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ORIGINAL RESEARCH

Characterization of the most common *embCAB* gene mutations associated with ethambutol resistance in *Mycobacterium tuberculosis* isolates from Iran

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Introduction: Ethambutol (Emb) is one of the first-line drugs in the standard combination therapy for tuberculosis; however, due to the rapid increase in Emb resistance among clinical isolates of *Mycobacterium tuberculosis* (MTB), early detection of Emb resistance is desirable. As the *embCAB* operon is considered involved in resistance to Emb, this study aimed to analyze the most common mutations within the *embCAB* operon among MTB isolates from Iran to find any correlations of these mutations with Emb resistance.

Methods: A total of 307 clinical isolates of MTB were screened for Emb resistance by phenotypic drug-susceptibility testing. PCR amplification was performed on extracted DNA from all Emb-resistant and randomly selected Emb-susceptible isolates using sets of primers for various gene loci of *embC*, *embA*, and *embB*, followed by sequencing for the detection of most common alterations.

Results: In total, ten isolates showed resistance to Emb by phenotypic susceptibility testing (3.25%). The mutation rate in ten Emb-resistant MTB strains was 20% (n=2), comprising one mutation in *embB* (10%), at codon 306 Met–Val and one in *embC* (10%) at codon 270 Thr–Ile. A nonsynonymous mutation in the *embA* gene in one of the randomly selected Emb-susceptible isolates located in codon 330 Leu–Leu was also noticed.

Conclusion: The majority of our Emb-resistant isolates (n=8, 80%) did not demonstrate the sequences investigated within the *embCAB* operon. As such, these mutations solely are insufficient for the development of complete resistance to Emb in MTB isolates. Additional mechanisms of resistance other than mutations in these sequences studied within the *embCAB* operon should also be considered.

Keywords: *Mycobacterium tuberculosis*, susceptibility testing, ethambutol, drug resistance, *embCAB*

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), remains a life-threatening disease and is still one of the most important causes of morbidity and mortality worldwide.[1] In the latest World Health Organization (WHO) global report on TB,[2] there were an estimated 1.3 million TB deaths in 2017 and an additional 300,000 deaths resulting from TB disease among HIV-positive people. The worldwide emergence of drug-resistant MTB (DR-TB), especially multi-DR TB (MDR-TB) and extensive DR-TB, is widely considered to be a serious challenge for TB control

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programs.[3,4] MDR-TB is caused by strains of MTB that do not respond to at least both isoniazid (Inh) and rifampicin (Rif), and extensive DR-TB is defined as an MDR isolate that is also resistant to a fluoroquinolone and at least one second-line injectable agent (amikacin, kanamycin, and capreomycin).[3] According to the WHO report, there were an estimated 558,000 cases of MDR Rif-resistant TB in 2017.[1] As such, the rapid detection of drug resistance is essential for appropriate treatment of the disease that avoids treatment failure and prevents the spread of DR strains.[5]

Ethambutol (Emb) is an important first-line drug that is widely used for the treatment of TB and MDR-TB.[6,7] The mechanism of action of Emb is inhibition of the synthesis of the arabinan component of lipoarabinomannan and arabinogalactan in the mycobacterial cell wall.[8] Arabinosyl transferase is encoded by the *embCAB* locus, which comprises the three genes of *embC, embA and embB. embA* and *embB* genes apparently contribute to the synthesis of arabinogalactan, whereas *embC* is reserved for the synthesis of lipoarabinomannan.[9,10] Mutations in the 10 kb *embCAB* operon are associated with resistance to Emb, especially the *embB* gene.[11] The most commonly detected mutations have occur at codons 306, 406, and 497 within the *embB* gene. Therefore, these codons represent promising diagnostic markers for the rapid detection of Emb resistance.[12] Five distinct missense mutations are found in codon 306 with changes in the first or third base (ATG, GTG, CTG, ATA, ATC, or ATT), resulting in the replacement of methionine by three different amino acids (Met to Val, Leu, or Ile).[13] As the *embCAB* operon (*embC*, *embA*, and *embB*) of MTB is considered involved in resistance to Emb,[14] this study aimed to analyze the most common mutations within he *embCAB* operon among MTB isolates from Iran to find any correlation of these mutations with Emb resistance.

