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Detection of Ethambutol-Resistant Associated Mutations in *Mycobacterium tuberculosis* Isolates from Iran Using Multiplex Allele-Specific PCR

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Tuberculosis is a serious global public health problem and its high prevalence is strongly associated with the enhancement of drug resistance. In this study we demonstrate a multiplex allele-specific polymerase chain reaction (MAS)-PCR assay to simultaneously detect mutations in the first and third bases of the *embB* gene codon 306 ATG in ethambutol (EMB) resistant isolates of *Mycobacterium tuberculosis*. A total of 5029 patients were sampled between 2010 and 2012. All the specimens were examined microscopically for acid-fast bacilli and cultured in Löwenstein-Jensen medium. The susceptibility tests were performed for culture positive samples and the EMB resistant *M. tuberculosis* isolates were subjected to MAS-PCR targeting *embB* gene in the codon 306 ATG. Frothy eight of 176 isolates were EMB-resistant. None of the isolates showed mutation in the first base of the codon 306 ATG, but mutation in third base of the codon 306 ATG was detected in 14 isolates. We observed a correlation between culture-based phenotypic drug susceptibility and MAS-PCR method. The absence of mutation in resistant isolates can be attributed to possible involvement of other codon position at the same gene or other genes.

Keywords: ***M .tuberculosis*, Ethambutol, Drug resistant, Multiple Allele Specific PCR.**

INTRODUCTION

According to the World Health Organization (WHO) about one third of the world population is infected with *Mycobacterium tuberculosis* and this infection remains as one of the biggest public health problems in the 21st century [1, 2]. A severe challenge to treatment of tuberculosis control is drug resistance, as it enhances the probability of a condition that no longer can be effectively treated with anti-tuberculosis drugs [3]. Multidrug-resistant (MDR) tuberculosis (TB) strains are resistant to at least rifampin (RIF) and isoniazid (INH) and infections with these strains must be treated with the second-line drugs [1, 3, 4]. In the last two decades, despite efficacy of second-line drugs, extensively drug-resistant (XDR) TB has emerged; XDR-TB is caused by the strains that are resistant to isoniazid and rifampin, plus any fluoroquinolone and at least one of three injectable second-line drugs [5]. The strains that are resistant to both first and second-line drug are called as totally drug resistant (TDR) or extremely drug resistant (XXDR) TB. The patients infected with XDR or XXDR-TB are treated difficulty and may increase the risk of drug resistant strains transmission among the community [6]. Ethambutol has been successfully used in multidrug therapy for the treatment of TB in combination with other drugs [7]. The *emb* operon of *M. tuberculosis,* which consist of three continuous genes namely, *embC*, *embA*, and *embB*, encode arabinosy l transferases.

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These gens exhibit 65% similarity to each other. Analysis of EMB resistant clinical isolates of *M. tuberculosis* has shown several mutations in these genes, especially in *embB*, and up to 90% of them were found in codon 306 ATG. Five different mutations have been discovered in this codon (ATG→GTG, CTG, ATA, ATC, and ATT), resulting in three different amino acid shifts (Met → Val, Leu, or Ile) [8]. The EMB action involves detrimental alteration of the *Mycobacterium* cell wall structure through inhibiting the polymerization of cell wall arabinan, arabinogalactan and lipoarabinomannan that are necessary for cell wall construction; this induces the accumulation of mycolic acids, which results in cell death [2, 8]. Ethambutol is a valuable drug for anti-tubercular therapy as resistance to this drug among patients with MDR-TB does not occur frequently, and EMB monoresistance is extremely rare. However, to preserve this valuable drug, even if its resistance development is not generally very high, necessitates rapid detection of resistance cases [8]. Molecular techniques such as sequencing and PCR restriction fragment polymorphism (PCR-RFLP) have been extensively used for detection of EMB resistance- related mutations. However, these methods are expensive and time consuming. In recent years, mutiplex allele-specific (MAS)-PCR has received a great attention for detection of EMB resistance in *M. tuberculosis* clinical isolates. This method can simultaneously detect two mutations in first and third bases of ATG codon of embB306 in *M. tuberculosis* [8].

This study is aimed to detect EMB resistant-associated mutations in the codon 306 ATG by MAS-PCR in EMB-phenotypic resistant *M. tuberculosis* isolates from Iran.

MARERIALS AND METHODS

Sample collection and culture. Samples were obtained from some 5029 patient admitted to Mycobacteriology and Pulmonary Research Department of Pasteur Institute of Iran between 2010 and 2012. The specimens included sputum, bronchioalveolar washing (BAL), gastric washing and lymph node. All the specimens were microscopically examined for the acid-fast bacilli and then decontaminated and cultured on Löwenstein-Jensen medium according to WHO guidelines [9].

