substitution at position 306 (M306V). A novel mutation at codon 366 was identified (S366L) in one isolate. Ural was the most predominant genotype in the studied population. Beijing genotype was associated with both MDR and EMB resistance in which all mutations occurred at codon 306 of the embB gene.

Conclusion: A significant association between Beijing genotype and EMB resistance was found, mainly due to mutations at embB306. Results of this study can be used as a basis to develop or improve rapid molecular tests to monitor drug-resistant strains in this country.

Key words: *Mycobacterium tuberculosis*; *embB*; ethambutol; spoligotyping; Beijing.

J Infect Dev Ctries 2016; 10(4):363-368. doi:10.3855/jidc.7215

(Received 06 January 2015 – Accepted 14 October 2015)

Introduction

Tuberculosis (TB) is a life-threatening disease and a major global health problem [1]. The emergence of multidrug resistance and insufficient laboratory ability for detection is a serious problem that leads to treatment failure [2,3]. In this regard, one of the major obstacles in TB control strategies is the transmission and spread of resistant strains from such patients [4].

Among anti-tuberculosis drugs, ethambutol (EMB) [dextro-2, 2-(ethylenediimino) di-1-butanol], an arabinose analogue, is one of the first-line drugs recommended by the World Health Organization (WHO) for the treatment of tuberculosis [5]. It is often used in combination with rifampin, isoniazid, pyrazinamide, and streptomycin to prevent the emergence of drug resistance [6]. However, the rate of resistance to EMB in retreated TB patients has increased by 50% in some regions [7]. EMB targets membrane-associated arabinosyl transferase that is well conserved in mycobacteria and involved in the biosynthesis of arabinan, a component of arabinogalactan existing in the cell wall [8]. Arabinosyl transferase, encoded by a 10kbpembCABoperon, encompasses three contiguous genes (*embC*, *embA*, and *embB*) that are involved in the biosynthesis of the cell wall. These genes are ubiquitous in mycobacteria and have no sequence similarity to any identified protein family in other bacteria. Consequently, the accumulation of mycolic acids due to a lack of arabinan receptors for mycolic acids results in cell death [9-11].

Point mutations in these three genes, particularly mutations in *embB* codon 306, which occur in 30%–

69% of ethambutol-resistant clinical strains, are associated with resistance to EMB [12]. One-fourth of EMB-resistant strains still lack any known mutation linked to EMB resistance, implying that multiple molecular pathways are required for its development [5].

Different mutations have been identified in this codon that change its first or third base (ATG, GTG, CTG, ATA, ATC, or ATT) and result in three amino acid shifts (Val, Leu, and Ile). Thus, the identification of mutations, particularly in *embB* codon 306, is thought to represent a rapid screening method for detection of ethambutol resistance in clinical isolates [13].

Conventional culture-based EMB susceptibility test methods are slow and make it difficult to firmly exclude the presence of EMB resistance. Therefore, the rapid detection of drug resistance in order to design a suitable treatment regimen can reduce the spread of drug-resistant isolates. In this way, a molecular assay can reduce the delay of conventional liquid media-based systems. Genetic assays can identify EMB resistance with high inter-assay reproducibility by detecting mutations within the *embB* gene [14].

Depending on the geographic area, a variation in the prevalence of mutations associated with EMB resistance can be determined. To our knowledge, there is no data about specific mutation patterns in ethambutol-resistant strains in Iran. The aim of the present study was to examine codon 306–497 mutations in the *embB* gene among multidrug-resistant (MDR) and non-MDR *Mycobacterium tuberculosis* isolates from Iran. Moreover, genetic diversity of strains and possible association between strain type and susceptibility to ethambutol was studied.

Methodology

Mycobacterium tuberculosis clinical strains

A total of 20 drug-resistant *M. tuberculosis* isolates obtained from TB patients in four regions (Tehran [n = 8], Zahedan [n = 3], Isfahan [n=2], and Khorasan [n = 7]) during 2011 and 2013 were included in this study. Obtained isolates were identified based on standard microbiological tests including morphology of colony, acid-fast staining, and biochemical tests.

Drug susceptibility testing (DST)

Strains were tested for susceptibility to at least four first-line anti-tuberculosis drugs including rifampin, isoniazid, ethambutol, and streptomycin using the Löwenstein-Jensen (LJ) proportional method. The drug concentrations to perform DST were as follows: 0.2µg/mL isoniazid (INH), 40µg/mL rifampin (RIF), 4.0µg/mL streptomycin (STR), and 2.0µg/mL ethambutol (EMB) (Sigma-Aldrich, Taufkirchen, Germany). *Mycobacterium tuberculosis* H37Rv reference strain was included as a control in all experiments [15].