Methods

Sample collection and bacterial strains

A total of 307 clinical isolates of MTB were detected in 906 samples during a 16-month period from February 2016 to June 2017. The samples were obtained from patients with suspected pulmonary TB diagnosed by infectious-disease specialists and referred to the regional tuberculosis laboratory of Khuzestan province, Iran. The preliminary proposal of the work was approved in joint by the institutional review board and ethics committee of Ahvaz Jundishapur University of Medical Sciences, Iran, and necessary permission was granted for sample collection. Apart from this, as part of the regional center policy, referred patients were requested to sign the informed consent in case their samples were used for research purposes apart from routine clinical investigation, and our study was conducted in accordance with the Declaration of Helsinki. Isolates were identified as MTB on the basis of acid-fast-stain microscopy, growth in modified Löwenstein–Jensen (LJ) medium, and performance of conventional biochemical tests, including niacin accumulation, nitrate reductase, and catalase at 37°C and 68°C.[15]

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) for first-line anti-TB drugs was performed on LJ medium using a proportional method according to the Clinical and Laboratory Standard Institute guideline.[16] The following critical drug concentrations were used for AST: 0.25 µg/mL for Inh, 0.02 µg/mL for Rif, and 4 µg/mL for Emb (Sigma-Aldrich, St Louis, MO, USA). H37Rv (ATCC 27294) was used as the control for AST. Susceptibility was defined as no or <1% growth on LJ medium-containing drugs compared with the control medium.

DNA extraction

DNA was extracted from MTB grown on LJ medium by a simple boiling method as previously described.[17] The concentration of extracted DNA was measured by biophotometry (Eppendorf, Hamburg, Germany) at 260 nm, and samples were stored at −20˚C until use.

PCR amplification

PCR amplification was performed in an Eppendorf thermocycler (Roche, Basel, Switzerland) by application of primers designed for various gene loci of *embC*, *embA*, and *embB*,[18,19] as presented in Table 1.

Amplification reactions in a total volume of 50 µL containing 5 µL 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µM of each primer, 2.5 U *Taq* polymerase, and 5 µL template DNA (10 ng) were prepared. Amplification conditions were: *embB* gene – initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 40 seconds, primer annealing at 48°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes; *embA* and *embC* genes – initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 40 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. H37Rv (ATCC 27294) was used as a reference strain. PCR products were loaded on a 1.5% (w:v) agarose gel with 0.5 µg/mL ethidium bromide,

Table 1 Oligonucleotide primer sequences employed for amplification of the *embCAB* genes

	Sequence	Annealing temperature (°C)	PCR-product size (bp)
embA	5'-GCCGGCTATGTAGCCAACTA-3'(F) 5'-GACCGTTCCACCAACACC-3'(R)	63	338
embB	5'-CTGCTCTGGCATGTCAT-3'(F) 5'-AGCGGAAATAGTTGGAC-3'(R)	48	103
embC	5'-GATACCCGCTACAGCAGCA-3' (F) 5'-GGTCGTAGTACCAGCCGAAA-3'(R)	63	334

analyzed by gel electrophoresis, and photographed using the gel-documentation system (ProteinSimple, San Jose, CA, USA). SPSS version 16 was used for data analysis. PCR products were sent to Bioneer (Daejeon, South Korea) for sequencing.

Results

From the total 906 sputa processed, 307 (33.88%) were identified as MTB on the basis of culture and biochemical criteria. These belonged to 233 men and 74 women with a mean age of 33 years. In total, 26 isolates (8.47%) showed resistance to the antimicrobial agents tested. The resistance profiles of the 307 MTB isolates according to AST were Inh 6 (1.95%), Rif 10 (3.25%), and Emb 10 (3.25%). The prevalence of MDR was four (1.30%), three of which showed coresistance to Emb (Table 2).