Susceptibility testing. Antibiotic susceptibility of isolates was measured by using the proportional method for the first line antibiotics including rifampin (40 µg/ml), isoniazid (0.2 µg/ml), streptomycin (4 µg/ml), ethambutol (2 µg/ml), kanamycin (20 µg/ml) and ethionamide (20 µg/ml)[10].

DNA preparation. Two or three *Mycobacterial* colony were resuspended in 200 µl of TE buffer (10 mMTris-HCl–1 Mm EDTA [pH 7.4] followed by boiling for 30 min, the DNA containing supernatant (cell lysate) was harvested after centrifugation and stored at -20°C until used [8].

MAS-PCR. Two inner primers, Emb306A, forward (5´-GACGACGGCTACATCCTGGGCA) and Emb306B, reverse (5´-GGTCGGCGACTCGGGC-C) were selected to stop at the first and third bases of codon306 wild-type allele ATG [8]. As a result, the strains that had no mutation in emb306 produced two allele specific bands of 160 and 210 bp and strains that had mutation in the first or third base in emb306ATG produced only either a 210 bp or 160 bp fragment, respectively (8). In addition, two outer primers Emb1F, forward (5´-GGGCGGGGCTCAATTGCC) and Emb2R, reverse (5´-GCGCATCCACAGACTGGCGTC) invariably amplified a 324 bp fragment [8]. An amount of 0.5 µl of purified DNA sample was added to the PCR mixture (final volume, 20 µl) containing 2 pmol of the Emb2R and Emb306A primers, 25 pmol of the Emb1F and Emb306B primers, 0.64 mM $MgCl_2$, 0.4U *Taq* DNA polymerase and 200 µM concentrations of each of the deoxynucleoside triphosphates. The amplifycation was performed with an initial denaturation at 95°C for 4 min; followed by 10 cycles of 94°C for 1 min, 75°C for 1 min, and 72°C for 20 sec; followed by 10 cycles of 94°C for 1 min, 74°C for 40 sec, and 72°C for 20 sec; followed by29 cycles of 94°C for 1 min, 73°C for 30 sec, and 72°C for 20 sec; with a final elongation at 72°C for 2 min.

The amplified fragments were electrophoresed in 2% standard agarose gels and visualized under UV light after ethidium bromide staining [8].

RESULTS

Out of 5029 patients that were sampled, 176 had positive culture for *M. tuberculosis*. Out of 176 M. *tuberculosis* isolates studied 48 were EMB resistant. We detected 10 MDR *M. tuberculosis* isolates, 8 with co-resistance to EMB. Moreover, 12 isolates showed co-resistance for EMB and INH, and 19 for EMB and RIF.

Out of 48 EMB resistant isolates, 34 yielded a three-band profile (160 bp, 210 bp, and 324 bp) with MAS-PCR indicating no mutation in *embB306* codon (Fig. 1). Fourteen EMB-resistant isolates showed a two-band profile (160 bp and 324 bp) implying a mutation in third base in *embB306* codon (Fig. 2). No isolate exhibited mutation in the first base of 306 codon.

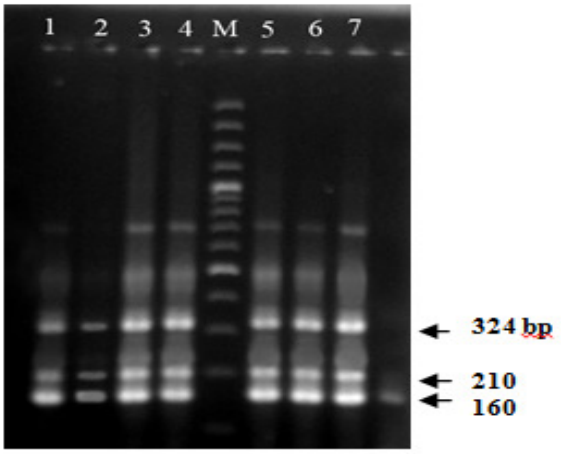

Fig. 1. MAS-PCR profiles of EMB resistant *M. tuberculosis* clinical isolates; lanes 1, 2, 3, 4, 5, 6, and 7, isolates with embB306 wild-type allele; M,100 bp molecular weight marker,Fermentas.

DISCUSSSION

Despite recent advances, TB diagnosis remains imperfect in resource-limited settings due to poor sensitivity of available tests. Although these methods have been continuously refined and improved, they still have severe limitations.