DNA extraction and amplification

The extraction of genomic DNA from *M. tuberculosis* was performed using the method of van Sooligen *et al*. [16]. Briefly, loopfuls of colonies of bacterium were transferred in screw-capped tubes with glass beads (size 180 lm) and 500 mL (TE) Tris-EDTA buffer (pH 8.0). Bacteria were lysed for 30 minutes at 95°C, followed by enzymatic degradation of the cell walls after incubation with lysozyme at a final concentration of 1µg/mL at 37°C for 1 hour, and 10% sodium dodecyl sulfate and proteinase K (8 mg/mL) at 56°C for 2 hours. CTAB-NaCl was used to extract genomic DNA at 65°C for 10 minutes. Finally, extracted DNA was purified by a mixture of phenol and chloroform (Sigma-Aldrich, Taufkirchen, Germany), precipitated by ethanol, and dissolved in TE buffer. The samples were stored at -20°C until use.

The primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). For assessment of EMB resistance, all isolates were screened for single nucleotide polymorphism in an 899 bp region of the *embB* gene encompassing codons 306–497, which have been found to be a main cause of EMB resistance. This hot spot region was amplified by polymerase chain reaction (PCR) with primers embBF (GCTCAATTGCCCAGCTCCTC) and embBR (GATCAAAAAGCCGAAGCGCC).

Amplification reactions were performed in a total volume of 50 µL mixture containing PCR master kit (Ampliqon, Odense M, Denmark), 0.2 µM of each primer, and 10 ng of template DNA. After initial denaturation at 94°C for 5 minutes, the reaction mixture was run through 30 sequential cycles of denaturation at 94°C for 30 seconds, primer annealing at 58°C for 45 seconds, and primer extension at 72°C for 45 seconds, followed by a final extension at 72°C for 5 minutes. Amplified DNA fragments were separated by electrophoresis at 3.5 V/cm on 1%agarose gel in 0.5XT BE buffer and stained with ethidium bromide.

Amplicon sequence analysis

Purified PCR products were sequenced by Macrogen Company (Macrogen, Seoul, Korea). Sequencing was performed in both directions using the same forward and reverse primers as those used in the

PCR amplification. Sequence data were assembled and the resulting chromatograms were analysed using ChromasPro (version 1.7.1) software (Technelysium, South Brisbane, Australia). Mutations were determined by comparing the obtained sequences with *M. tuberculosis* H37Rv strain sequence of *embB* from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/).

Spoligotyping

Spoligotyping was performed as previously described by Kamerbeek *et al*. [17], and results were compared with the SITVIT2 database (Pasteur Institute of Guadeloupe, http://www.pasteur-guadeloupe.fr:8081/SITVITDemo). A cluster was defined as two or more isolates from different patients with identical spoligotype patterns.

Results

The results of susceptibility testing for ethambutol are shown in Table 1. Of the 20 studied *M. tuberculosis* isolates, 13 were phenotypically resistant to EMB; among them, 9 were MDR (ethambutol-resistant MDR [ER-MDR]), and 4 strains were non-MDR.

Among 9ER-MDR strains, 4 had valine substitutions at *embB*306, while the remaining 5 ER-MDR strains lacked any amino acid substitutions at codon 306 or other parts of the amplified region of the *embB* gene. Moreover, as we expected, no mutation of *embB* was found in ethambutol-sensitive MDR (ES-MDR) strains. Among 4 ethambutol-resistant non-MDR strains, 2 were genetically resistant, each showing mutation at codons 306 and 366, respectively. Interestingly, a mutation at codon 423 was identified in 1ethambutol-sensitive strain.

Amino acid changes at codon 306 were the most common mutations, occurring in 5 (38.4%) phenotypically ethambutol-resistant isolates.

The Met306Val substitution resulting from a transition of A to G at nucleotide position 916 was detected in 5 EMB-resistant isolates, while the Ser366Leu substitution, due to a transition from C to T at nucleotide position 1097, was detected in 1 non-MDR EMB-resistant isolate, and 1 EMB-sensitive isolate had a G→A alteration (Met423Ile) at position 1269 in the analyzed region of the *embB* gene.

In total, alterations in the examined region of the *embB* gene were identified in 1 of the 7 EMB-susceptible isolates.

Among the isolates, 40% (4/10) of MDR-TB isolates carried the *embB*306 mutation. Indeed, the proportion of *embB*306 mutants among MDR strains (40%) was much higher than that in non-MDR strains (10%, 1/10). An S366L mutation (TCG → TTG, nucleotide position 1097) was found as a new *embB* mutation in EMB-resistant *M. tuberculosis* strains in this study (Table 2).