The ten Emb-resistant and 28 randomly selected Emb susceptible isolates were processed for characterization of *embCAB* genes. According to the sequencing analyses, the mutation rate in the Emb-resistant MTB strains was 20% (n=2), comprising one mutation in *embB* (10%) at codon 306 Met–Val (ATG→GTG) and one in *embC* (10%) at codon 270 Thr–Ile (ACC→ATC). No mutation in *embA* was demonstrated in Emb-resistant isolates in this study (Table 3). Only one of the MDR strains with coresistance to Emb showed a mutation in *embB*. Moreover, we noticed a nonsynonymous mutation in the *embA* gene in one of the randomly selected Emb-susceptible isolates, located in codon 330 Leu–Leu (CTG→TTG). In Table 4, the mutations detected in all Emb-resistant and selected Emb-susceptible isolates are presented.

Table 2 Drug-susceptibility profile of resistance phenotypes among confirmed MTB isolates (n=307)

Resistant-isolate code	Inh	Rif	Emb
274	S	R	S
281	S	R	S
341	S	R	S
410	S	R	S
475	R	S	S
577*	R	R	R
731*	R	R	S
924	S	R	S
32	R	S	S
646*	R	R	R
108	S	R	S
67*	R	R	R
196	S	S	R
642	S	S	R
658	S	S	R
288	S	S	R
624	S	S	R
41	S	S	R
684	S	S	R
Resistance rate, n (%)	6 (1.95%)	10 (3.25%)	10 (3.25%)

Note: *MDR isolates.
Abbreviations: Emb, ethambutol; Inh, isoniazid; R, resistant; Rif, rifampicin; S, sensitive; MDR, multidrug-resistant.

Discussion

Emb is used as an alternative drug to streptomycin in the standard four-drug combination TB therapy; however, due to the rapid increase in Emb resistance among clinical isolates of MTB, early accurate detection of Emb resistance is essential to avoid the risk of adverse reactions, particularly optic neuritis.[20] In this study, molecular methods based on *embCAB*-gene mutations were used to investigate the nature of Emb resistance in MTB isolates. DNA-sequencing analysis of *embCAB* loci in ten Emb-resistant and 26 Emb-susceptible isolates revealed three polymorphic nucleotide sites, each located in different regions of the three gene loci. We demonstrated only two mutations in Emb-resistant strains (20%), one a common mutation with alteration of methionine–valine at codon 306. Alterations at codon 306 of *embB* have been demonstrated as the most common alterations in Emb-resistant MTB clinical isolates.[19,21] Moreover, high detection rates of mutations at codon *embB*306 among Emb-resistant MTB isolates have been reported in recent years from China (55%),[22] Cuba and the Dominican Republic (70%),[23] and Germany (68%).[24]

The second mutation occurred at codon 270 of *embC,* with alteration in threonine–isoleucine. This mutation has also been described previously with less frequency among

Table 3 Mutation pattern of *embCAB* genes in ten ethambutol-resistant isolates with different drug-resistance phenotypes

Resistant-isolate code (S)	Affected codon	Resistance phenotype, Inh/Rif	Nucleotide change, *embA*	Nucleotide change, *embB*	Nucleotide change, *embC*
67	306	+/+ (MDR)	–	Met to Val (ATG→GTG)	–
577	None	+/+ (MDR)	–	–	–
646	None	+/+ (MDR)	–	–	–
658	270	–/–	–	–	Thr to Ile (ACC→ATC)
196, 642, 288, 624, 41, 684	None	–/–	–	–	–

Abbreviation: MDR, multidrug-resistant.

