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Point-Mutations in *emb*B306 Gene and Their Association with Resistance to Ethambutol in *Mycobacterium tuberculosis* in Clinical Isolates

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ABSTRACT

Background & Objective: Mutations in *emb*B306 gene and their association with resistance to ethambutol (EMB) in *Mycobacterium tuberculosis* (*M. tuberculosis*) have not been fully investigated. The aim of this study was to investigate the point-mutations in *emb*306B gene and their association with resistance to EMB in *M. tuberculosis*.

Materials & Methods: This case *(M. tuberculosis* resistant to EMB) -control *(M. tuberculosis* sensitive to EMB) study was performed in the West of Iran (2014-2015), in order to determine the sensitivity of *M. tuberculosis* strains. Polymerase chain reaction (PCR)-DNA sequencing was used for determining the point-mutations of *emb*B306 gene in both groups (sensitive and resistant to EMB). Data was analyzed by SPSS 16 and Fisher's exact test.

Results: Fifty *M. tuberculosis* strains were isolated from 1019 patients that were suspected to have tuberculosis (TB). 86% of the isolates were sensitive and 14% were resistant to EMB. *Emb*B306 gene sequencing showed no mutation in control samples; but mutation was observed in 85.71% of resistant samples in case samples. The *emb*B306 mutation showed a significant relationship with EMB resistance (P= 0.00).

Conclusion: Mutations in *emb*B306 were observed in the strains resistant to EMB; however, there was no mutation in the sensitive group. There is a direct relationship between these mutations and this type of resistance, so it is an indicator of creation of resistance to EMB in *M. tuberculosis*.

Keywords: *embB306 Gene, Ethambutol, Mycobacterium tuberculosis, Point-Mutations*

Introduction

Occurrence and dispersion of multidrug-resistant (MDR) and drug-resistant (DR) strains of Mycobacterium tuberculosis (M. tuberculosis) are the biggest problems in tuberculosis (TB) treatment. Ethambutol (EMB) is a bacteriostatic drug that physicians have been using for treating TB since the mid1960s. Resistance to ethambutol (EMB) may cause resistance to other anti-TB drugs in M. tuberculosis cases (1). Different factors cause resistant to bacteria, one of which is mutation. Types of mutation are spontaneous mutations (occur without mutation induction and is the result of errors during DNA replication) such as silent mutation, missense mutation, nonsense mutation and mutation induction (mutagens may be of physical, chemical, or of biological origin) (2). The main mechanism of acquiring resistance to EMB in M. tuberculosis depends on point mutations (nonsense mutation) in emb operon encoding arabinosyltransferase (point mutation or substitution is defined as a genetic mutation, in which a single nucleotide base is deleted, inserted, or changed in a DNA or RNA sequence. This type of mutation has different effects on the downstream protein product consequences that are predictable to a certain extent based on the specifics of the mutation). The emb operon encodes various arabinosyltransferases that include three adjacent genes (embC, embA, and embB) (3,4). Five different mutations have been found in this codon where translocations have intermittently occurred in the first

or the third base (ATG to GTG, CTG, ATA, ATC, or ATT) (5). Mutations in embB497, embB406, and embB306 codons bring about moderate and low levels of EMB resistance (1). Having utilized direct sequencing and polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP), Nasr Esfahani et al. demonstrated that out of 32 M. tuberculosis isolates, 6.25% had resistance to EMB and in 2 EMB-resistant isolates, mutations were observed on codons 309 and 299 (6). Ahmad *et al.* (2017) using PCR and sequencing, reported that out of 163 isolates, 19% were resistant to EMB and only 10 isolates showed embB mutation at either codon 281 or 306, or both (7).

While having respect for the above-mentioned investigations, this relationship is unclear; because all of these codons have been mutated in some of the M. tuberculosis that is sensitive to EMB. According to this contradictory result, it was decided to examine this gene once again in order to study the possible role of mutation in the created resistance to EMB. Variation in the prevalence of specific mutations in relation to resistance to EMB is likely to be due to different geographical areas. Rarely any research has been conducted on the relationship between point mutation and antibiotic resistance to EMB in the Western region of Iran. Moreover, many researchers disagree on the validity of this matter. Therefore, numerous studies in different areas are needed to investigate this issue and to research the relevance or possible association of point mutation and antibiotic resistance to EMB in order to be able to devise better plans for preventing antibiotic resistance in M. tuberculosis cases. Considering the geographic area of the present study, it is expected to obtain valuable results.

