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Genetic Patterns of *rpsL* and *rrs* Genes in Clinical Isolates of *Mycobacterium tuberculosis*, Isfahan, Iran

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Abstract

Drug-resistant tuberculosis is considered a major universal problem. Based on knowledge on certain mutations occurring in *Mycobacterium tuberculosis* genome, drug resistance could be detected timely. The goal of this study was to determine the prevalence of the most common mutations likely to result from resistance to streptomycin in *M. tuberculosis* isolates, as well as genetic patterns of *rpsL* and *rrs* genes, in the province of Isfahan, Iran.

Clinical specimens were collected from individuals suspected of tuberculosis who referred to the Tuberculosis Center of Isfahan among whom 205 isolates were diagnosed with *M. tuberculosis* by conventional methods. The minimum inhibitory concentration of streptomycin in these isolates was determined with proportion method using Lowenstein- Jensen medium from which 10 isolates were recognized with streptomycin-resistant tuberculosis. The nucleotide sequence of *rpsL* and 530 loop of *rrs* genes were analyzed in all streptomycin-resistant isolates, in addition to five randomly selected streptomycin-susceptible isolates.

Six (6/10, 60%) streptomycin-resistant isolates represented a mutation in either *rpsL* gene and/or *rrs*530 loop. Four (40%) isolates showed *rpsL* mutations (codons 43 and 88), and two (20%) of them alterations in *rrs* gene (A514C and C517T). However, no mutation was found in streptomycin-susceptible isolates in either of the genes.

The study could successfully highlight the positive effects of rpsL and rrs mutations as molecular markers of streptomycin resistance in *M. tuberculosis strains.* Diversity and presence or absence of mutations suggested possible circulation of a variety of strains and the role of additional mechanisms contributing to strstreptomycin resistance in various regions.

Keywords: *M. tuberculosis*; *rpsL* gene; *rrs* gene; streptomycin resistant; PCR

Introduction

Tuberculosis (TB) is one of the main lethal infectious diseases worldwide, with 9.6 million people affected and 1.5 million deaths, in 2014 [1]. Over 95% of TB deaths tend to occur in low- or middle-income countries (http://www.who.int/mediacentre/factsheets/fs104/en/). In Iran, the estimated incidence of all TB types was 17,000, case detection rate was 22 (per 100,000 population), and mortality rate of TB cases (all forms, excluding in HIV positive patients) was 3.5 (per 100,000 population), in 2014 (http:// who.int/tb/country/data/profiles/en/index.html). The disease still remains a global concern with an increasing rate due to drug-resistant TB [2]. Globally, about 3.3% of new and 20% of formerly treated cases were diagnosed as Multi-drug-resistant Tuberculosis (MDR-TB), with the rates remaining almost unchanged in recent years. In 2014, there were approximately 480,000 (range: 360,000–600,000) new cases of MDR-TB worldwide, and roughly 190,000 (range: 120,000–260,000) deaths from MDR-TB [3]. Among patients with pulmonary TB who were diagnosed in 2014, on average 300,000 (range: 220,000–370,000) suffered from MDR-TB. More than half of these patients were from India, China, and Russia. In Iran, there was approximately 0.8% (0.30–1.4%) of new TB cases with MDR-TB in the same year [3].

Streptomycin (STR), an aminocyclitol glycoside antibiotic, was the first drug to treat TB in the 1940s becoming the first-line antibiotic in all forms of TB. Two years after taking STR, in the same decade, *M. tuberculosis* showed resistance to STR due to the drug's unique use and monotherapeutic application [4-6]. The resistance together with the drug's strong adverse effects, such as ototoxicity, nephrotoxicity, fetal auditory toxicity and neuromuscular paralysis, resulted in declines in its prescription in recent years [7,8]. Nevertheless, due to increases in MDR-TB strains worldwide, STR is still considered an integral component of TB treatment regimens. The medicine is still regarded as an important anti-TB drug in China and many third-world countries. It is the first-line agent in drug-resistance TB cases, susceptible to aminocyclitol glycoside antibiotic [2,8-10].