Table 4 Characteristics of ten ethambutol-resistant and 26 randomly selected ethambutol-susceptible MTB isolates with *embCAB* mutations

Phenotype	Isolate code	*embA*	*embB*	*embC*
R (MDR)	67	Wild type	ATG→GTG (Met306Val)	Wild type
R	658	Wild type	Wild type	ACC→ATC (Thr270Ile)
R	196	Wild type	Wild type	Wild type
R	642	Wild type	Wild type	Wild type
R	288	Wild type	Wild type	Wild type
R	624	Wild type	Wild type	Wild type
R	41	Wild type	Wild type	Wild type
R	684	Wild type	Wild type	Wild type
R (MDR)	646	Wild type	Wild type	Wild type
R (MDR)	577	Wild type	Wild type	Wild type
S	732	CTG→TTG (Leu330Leu)	Wild type	Wild type
S	427	Wild type	Wild type	Wild type
S	71	Wild type	Wild type	Wild type
S	731	Wild type	Wild type	Wild type
S	753	Wild type	Wild type	Wild type
S	893	Wild type	Wild type	Wild type
S	520	Wild type	Wild type	Wild type
S	416	Wild type	Wild type	Wild type
S	30	Wild type	Wild type	Wild type
S	848	Wild type	Wild type	Wild type
S	66	Wild type	Wild type	Wild type
S	919	Wild type	Wild type	Wild type
S	668	Wild type	Wild type	Wild type
S	969	Wild type	Wild type	Wild type
S	924	Wild type	Wild type	Wild type
S	303	Wild type	Wild type	Wild type
S	109	Wild type	Wild type	Wild type
S	104	Wild type	Wild type	Wild type
S	128	Wild type	Wild type	Wild type
S	20	Wild type	Wild type	Wild type
S	744	Wild type	Wild type	Wild type
S	86	Wild type	Wild type	Wild type
S	14	Wild type	Wild type	Wild type
S	935	Wild type	Wild type	Wild type
S	108	Wild type	Wild type	Wild type
S	600	Wild type	Wild type	Wild type

Abbreviations: MTB, *Mycobacterium tuberculosis*; MDR, multidrug-resistant.

Emb-resistant isolates of MTB. We have also identified a nonsense mutation in one Emb-susceptible isolate in the *embA* gene as Leu330Leu. The region of *EmbB* containing residue Met306 is highly conserved in MTB, *Mycobacterium avium*, *Mycobacterium leprae*, and *M. smegmatis* strains,[25] and the data generated from the Emb-susceptibility testing

indicate a strong nonrandom association between certain amino-acid substitutions at *embB* position 306 and the level of resistance to Emb.[26] We did not find any other mutations in the rest of the Emb-resistant MDR strains, and our results suggest that *embB*306 and *embC270* mutations do not cause Emb resistance in any MTB isolates, and other mutations, including the involvement of less common mutations in codons 406 and 497, should be considered for resistance to this antibiotic in isolates. Moreover, we cannot rule out a contribution of mutations outside the *embCAB* operon that could have been coselected during growth in the presence of Emb. Gene mutations only modestly increase resistance to Emb in MTB isolates. Our findings were in line with a previous study conducted by Safi et al,[27] demonstrating that although *embB*306 mutations are necessary for Emb resistance, no resistant strains were detected by these mutations.

Although in this study the large number of MTB isolates (n=307) was investigated, the number of Emb-resistant isolates was quite low (n=10, 3.25%). However, according to previous reports from Iran, the resistance rate of MTB to Emb varied widely: from 1% in Tavanaee et al[28] and 4.2% in Seif et al[29] to as high as 14% in Farazi et al.[30] Similarly, the prevalence of MDR in our study was also low (n=4, 1.30%), three of which showed coresistance to Emb as well, and in only one Emb-resistant MDR isolate did we find the 306 *embB* mutation.

Among the Emb phenotypic resistant isolates, only two were detected by mutations within the *embCAB* operon. The discordance between phenotypic AST of Emb and mutations in the *embCAB* operon has also been reported by investigators from other countries and Iran.[31–33] In the study from Iran, *embB* mutations were investigated in a few Iranian cities, and based on their results from four Emb-resistant isolates recovered from Tehran, only one 306 mutation was detected, and in some cities no 306 mutation was demonstrated among the phenotypic Emb-resistant isolates.[33] In the current study, we investigated the most common mutation sites within the three genes *embA, embB*, and *embC*. Further studies should investigate other mutations within and outside this operon over an extended period, as other investigators have concluded that a few mutations conferring Emb resistance may occurred outside this operon.[13]