Table 1. Drug susceptibility profile, spoligotyping genotype, and mutational changes among isolates of *M. tuberculosis.*

Strain	Origin	INH	RIF	STR	EMB	*embB* mutations	Spoligotype family/SIT
1	Tehran	R	S	R	S		H37rv/451
2	Tehran	R	S	R	R		H4/127
3	Tehran	R	R	R	R	Codon 306	H4/777
4	Tehran	R	R	R	R		H4/361
5	Tehran	R	R	R	S		H4/127
6	Tehran	S	S	R	S		H4/127
7	Tehran	S	S	R	S	Codon 423	H4/361
8	Tehran	S	S	S	R	Codon 366	CAS/26
9	Zahedan	R	S	R	R		CAS/26
10	Zahedan	R	R	R	R		H4/127
11	Zahedan	R	R	R	R		CAS/26
12	Isfahan	R	R	R	R		H4/361
13	Isfahan	S	R	S	S		T1/53
14	Khorasan	R	S	R	R	Codon 306	T1/284
15	Khorasan	R	R	R	R	Codon 306	Beijing/1
16	Khorasan	R	R	R	R		H4/127
17	Khorasan	S	R	S	S		H4/127
18	Khorasan	R	R	S	R	Codon 306	Beijing/1
19	Khorasan	R	S	R	S		Orphan
20	Khorasan	R	R	R	R	Codon 306	Beijing/1

INH: isoniazid; RIF: rifampin; STR: streptomycin; EMB: ethambutol; SIT: spoligotype international type; R: resistant; S: sensitive

Spoligotyping of 20 isolates produced 9 spoligotype patterns that are shown in Table 1.A total of 19 isolates were distributed in 5 families including Ural (former H4)/SITs 127, 361, and 777 (10/19); Beijing/SIT1 (3/19); Central Asian strain (CAS)/SIT26 (3/19); T/SITs 53 and 284 (2/19) and H37Rv/SIT451 (5%, 1/19). One isolate was not found in theSITVIT2 database and was considered a novel genotype. Beijing (3/3), CAS (3/3), Ural (6/10), and T (1/2) families were characterized with phenotypic EMB resistance. Beijing genotype was associated with MDR, phenotypic and genotypic EMB resistance, and all strains characterized with this genotype had mutation at codon 306 of the *embB* gene.

Discussion

Infection with MDR *M. tuberculosis* is associated with high mortality. The WHO has called for research into a fast and accurate drug susceptibility testing method in order to reduce drug-resistant TB and therefore the TB burden worldwide [18]. The use of molecular methods has been suggested as an effective way to decrease the delay in the detection of drug resistance in *M. tuberculosis* [12].

The impact of *embB* substitutions on ethambutol resistance might vary depending on other mutations or polymorphisms and the genomic background of the strain [19]. Although the association between *embB*306 mutation and EMB resistance has been identified, the exact role of *embB*306 mutations in the development of ethambutol resistance and multidrug resistance in *M. tuberculosis* is not fully understood. Since the majority of EMB resistance occurs in MDR strains of *M. tuberculosis*, it is likely that *embB*306 substitutions were not directly associated with ethambutol resistance but rather with MDR-TB, and it is concluded that *embB*306 mutations can serve as a marker for development of drug resistance [20,21].

The rates of resistance to this drug have been reported from 4.0% among new cases to 31.0% in previously treated cases [22]. In our study, we detected 10 MDR *M. tuberculosis* isolates; of those, 9 had co-resistance to EMB. Moreover, 12, 9, and 11 isolates demonstrated co-resistance to EMB/INH, EMB/RIF, and EMB/STR, respectively. Because mono-resistance with EMB is rare, EMB is still a valuable drug for TB treatment.

The inconsistency between the results of ethambutol DST and *embB*306 mutation can be related to other mutations occurring outside the *embB* gene in the genome of these clinical strains. Previous studies demonstrated that ethambutol resistance is a process containing multi-gene mutation that requires mutations in the *embB* gene and other currently unknown loci.

The high detection rates of mutations at codon *embB*306 among EMB-resistant *M. tuberculosis* isolates were reported from Cuba and the Dominican Republic (70%), Germany (68%), China (55%), and Russia (48%). However, some reports have indicated that only less than 35% of EMB-resistant *M. tuberculosis* isolates harbored mutations in the *embB* codon 306 [23,24]. Bahrami *et al*. found that 29% (14/48) of the EMB-resistant isolates from Iran had a mutation at *emb*306 codon determined by a multiplex allele-specific PCR method [25]. In our study, we detected *emb*306 mutations in 38.4% (5/13) of EMB-resistant isolates using the DNA sequencing method, which is considered to be the gold standard method for genotypic DST.