Materials and Methods

Study Population

The present research was conducted as a case-control study. Case group included isolates of *M. tuberculosis* that were resistant to EMB at least in terms of phenotype and had mutation in *emb*306B gene; and the control group was comprised of those types of *M. tuberculosis* isolates that were susceptible to EMB in phenotype and had no mutation in *emb*B. According to a census carried out by the country's Drug Resistance Surveillance (DRS) program in Western part of the country in a period from 2014 to 2015, only seven samples of antibiotic-resistant *M. tuberculosis* were identified from all the antibiotic susceptibility tests. The study population included samples sent from Western provinces of Iran to the Mycobacterium Research Center as the reference laboratory, which is located in the city of Kermanshah in Kermanshah province.

Identification of Samples

Initially, sputum samples that were suspected of having TB were sanitized with Modified Petroff Method. The samples were colored using Auramine and Ziehl-Neelsen technique. Löwenstein Jensen (LJ) agar medium (Merck, Germany) was used for culturing the samples and the colonies were studied after incubation at temperature of 37°C for 4 weeks. Suspicious colonies were assessed based on the speed of growth of colonies (slow growing) and by biochemical assays (positive niacin production, positive nitrite reduction, positive urease, positive thiosemicarbazon, sensitive cycloserine 20 µL/mL in LJ, positive oxygen performance, positive pyrazinamide activity, catalase activity at 68°C, negative NaCl tolerance, and pigment production) (8).

Determining the Medicinal Sensitivity of *Mycobacterium tuberculosis*

A bacterial suspension was added to each of the LJ culture media containing EMB (2 µg/mL) antibiotic, and the results were examined after 28 and 48 days (in case of growth of bacteria in these media, they were considered as resistant isolates, and if no growth was observed, they were considered as sensitive isolates). Concentration rate of EMB was 0.2 µg/mL. The standard *M. tuberculosis* strain (H37RV) (SinaClon, Iran) was used as quality control of the test of sensitivity to EMB (9).

Identification of *emb*B306 Gene in *M. tuberculosis* Isolates

DNA was extracted using CTAB (Cetyltrimethylammonium Bromide) after deactivation at 80°C for 1 h. A piece including 863 base-pairs (bp) of *emb*B306 gene was reduplicated by PCR to determine the presence of *emb*B306 gene in isolates sensitive and resistant to EMB. Oligonucleotides included *emb*B306-F (5'-CGACGCCGTGGTGATATTCG-3') and *emb*B306-R (5'-CCACGCTGGGAATTCGCTTG-3) (SinaClon, Iran). The final PCR mixture (final volume of 50 µL) consisted of 0.2 µmolar of every primer, 25.1 units of Taq-polymerase enzyme (SinaClon, Iran), 0.2 mmolar dNTP, and 1 mmolar of 10X buffer. PCR reaction was conducted as follows: Primary denaturation for 5 min at 95°C, secondary denaturation at 94°C for 30 s, annealing phase at 70°C for 40 s, extension at 95°C for 72 s, in 30 cycles. The final extension phase was carried out at 72°C for 10 m. The *emb*B gene sequence was determined by means of sequence analysis (Gene Faravaran, Iran) and analysis of created point mutations in sequence of *emb*B306 gene was done through comparing them with the sequence of *emb*B gene sequence derived from the standard strain of *M. tuberculosis* H37RV provided by GeneBank (http://www.ncbi.nlm.nih.gov/genbank) using BLASTn algorithm (http://blast.nbci.nlm.nih.gov). Presence of mutations of *emb*B gene and point mutations were examined in sensitive and resistant strains and finally, the relationship between *emb*B306 gene mutation and resistance to EMB was analyzed (10).

Statistical Analysis

Frequency and percentages were determined by means of Fischer's test and SPSS 16 (SPSS Inc., Chicago, IL., USA), which were used to compare mutations in both resistant and nonresistant groups to EMB ($P \leq 0.05$).

Results

Patients

Pulmonary specimens were obtained from 1019 patients with suspected TB. After identification of samples, 50 *M. tuberculosis* strains (from 22 men and 28 women with the age range of 23-86 and a median age of 54.5) were identified and then isolated to the species level. Afterward, drug susceptibility tests were carried out.

Results of Antibiotic Resistance

The number of isolates sensitive to EMB was 43 (86%) and the number of samples resistant to EMB was 7 (14%). The results of antibiotic resistance test are provided in Table 1 for both the case and control groups. The results of mutation analysis for embB306 gene in the case and control groups showed that no mutation had occurred in the control samples, but embB306 mutation was observed in six (85.71%) out of seven samples in this gene, which were resistant to EMB (Figure 1, Table 1). Presence of mutation in embB306 gene was directly correlated with EMB and mutation in emb306 gene (P=0.000).