Conclusion

Few of our Emb-resistant isolates (n=8, 80%) were demonstrated by mutations in investigated sequences within the *embCAB* operon. As such, these mutations alone are insufficient for the development of full resistance to Emb in MTB strains. Additional mechanisms of resistance other than mutations in the current studied sequences within the *embCAB* operon should also be considered. Although this study demonstrates a low level of Emb resistance in our region, for the control of drug-resistance spread, regular monitoring is necessary to maintain a lower resistance rate, which is crucial for the treatment strategy and management of TB.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. WHO. Drug resistant tuberculosis. Geneva: World Health Organization; 2018. Available from: https://www.who.int/tb/areas-of-work/drug-resistant-tb/en. Accessed September 26, 2018.
2. WHO. Global tuberculosis report. Geneva: World Health Organization; 2018. Available from: https://www.who.int//iris/bitstream/handle/10665/274453/9789241565646-eng.pdf. Accessed September 26, 2018.
3. Lange C, Abubakar I, Alffenaar JW, et al. Management of patients with multidrug-resistant/extensively drug-resistant tuberculosis in Europe: a TBNET consensus statement. *Eur Respir J*. 2014;44(1):23–63.
4. Kerantzas CA, Jacobs WR. Origins of combination therapy for tuberculosis: lessons for future antimicrobial development and application. *MBio*. 2017;8(2):e01586–16.
5. Brossier F, Veziris N, Aubry A, Jarlier V, Sougakoff W. Detection by GenoType MTBDRsl test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol*. 2010;48(5):1683–1689.
6. Caminero JA, Sotgiu G, Zumla A, Migliori GB. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. *Lancet Infect Dis*. 2010;10(9):621–629.
7. Daley CL, Caminero JA. Management of multidrug resistant tuberculosis. *Semin Respir Crit Care Med*. 2013;34(1):44–59.
8. Jackson M, Mcneil MR, Brennan PJ. Progress in targeting cell envelope biogenesis in *Mycobacterium tuberculosis*. *Future Microbiol*. 2013;8(7):855–875.
9. Mikusová K, Slayden RA, Besra GS, Brennan PJ. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. *Antimicrob Agents Chemother*. 1995;39(11):2484–2489.
10. Jankute M, Grover S, Rana AK, Besra GS. Arabinogalactan and lipoarabinomannan biosynthesis: structure, biogenesis and their potential as drug targets. *Future Microbiol*. 2012;7(1):129–147.
11. Parsons LM, Salfinger M, Clobridge A, et al. Phenotypic and molecular characterization of *Mycobacterium tuberculosis* isolates resistant to both isoniazid and ethambutol. *Antimicrob Agents Chemother*. 2005;49(6):2218–2225.
12. Wang T, Jiao WW, Shen AD. Progress on mechanism of ethambutol resistance in *Mycobacterium Tuberculosis*. *Yi Chuan*. 2016;38(10):910–917.

