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ORIGINAL RESEARCH

Investigation of Drug Resistant *Mycobacterium tuberculosis* Strains with Molecular Methods

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ABSTRACT

The aim of this study was to investigate mutations in the ***rpo*B** and ***emb*B** genes using molecular methods in *Mycobacterium tuberculosis* isolates phenotypically resistant to rifampicin and/or ethambutol. Drug susceptibility testing of 110 isolates collected in Turkey between 2005 and 2014 was performed using the modified agar proportion method. Molecular analysis was conducted on 28 selected isolates, including 7 rifampicin-resistant strains. Mutations were detected within the Rifampicin Resistance-Determining Region (RRDR) between codons 507–533 of the ***rpo*B** gene. The most common mutation was **Ser531Leu (TCG→TTG)**, found in 10 strains; one strain had a **His526Asn (CAC→AAC)** mutation. These mutations were considered important molecular markers of rifampicin resistance. Rapid molecular detection of these mutations is important for early and effective tuberculosis treatment planning

Keyword: *Mycobacterium tuberculosis*. Drug resistance. Molecular analysis.

Moleküler Yöntemlerle İlaç Dirençli *Mycobacterium tuberculosis* Suşlarının Araştırılması

ÖZET

Bu çalışmanın amacı, fenotipik olarak rifampisin ve/veya etambutol dirençli *Mycobacterium tuberculosis* izolatlarında, ***rpo*B** ve ***emb*B** genlerindeki mutasyonları moleküler yöntemlerle araştırmaktır. 2005–2014 yılları arasında Türkiye'den toplanan 110 izolatın ilaç duyarlılıkları modifiye agar proporsiyon yöntemiyle test edilmiştir. Seçilen 28 suşta moleküler analiz yapılmış, 7'si rifampisin dirençli bulunmuştur. ***rpo*B** geninde, Rifampisin Direnç Belirleyici Bölge (RRDR) olan 507–533. kodonlar arasında mutasyonlar tespit edilmiştir. En yaygın mutasyon, 10 suşta görülen **Ser531Leu (TCG→TTG)** mutasyonu, bir suşta ise **His526Asn (CAC→AAC)** mutasyonudur. Bu mutasyonlar, rifampisin direncinin moleküler göstergeleri olarak önemli bulunmuştur. Moleküler yöntemlerle bu mutasyonların hızlı tespiti, tüberküloz tedavisinin erken planlanması için önemlidir.

Anahtar Kelimeler: *Mycobacterium tuberculosis*. Ilaç direnci. Moleküler analiz.

Tuberculosis is an important public health problem all over the world despite modern diagnostic, treatment, and control methods.[1] The gold standard for diagnosis is still *Mycobacterium tuberculosis* culture, which is essential for identification and drug susceptibility testing.[2]

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Rifampicin (RIF) and ethambutol (ETB), both of which have antimycobacterial effects, are major anti-tuberculosis drugs used in the treatment of the disease. RIF affects bacterial protein synthesis, while ETB inhibits arabinogalactan synthesis through inhibition of the arabinosyl transferase enzyme.[3]

Multidrug resistance in *Mycobacterium tuberculosis* represents a global health burden.[4] Resistance to RIF in the *M. tuberculosis* complex is caused by mutations in the 81-base pair (bp) region of the **rpoB** gene, which encodes the β-subunit of RNA polymerase.[4–7] The frequency of codon mutations in the *rpoB* gene among RIF-resistant *M. tuberculosis* isolates varies by geographic region. Sequence analysis of the *rpoB* gene in 37 isolates from Italy revealed mutations in codons 531 (59.4%), 526 (35.1%), and 516 (8.1%).[7] Data from 86 isolates in China showed mutations at codons 531 (41.0%), 526 (40.0%), 516 (4.0%), 513 (2.0%), and 533 (2.0%).[8] Two reports from Taiwan in the past decade also analyzed the prevalence of *rpoB*

mutations.[8–10] Further surveillance is clearly needed to monitor the evolution of mutations and their associated resistance.

This study aimed to investigate the presence of mutations in the **embB** and **rpoB** gene regions, as well as mutation patterns, through DNA sequence analysis of isolates that exhibited phenotypic resistance to RIF and/or ETB in drug susceptibility tests.

Material and Method

M. tuberclosis isolates

In our study, a hundred ten *M. tuberculosis* samples, which detected at least one phenotypic anti-tuberculosis drug resistance, were collected the Tuberculosis Laboratory of the Medical Microbiology Department of the Uludağ University Health Practice and Research Center between 2005 and 2014. Seven of these strains were only resistance to RIF, 15 were ETB, 6 were both. Sterile clinical specimens were centrifuged and then centrifuged at the bottom of the centrifuge tube. Non-sterile specimens were inoculated to Löwenstein-Jensen and MGIT media after homogenization-decontamination and incubated at 37 ° C in an incubator for 6 weeks. Strains were separated from MOTT using the PNB test and the MGIT TBc test.