Previous studies recommend rapid PCR-DNA sequencing for multiple-site (*embB*306, 406, and 497) detection of resistant mutants. The level of *embB*406 mutations among EMB-resistant isolates was rather low, whereas mutations in codon *embB*497 were twice as frequent [11]. In our study, no mutation at *embB* codons 406 and 497 was found, which may be due to the limited sample size. However, a novel mutation in

Table 2. Nucleotide alterations within studied region of the *embB* gene among MDR and non-MDR strains of *M. tuberculosis* determined by DNA sequencing method**.**

Phenotype (N)	Codon	Amino acid change (s)	Nucleotide change (s)	N (%)
MDR (10)				
EMB resistant (9)	306	Met → Val	ATG → GTG	4 (44.4%)
EMB sensitive (1)	306	None	None	0
Non-MDR (10)				
EMB resistant (4)	306	Met → Val	ATG → GTG	1 (25%)
	366	Ser → Leu	TCG → TTG	1 (25%)
EMB sensitive (6)	306	None	None	0
	423	Met → Ile	ATG → ATA	1 (16.6%)

MDR: multidrug resistant

the examined fragment of the *embB* gene (substitution at codon 366) was detected in our study.

In spite of the existing mutation in the *embB* gene (*embB*423*)*, one strain was sensitive to ethambutol. This suggests that this mutation will not confer phenotypic resistance unless a second mutation in an unknown site occurs.

Several studies have demonstrated a strong association between resistance to INH or RMP, or MDR phenotype and *embB*306 mutations. It is likely that *embB*306 mutations may have selective advantage upon treatment with multiple drugs. On the other hand, these mutations prevent the synergistic effect of combination of multiple anti-TB drugs. The exact molecular mechanism of this phenomenon is not clear, and it can only be speculated that changes in the cell wall permeability are a result of *embB*306 mutations [26,27].

Safi *et al*. suggested that all *M. tuberculosis* isolates with any form of drug resistance should be tested for the presence of *embB*306 mutations. Patients infected with *embB*306 mutants should be carefully monitored for treatment failure and the possible emergence of MDR [28]. In an investigation between EMB resistance and strain genotype, we found Beijing and CAS genotypes to be resistant to EMB. Interestingly, all three EMB-resistant Beijing genotypes had *embB*306 mutation and were MDR as well. These strains were from the same city in Iran, tempting us to speculate that these three strains might be related to each other or originated from the same source. In spite of having significant association with phenotypic resistance to EMB, only one strain from three CAS genotypes had an *emb*306 mutation and the remaining two isolates lacked any mutation in the studied region of the *embB* gene.

The results of this study essentially corroborate all of the findings reported in prior studies. First, the *embB*306 substitutions found in MDR-TB maybe due to a proposed benefit these mutations confer for growth in the presence of isoniazid and rifampicin. Second, valine substitutions at *embB*306 predominated in ethambutol-resistant MDR strains. Third, there must be other sites for mutations conferring ethambutol resistance, as some ethambutol-resistant strains do not have any *embB* mutations. Finally, Beijing and CAS genotypes were found to be associated with EMB resistance. Also, EMB resistance in all Beijing strains was due to mutations at *embB*306 codon. However, due to the small size of strains characterized with these two genotypes, association between these genotypes and EMB resistance should be interpreted with caution.

Conclusions

In summary, we found mutations conferring resistance to EMB in 46.15% (6/13) of EMB-R isolates, which were mainly (83.33%, 5/6) due to alterations at codon 306 of the *embB* gene. Also, the proportion of *embB*306 mutants among MDR strains (40%) was higher than that in non-MDR strains (10%), suggesting that *embB*306 could serve as a marker for tuberculosis cases that are at increased risk for developing MDR. Furthermore, the Beijing genotype, a hyper-virulent *M. tuberculosis* genotype, was found to be associated with both MDR (100%) and *embB*306 mutation (100%), indicating that substitutions at this locus may possibly contribute to the emergence of highly pathogenic strains of *M. tuberculosis*. Further studies with additional genetic loci and larger sample size are required to elucidate the association between *emb*306 mutations and drug resistance emergence in *M. tuberculosis.*

Acknowledgements

We thank Tehran University of Medical Sciences (Project No. 20216).

Ethical approval: The research has been approved by the Ethics committee of Tehran University of Medical Sciences

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Conflict of interests: No conflict of interests is declared.