13. Sugawara I, Otomo K, Yamada H, et al. The molecular epidemiology of ethambutol-resistant *Mycobacterium tuberculosis* in Henan Province, China. *Jpn J Infect Dis*. 2005;58(6):393–395.
14. Zhao LL, Sun Q, Liu HC, et al. Analysis of *embCAB* mutations associated with ethambutol resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates from China. *Antimicrob Agents Chemother*. 2015;59(4):2045–2050.
15. Kent PT, Kubica GP. *Public Health Mycobacteriology: A Guide for the level III Laboratory*. Atlanta, GA: Centers for Disease Control, U.S. Department of Health and Human Services; 1985.
16. Desmond E. *Susceptibility testing for Mycobacteria, Nocardiae, and other Aerobic Actinomycetes; Approved Standard*. CLSI Document M24-A2. 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2011.
17. Hosek J, Svastova P, Moravkova M, Pavlik I, Bartos M. Methods of mycobacterial DNA isolation from different biological material: a review. *Veterinarni Medicina*. 2012;51(No. 5):180–192.
18. Xu Y, Jia H, Huang H, Sun Z, Zhang Z. Mutations found in embCAB, embR, and ubiA genes of ethambutol-sensitive and -resistant Mycobacterium tuberculosis clinical isolates from China. *Biomed Res Int*. 2015;2015:1–8.
19. Tracevska T, Jansone I, Nodieva A, Marga O, Skenders G, Baumanis V. Characterisation of *rpsL*, *rrs* and *embB* mutations associated with streptomycin and ethambutol resistance in *Mycobacterium tuberculosis*. *Res Microbiol*. 2004;155(10):830–834.
20. Frieden TR, Sterling TR, Munsiff SS, Watt CJ, Dye C. Tuberculosis. *Lancet*. 2003;362(9387):887–899.
21. Ramaswamy SV, Dou SJ, Rendon A, Yang Z, Cave MD, Graviss EA. Genotypic analysis of multidrug-resistant *Mycobacterium tuberculosis* isolates from Monterrey, Mexico. *J Med Microbiol*. 2004;53(Pt 2):107–113.
22. Shi D, Li L, Zhao Y, et al. Characteristics of *embB* mutations in multidrug-resistant *Mycobacterium tuberculosis* isolates in Henan, China. *J Antimicrob Chemother*. 2011;66(10):2240–2247.
23. Guerrero E, Lemus D, Yzquierdo S, et al. Association between *embB* mutations and ethambutol resistance in *Mycobacterium tuberculosis* isolates from Cuba and the Dominican Republic: reproducible patterns and problems. *Rev Argent Microbiol*. 2013;45(1):21–26.
24. Plinke C, Rüsch-Gerdes S, Niemann S. Significance of mutations in embB codon 306 for prediction of ethambutol resistance in clinical *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Chemother*. 2006;50(5):1900–1902.
25. Cui Z, Li Y, Cheng S, et al. Mutations in the *embC-embA* intergenic region contribute to *Mycobacterium tuberculosis* resistance to ethambutol. *Antimicrob Agents Chemother*. 2014;58(11):6837–6843.
26. Srivastava S, Ayyagari A, Dhole TN, Nyati KK, Dwivedi SK. emb nucleotide polymorphisms and the role of embB306 mutations in *Mycobacterium tuberculosis* resistance to ethambutol. *Int J Med Microbiol*. 2009;299(4):269–280.
27. Safi H, Sayers B, Hazbón MH, Alland D. Transfer of *embB* codon 306 mutations into clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol, isoniazid, and rifampin. *Antimicrob Agents Chemother*. 2008;52(6):2027–2034.
28. Tavanaee Sani A, Shakiba A, Salehi M, Bahrami Taghanaki HR, Ayati Fard SF, Ghazvini K. Epidemiological Characterization of Drug Resistance among *Mycobacterium tuberculosis* Isolated from Patients in Northeast of Iran during 2012–2013. *BioMed Res Int*. 2015;2015(1):1–6.
29. Seif S, Malekshahian D, Shamsi M. Prevalence of primary ethambutol resistance in new smear-positive pulmonary TB cases. *Int J Mycobacteriol*. 2015;4:171–172.
30. Farazi A, Sofian M, Zarrinfar N, Katebi F, Hoseini SD, Keshavarz R. Drug resistance pattern and associated risk factors of tuberculosis patients in the central province of Iran. *Caspian J Intern Med*. 2013;4(4):785–789.
31. Aung HL, Tun T, Moradigaravand D, et al. Whole-genome sequencing of multidrug-resistant *Mycobacterium tuberculosis* isolates from Myanmar. *J Glob Antimicrob Resist*. 2016;6:113–117.
32. Casali N, Nikolayevskyy V, Balabanova Y, et al. Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nat Genet*. 2014;46(3):279–286.
33. Rezaei F, Haeili M, Mohajeri P, et al. Frequency of mutational changes in the *embB* among the ethambutol-resistant strains of *Mycobacterium tuberculosis* in Iran. *J Infect Dev Ctries*. 2016;10(4):363–368.