DNA isolation and Polymerase chain reactions (PCR)

DNA was extracted from the *M. tuberculosis* using a Tris-EDTA. DNA from the samples was amplified with selected primers designed with Ensembl (Table I).

Table I. Primers for the amplification of *rpo*B and *emb*B.

Gene	Primers	Base-pair
*rpo*B-F	CTTGCACGAGGGTCAGACCA	543
*rpo*B-R	ATCTCGTCGCTAACCACGCC	
*emb*B-F	CTGACCGACGCCGTGGTGATAT	490
*emb*B-R	TGAATGCGGCGGTAACGACG	

Amplifications with Taq polymerase were performed in 25-µl reaction mixtures containing 0.15 mM of deoxyribonucleoside triphosphate (dNTP, Promega-U1515, USA), 10 pmol of each primer, 5 U/ml of Taq DNA polymerase (Promega-M8305, USA), and 150 ng of genomic DNA. Polymerase chain reactions (PCR) were performed with a 2-min initial denaturation at 94°C, followed by 30 cycles of 1 min at 94°C, 30 s at 55°C, 1 min at 72°C, and a 10-min final extension at 72°C. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

Sequencing analysis

The PCR products were purified according to the manufacturer's instructions by OMEGA. The RIF-resistance-determining hot-spot region of *rpo*B and *emb*B samples was sequenced by Dye Terminator Cycle Sequencing (DTCS) and analyzed using a CEQ-8000 Automated DNA Sequencing System (Beckman Coulter, Inc., Fullerton, CA, USA). The results of the sequencing analysis were compared with wild-type samples and the wild-type sequences of these genes. The relationships between the defined alterations and the risk of resistance were verified using the Ensembl Genome Browser (http://www.ensembl.org).

This study was conducted with the approval of the Clinical Research Ethics Committee of Uludağ University Faculty of Medicine (decision dated 09.06.2015, no. 2015-12/17) and was supported by the Uludağ University Scientific Research Projects Unit (project no. KUAP(T)-2015/43).

Results

In our study, *M. tuberculosis* complex strains that detected at least one phenotypic anti-tuberculosis drug resistance were investigated in the Tuberculosis Laboratory of the Medical Microbiology Department of Medical Utilization Research Center of Uludağ University between 2005 and 2014. These tuberculosis complex strains examined belong to 105 patients. The samples were mostly sent from the Department of Chest Diseases (60%) followed by the Department of Infectious Diseases and Pediatric Infectious Diseases. A total of 110 strains were tested; 75 strains (68.2%) had single-strand resistance, 23 (21%) had two strains, and 12 strains (11%) had very little resistance. Molecular analysis included a total of 28 strains, 7 with RIF resistance, 15 with ETB resistance, and 6 with both RIF and ETB resistance, and investigated the presence of mutations in the *rpo*B and *emb*B gene regions. In our study, a mutation in the *rpo*B gene was detected in 11 (84.6%) of 13 strains phenotypically resistant to RIF. Two of them (16.4%) were phenotypically resistant to RIF, but no mutations were observed in the examined gene locus. Mutations detected were found between codons 507 and 533, which are defined as the RRDR region. Mutation is detected in 11 strains of 10 strains, with the Ser531Leu (TCG-TTG) mutation found in 531. (Figure-1). In one strain, the His526Asn (CAC-AAC) mutation was detected (Figure-2).

In 21 strains resistant to ETB phenotypically, mutation in *the embB* gene was detected in 14 (66.6%). (Figure 3). Seventy-three percent (33.3%) were phenotypically resistant to ETB, but no mutation was observed in the examined gene locus. Mutations were detected in 14 strains, 3 in binary mutation and 11 in single mutation.

The most frequently identified mutation was the Ser297Ala (TCG-GCG) mutation in the 297th codon and was detected in 8 strains. In four strains, the Met306Val (ATG-GTG) mutation was detected at the 306th codon.

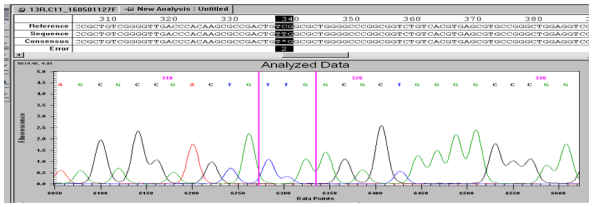

Figure 1.
Codon 531 mutation (Mutation is shown in black).