STR tends to exert its effect by inhibiting protein translation. It binds to ribosomal protein S12 and *16S rRNA* gene, the components of 30S subunit of bacterial ribosome, by disrupting the relationship

between the two components, interfering with proofreading system, and, finally, inhibiting protein synthesis. *M. tuberculosis* resistance to STR is generally associated with mutations in genes encoding these two constituents, i.e rpsL (encoding ribosomal protein S12), and *rrs* genes (encoding 16S rRNA). The most common mutations of rpsL and rrs genes have been showed in Table 1.

Gene	Position
rpsL	K43R (AAG→AGG)
	K88R (AAG→AGG)
rrs	530 Loop
	912 region

Table 1: The most common mutations in *rpsL* and *rrs* genes.

The most common mutation is likely to relate to *K43R* in *rpsL* gene. Approximately, 70% of STR-resistant *M. tuberculosis* strains have shown mutations in one of these genes. However, in the remaining strains, the cause of drug resistance remains unknown [11-23].

Rapid identification of drug resistance patterns of *M. tuberculosis* clinical isolates would be a requisite in prompt management of effective chemotherapy and proper initiation of TB treatment, and, as a result, prevention of DR-TB strain transmission. Since mutation pattern of the mentioned genes of *M. tuberculosis* strains isolated from patients in Isfahan was not determined prior to this study, the main goal here was to assess the frequency and types of mutation in *rpsL* and *rrs* genes of STR-resistant mycobacteria isolates, in this province.

Materials and Methods

Clinical specimens were collected from individuals suspected of TB who referred to the Tuberculosis Center of Isfahan from 2014 to 2015. Specimens were then decontaminated using N-acetyl-L-cysteine-NAOH and cultured on Lowensteine Jensen (L.J) medium [24].

M. tuberculosis colonies were identified with primary conventional standard phenotypic methods, considering features such as characteristics colony morphology, acid-fast staining, nitrate reduction, and niacin tests. *M. tuberculosis H37Rv* (ATCC 27294) was used as reference strain.

First-line drug susceptibility testing (DST) was carried out using conventional proportion method on L-J medium [25].

DNA of clinical isolates was collected from scraped colonies in 400 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and boiled at 80°C for 30 minutes to inactivate bacteria. Then, lysozyme/proteinase K cetyl-trimethyl ammonium bromide (CTAB) method was used to extract DNA [26].

Subsequently, the extracted DNA was dissolved in TE buffer, its concentration was measured by spectrophotometry using Nanodrop (Biowave II, made in British), and stored at -20°C until being used [27].

Oligonucleotide primers used in PCR amplification genes were: *rpsL*-F 5′ -ATGCCAACCATCCAGCAGCT-3′ and rpsL-R 5′ -CTTAGCGCCGTAACGGCTGC-3′ for *rpsL* gene (12); *rrs*-F 5′-GTTGTAAACCTCTTTCACCATC-3′ and *rrs*-R 5′ -GTTGCATCGAATTAATCCAC-3′ for *rrs* gene [28]. PCR mixtures contained 10 µl of HotStarTaq Master Mix (Amplicon, Denmark), 1 µM of each primer, 0.5 µl of template DNA and dd-water up to 20 µl reaction volume.

Amplifications were performed in T100Tm Thermal Cycler (Bio Rad, Hercules, CA, USA) under the following conditions: an initial step of 94°C for 5 min, followed by 30 cycles of 15 s at 94°C, 60°C for 30 s, 72°C for 60 s, and a final extension step for 7 min at 72°C. PCR products were loaded onto 1.5% agarose gel.

Negative (water instead of DNA(and positive (*M. tuberculosis H37Rv*) controls were used in each set of PCR reactions. After purification, PCR products were sequenced using both forward and reverse primers of each gene, and the results were analyzed with ABI PRISM 370 × 1 Genetic Analyzer (Developed Biosystems, USA).

The sequencing results were analyzed with ChromasPro (ver. 2.4.4) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) software. BLAST program was used to compare the sequences with those deposited in GenBank database corresponding with wild-type sequences of reference H37Rv strain at NCBI (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/).