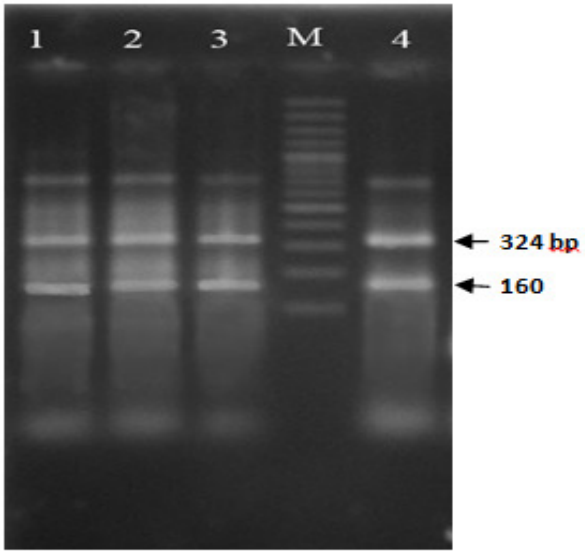

Fig. 2. MAS-PCR profiles of EMB- resistant *M. tuberculosis* clinical isolates; lanes 1, 2, 3, and 4 isoltes with mutation in third base of *embB306* codon; M: 100 bp molecular weight marker, Fermentas.

Culture is the most accurate and the gold standard method, but is labor-intensive and slow. Detection of clinical isolates of *M. tuberculosis* is initially determined by culture characteristics and biochemical tests. Such tests need more than a few weeks and eventually may not lead to a valid differentiation. Sensitivity of culture method varies from 37-56% [10]. On the other hand sensitivity of microscopic examination of sputum for TB has reported to range from 61.8% to 70% when compared to culture [4]. The estimated rate for incidence of all forms of tuberculosis is 50-149 per 100,000 populations [11]. In the present study *M. tuberculosis* was isolated from 176 (3.4%) cases of studied patients.

The initial and acquired resistance to EMB varies in different countries ranging from 1.3% to 23.3%, and is predominant among patients with MDR tuberculosis [12]. Ethambutol resistance seems to be closely linked to MDR resistance [3]. In the present study we detected 10 MDR *M. tubercu-*

losis isolates, which 8 of them had co-resistance to EMB. Moreover, 12 isolates showed co-resistance for EMB and INH and 19 for EMB and RIF. Since mono-resistance of EMB is rare, EMB is still a valuable drug for TB treatment and is being used extensively [3]. In *M. tuberculosis*, *embB* is organized into a 10 kbp operon with *embC* and *embA* genes named *embCAB*. Mutations in *embCAB* operon, in particular *embB*, are responsible for EMB resistance. About 35% of EMB-resistant isolates do not show mutations in *embB* suggesting that there may be other mechanisms for EMB resistance [2].

We have limited reports of mutation in codon *embB* 306 of EMB-resistant *M. tuberculosis* isolates; the result of present study demonstrates a high frequency of other probable mutations which may be due to the geographic conditions. Morousov *et al.* [8] studied 34 *M. tuberculosis* isolates in the St. Petersburg region of the Russian Federation and reported 24 EMB-resistant strains, which showed a two-band profile and 10 EMB sensitive that showed three-band profile. Yang *et al.* [13] studied 174 *M. tuberculosis* clinical isolates obtained from 4 different geographic regions in Turkey and reported 99.4% correlation between MAS-PCR and sequencing findings with culture-based phenotypic drug susceptibility testing as the reference standard. The sensitivity and specificity of a newly developed MAS-PCR assay for detection of EMB resistance-related mutations were determined to be 54.5% and 68.0 %, respectively [12]. The absence of mutation in 70.8% of the resistant isolates could be attributed to possible involvement of other codon position at the same gene or other genes, rather than the studied *embB*. So, the major limitation with MAS-PCR technique is that it generally detects only the known mutations. Regarding this limitation, MAS-PCR and other molecular method cannot yet completely replace the culture-based phenotypic susceptibility testing. However, since about 60% of EMB resistant clinical isolates of *M. tuberculosis* are result of mutations in *embB 306*, the MAS-PCR assay can be used as a reliable tool to detect these mutations with high probability and screen the majority of the resistant isolates, which, in turn, allows reducing the amount of phenotypic drug susceptibility testing by 29.1%, for the detection of EMB resistance. Further optimization of this method for direct examination of *M. tuberculosis* ETM (EMB) resistance mutations in clinical samples, e.g., sputum of TB patients will enable us to detect a large number of EMB resistant clinical isolates without culture.

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