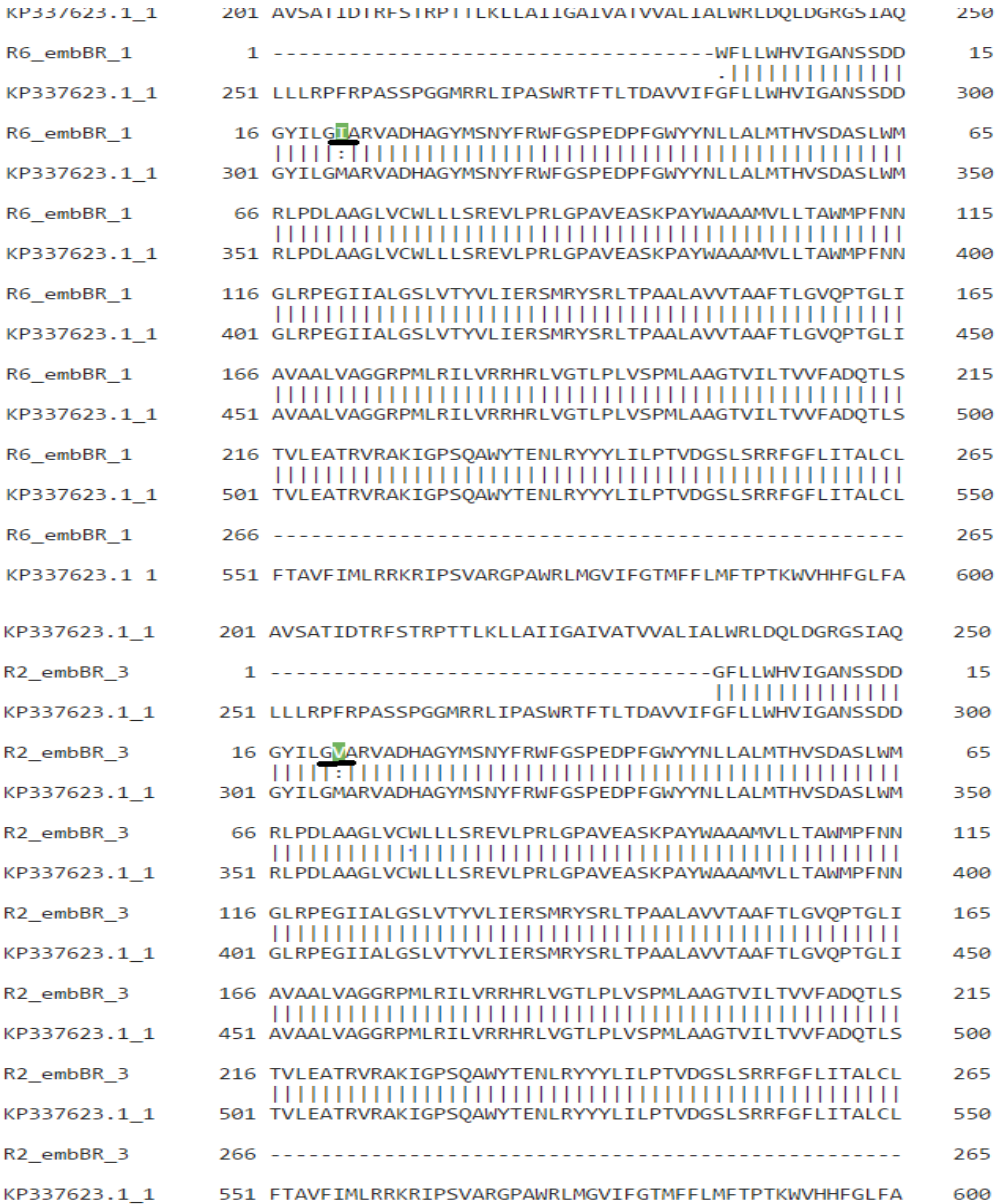

Figure 1. Mutated amino acid isoleucine (I) instead of methionine (M) and valine (V) instead of methionine (M) at codon 306, sequence of these amino acid was checked with H37RV according to European molecular biology laboratory (EMBL)-European Bioinformatics Institute (EBI)) nBLAST algorithms

Table 1. Point mutations (missense mutation) detected in studied EMB-resistant and EMB-susceptible *M. tuberculosis* isolates