Results

A total of 205 *M. tuberculosis* isolates were examined, among whom 10 isolates (4.8%, including MDR isolates) were phenotypically STR-resistant.

Among STR-resistant isolates, resistance to rifampin was identified in 4 (40%), to isoniazid in 6 (60%) and to ethambutol in 3 (30%) isolates (Figure 1). Finally, Multi-drug-resistant tuberculosis was found in 4 (40%) of the isolates (Figure 1).

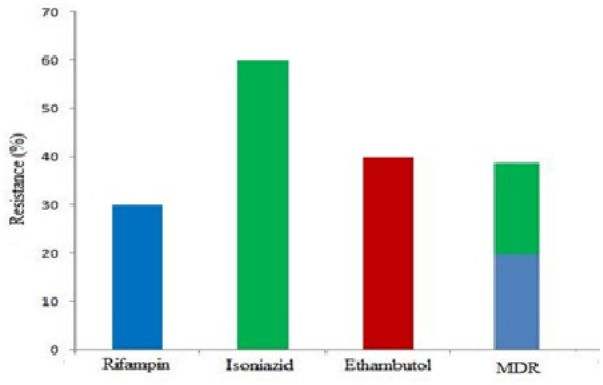

Figure 1: The frequency of drug resistance by proportion method in clinical STR-resistant isolates.

DNA fragments of 360 bp and 540 bp were seen in *rpsL* and *rrs* genes, respectively. After analyzing the sequences, in *rpsL* gene, one point mutation was identified in four (40%) of the isolates. Three isolates showed a substitution mutation at codon 43 (A→G, K43R), and one a substitution mutation at codon 88 (A→G, K88R) (Figure 2). In rrs gene, one point mutation was seen in two isolates. One isolate represented an A→C transversion at nucleotide position 514, and one a C→T transition at position 517 (Figure 3). In five STR susceptible isolates that were investigated randomly, no mutation was found in *rpsL* or *rrs* genes.

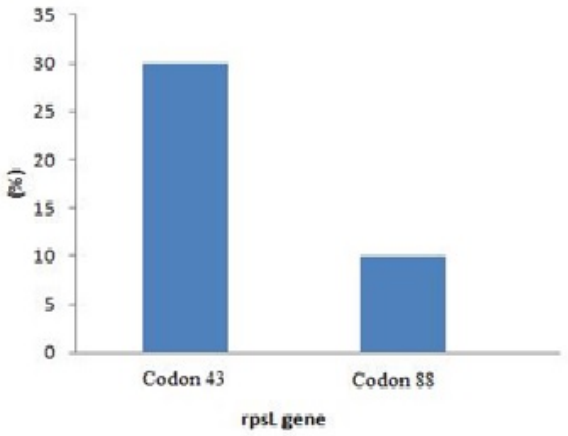

Figure 2: The frequency of mutations of the *rpsL* gene identified by sequencing in clinical isolates.

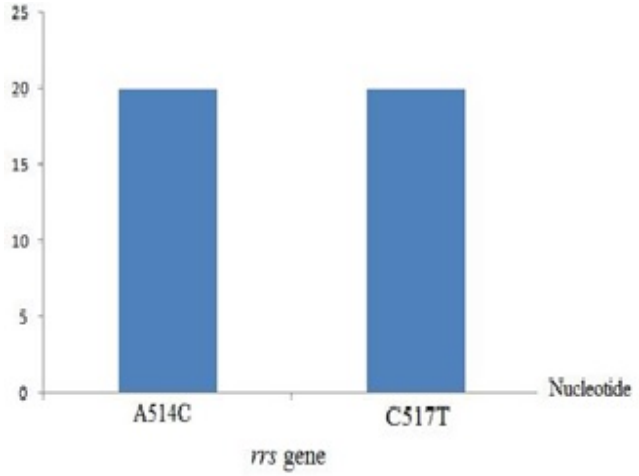

Figure 3: The frequency of mutations of the *rrs* gene identified by sequencing in clinical isolates.