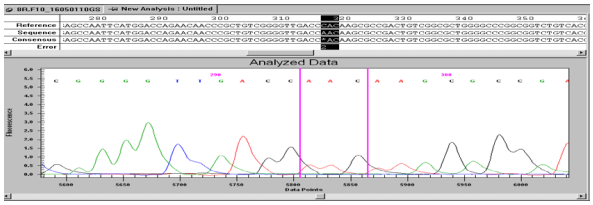

Figure 2:
Codon 526 mutation (Mutation is shown in black)

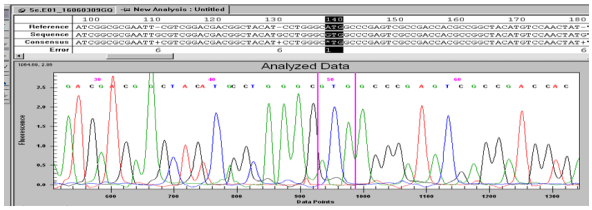

Figure 3:
Codon 306 mutation. The location of the mutation is shown in black.

Discussion and Conclusion

Although tuberculosis has a very long history, it remains an infectious disease that continues to evolve. Despite precise knowledge of its cause and the availability of effective medications, high morbidity rates still contribute to significant mortality.[11,12] Early diagnosis, effective and regular treatment of tuberculosis patients, and appropriate follow-up of treatment are the foundations of the tuberculosis control program.[13] One of the most important roles of the microbiology laboratory in tuberculosis control is the rapid and reliable detection of drug resistance. Culture-based phenotypic susceptibility testing for *Mycobacterium tuberculosis* is time-consuming and labor-intensive, which has led to the widespread use of molecular methods.

Rifampicin (RIF) is one of the most effective drugs in tuberculosis treatment and forms the basis of combination therapy alongside isoniazid. Numerous studies have shown that RIF resistance-associated mutations most frequently occur in codons 531, 526, and 516.[14–19]

Ethambutol (ETB) is a narrow-spectrum, first-line antimycobacterial agent used in tuberculosis treatment. It plays an important role in combination therapy and enhances the efficacy of RIF, aminoglycosides, and quinolones.[20] The most frequent mutations in the *embB* gene of ETB-resistant strains are missense mutations at codon 306.[21] However, Mokrousov et al. identified the embB 306 mutation in both ETB-sensitive and ETB-resistant strains.[22]

In our study, a total of 21 strains phenotypically resistant to ETB were examined, and mutations in the *embB* gene were found in 14 of them (66.6%). No mutations were detected in the examined gene region in 7 strains (33.3%) despite phenotypic resistance. Among the 14 mutated strains, 11 had single mutations and 3 had double mutations. The most frequently observed mutation was Ser297Ala (TCG → GCG) at codon 297, found in 8 strains. Four strains had the Met306Val (ATG → GTG) mutation at codon 306. Additional mutations were detected at codons 405 (Glu405Asp: GAG → GAC), 406 (Glu408Asp: GGC → GAC), and 378 (Glu378Ala: GAG → GCG). Our findings of mutations at codons 378, 405, and 406 are consistent with the results of Campbell et al.[23]

Mutations at different *rpoB* codons are associated with varying levels of RIF resistance. Mutations at codons 531 and 526 are typically linked to high-level resistance (MIC > 64 µg/mL) and cross-resistance to all rifamycins, whereas mutations at codon 516 are associated with moderate-level resistance (MIC = 32 µg/mL) and susceptibility to rifabutin.[24–27] However, in India, some isolates with mutations at codons 516 or 533 were shown to have high-level resistance (MIC > 128 µg/mL).[13] Although MIC testing was not performed in our study, multidrug resistance was observed in all strains with mutated alleles.[11,23]

In previous studies, strains with the same *rpoB* genotype from different geographic regions showed similar drug resistance patterns.[14,23] In contrast, the 14 isolates with allele 2 in our study demonstrated three different resistance profiles. These differences may result from the selective pressure of therapeutic regimens over time in various geographic regions.[12]

As a result, the presence of genes and mutations associated with RIF and ETB resistance in *M. tuberculosis* strains isolated from Bursa and surrounding regions was investigated for the first time. We believe that these findings will contribute to the literature not only for the Marmara Region but also for our country, even though the study was conducted with regional strains.

Molecular methods are important for the rapid diagnosis of *M. tuberculosis* and the identification of resistance. DNA sequence analysis of drug resistance-related genes is recommended as a reference method for establishing proper treatment protocols. However, the absence of mutations in frequently studied gene regions of phenotypically resistant strains complicates diagnosis and suggests the need to study additional gene regions. Conversely, detecting mutations in strains that are phenotypically susceptible may be the only way to reveal existing genetic alterations. Still, such findings are only meaningful if the corresponding proteins encoded by the mutated genes are also present and functional. Therefore, resistance to antituberculous drugs may open new research areas in proteomics.

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Idea and design: M.P.; Data collection and processing: S.A.; Analysis and interpretation of data: C.Ö.; Writing of significant parts of the article: M.P., C.Ö., S.S.
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