Nucleotide	Mutation Amino Acid	No. (%) of EMB-resistant mutant isolates in emb306 (n=6)
ATG → ATA	Met→ Il (306)	3 (42.85)
ATG → GTG	Met→Val (306)	3 (42.85)

Discussion

In the present study, the maximum rate of identified mutations was found in *emb*B306 gene, which belonged to the case group (EMB-resistant strains), while no mutation was found in the strains sensitive to this medication. In a study conducted in Iran, Derakhshani Nezhad *et al.* (2012) showed that among 106 *M. tuberculosis* samples, 33.9% were resistant to EMB using the proportion method. Using Allele-specific PCR (ASPCR), 27.6% of strains were EMB resistant. This method showed that mutation in the first base was 61.5% (Met→Val or Il) and it was 38.5% in the third base (Met→ Il) **(11)**. In the present research, six case samples out of 50 strains had mutation in the *emb*B306 region. Determination of possible mutations with the help of PCR-sequencing is the main technique for mutation analysis in resistance-related genes. Although this technique is a time-consuming and burdensome method, it has high efficiency in terms of sensitivity and property. An accurate laboratory diagnosis of TB is the most important step in diagnosis of the disease. One of the reasons for differences in various studies is the application of different techniques with varying degrees of differentiation and sensitivity, which may lead to different results **(8)**. In the study conducted by Bakula *et al.* (2013), who used PCR-sequencing, out of 50 studied strains (33 EMB-sensitive strains and 17 EMB-resistant strains), six types of mutations were found in 27 (54%) samples. Mutation in *emb*B gene was observed among 13 (76.5%) EMB-resistant strains and 14 (42.4%) EMB-sensitive strains **(1)**. Unlike Bakula's study, the current work showed no mutation in EMB-sensitive strains; and among the seven EMB-resistant samples, all of them had mutation in the *emb*B306 region. A strong relationship was observed between *emb*B306 mutants and resistance to EMB. The study's results showed that mutation in *emb*B306 gene is an important indicator for detection of resistance to EMB. In conclusion, it was demonstrated that *emb*B306 mutation is a common polymorphism in the strains resistant to EMB *M. tuberculosis*. Considering that only isolates resistant to EMB were studied in this work, further evaluation of a number of EMB-susceptible and resistant isolates from different geographic regions is required to explicate the role of these mutations in resistance to EMB. In addition, even though under normal conditions, definitive diagnosis of resistance to a drug requires about 42 days, it can be diagnosed in only 6-7 days by means of molecular techniques. Quick diagnosis of drug resistance may prevent wrong administration of drugs and it also leads to employing the proper therapeutic diet that prevents the spread of multiple drug-resistant (MDR) strains, which is a large issue **(12,13)**. Using phenotypic susceptibility testing, Khosravi *et al.* showed that resistance to *emb* was 3.25% in Iran. Rate of mutation in *embB* was 10% at codon 306 Met→Val **(12)**. It has been found that mutations in *embB*306 of *embB* gene very often occur among other codons. High detection rates of mutation have been reported in codon *embB*306 among EMB-resistant *M. tuberculosis* isolates in various countries **(1)**. Therefore, in this study, *embB*306 was investigated among other genes by PCR-Sequencing. PCR is a rapid method with high discriminatory capabilities, and it is easy to use while being obtainable at a low cost **(14,15)**. Moreover, limitations of this research are as follows: The small number of antibiotic-resistant specimens, especially samples with resistance to EMB in the studied area, the high cost of molecular testing equipment and tools, slow growth of bacteria in a specific culture medium and special incubation conditions, and due to the high contamination level of *M. tuberculosis*, sampling and DNA extraction should be performed under conditions of high immunity and level 3 safety. Given that *emb*B gene mutations, especially those located in ERDR, are considered as "hot-spots", they have been repeatedly investigated in studies concerned with EMB-resistance in *M. tuberculosis* cases. Therefore, the PCR-Sequencing method is a precise method for determining these hot-spots. Moreover, mutation rates in first and third bases were very similar to each other. In addition, it was observed that *embB*306 mutations were present only in isolates that were resistant to EMB and had a significantly higher proportion of *emb*B306 mutants.

Conclusion

The majority of EMB-resistant isolates showed embB306 mutation. No embB306 mutant was detected among the susceptible strains. The current study demonstrated that mutation in embB306 can be considered as an indicator of resistance to EMB in isolates of M. tuberculosis, and most of the found mutations were located in this codon. However, mutations in embB306 are not the main reason for resistance to EMB in isolates; other mechanisms of resistance in M. tuberculosis should be considered as well.

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Conflict of Interest

Authors declared no conflict of interest.

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