Discussion

STR is one of the main antibiotics prescribed for treating TB. Correlation of molecular resistance mechanisms with mutation in *rpsL* and *rrs* genes, regarded as genetic markers of resistance to SRT, has been reported in different areas of the world. In 2014, the total number of TB cases notified in Iran was 10395 [1]. The incidence of TB in Isfahan, one of the biggest provinces of Iran, was close to 7 cases per 100,000. In this study, 6 of the 10 (60%) examined isolates showed resistance to at least one of the first-line drugs and 4 (40%) were TB-MDR. All rpsL and rrs gene mutations found in this study were reported in *STR*-resistant *M. tuberculosis* isolates previously, most of which were also reported with MDR. In *rpsL* gene, mutation at codon 43 accounted for 3 (30%) isolates and at codon 88 for 1 (10%). All four strains showed a similar amino acid change (Lys/Arg), due to an AAG AGG mutation. In *rpsL*, mutations were reported at codons 43 and 88, with K43R mutation being the most common. The detection rate of this mutation would vary considerably in different geographical areas, i.e 70.4% in China [16], 80.4% in Singapore [19], 52.8% in Korea [28,29], 42.9% in the North of India [30], 13.2% in Mexico [24], and 25% in Brazil [30], whereas in this study the rate was 30%. Mutations at codons 43 and 88 were frequently described in other investigations [19,22,31,32]. In rrs gene, the most common mutations were detected in two specific regions, i.e 530 stem-loops and 912 region [5,22,33-35]. In previous investigations, many different mutations were found in *rrs*. Although one of the most hot- spot regions of *rrs* gene (530 loop) was analyzed here, it seems possible for resistant isolates to experience mutations in other regions of this gene, as well.

Mutations in both of these genes were rarely observed to occur concurrently [11,19,24,]. Double mutants in both *rpsL*, and *rrs* genes were absent from the sample analyzed in this work. This could suggest that mutational variations in either *rrs* or *rpsL* might reduce the need for the modification of the other gene. Interestingly, 40% (4/10) of STR-resistant isolates showed no mutations in either of the two loci investigated in this study. This would be congruent with several other geographical areas such as Portugal (33.3%) [36], Mexico (52%) [37], Spain (62.3%) [38] and Poland (51%) [13].

In addition to *rpsL* and *rrs*, mutation in gidB gene (encoding ribosome methyltransferase) could be regarded as another reason for STR resistance. According to previous studies, mutations in *rpsL* gene were likely to be associated with a high-level of STR resistance, whereas mutations in *rrs* gene were shown to confer to an intermediate-level, and in *gidB* gene a low-level of STR resistance [11,17,20,7,35,38-40]. Mutations in rpsL, rrs and *gidB* genes were found in circa 70% of *M. tuberculosis* isolates that were resistant to STR [21,23]. In the research investigating the three-gene panel (*rrs*, *rpsL*, and *gidB* genes), the proportions of STR-resistant isolates with wild-type alleles at all three loci were almost similar, i.e 11.8% in Korea [41], 12.5% in Sierra Leone [42], 6.9% in Vietnam [27], 22% in Brazil [30] and 12.5% in Poland [7]. The portions were much lower than all studies cited above. It was suggested that variety, and presence or absence of *rrs*, *rpsL* and *gidB* mutations might be the result of variations in treatment schemes implemented by health authorities, design and duration of study, and study isolate collection period [16]. In addition, it has recently been shown that TB-Drug resistant genotypes circulating in any region could be significant contributors to variations in these mutations [19,20].

From the mentioned studies, it could be inferred that mechanisms except mutation in *rrs*, *rpsL* or *gidB* genes might result in STR resistance. One mechanism regarded changes in cell wall as leading to decreased permeability, and uptake or increased efflux of the drug [11]. However, further studies would be needed to confirm this mechanism and find other mechanisms causing STR resistance in *M. tuberculosis* strains.

Concluding Remarks

In the present study, 60% of STR isolates would experience mutation in *rpsL* and *rrs* genes. This might negatively affect the development of a molecular test on diagnosis of STR resistance. The occurrence of further mechanisms associated with STR resistance in studied isolates regions could also be suggested.

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