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Frequency of *rrs* and *rpsL* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from Iranian patients

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ABSTRACT

Objectives: Streptomycin (SM) is one of the most effective drugs for the treatment of multidrug-resistant (MDR) tuberculosis. However, resistance to SM is increasingly reported, mainly due to mutations in the *rpsL* and *rrs* genes. This study was designed with the aim of determining the nature of SM resistance and the type and frequency of *rpsL* and *rrs* mutations among SM-resistant *Mycobacterium tuberculosis* (MTB) isolates from Iran.

Methods: A total of 100 clinical monoresistant and MDR MTB isolates were subjected to drug susceptibility testing (DST) for SM. SM-resistant isolates were genotyped by MIRU-VNTR typing. Fragments of the *rpsL* and *rrs* genes were amplified to investigate the most common mutations, with subsequent sequence analysis.

Results: By DST, 32 isolates (32%) were identified as SM-resistant, of which 50% (16/32) were MDR. By MIRU-VNTR typing, the SM-resistant isolates were classified into 20 different MIRU types and 8 clusters, with Beijing (22%) being the most prevalent genotype. Mutations in the *rrs* and *rpsL* genes were identified in 14 (44%) and 10 (31%) of the 32 SM-resistant isolates, respectively. The most common mutations were at *rpsL* nucleotide 128 (AAG→AGG, Lys43Arg), found in 7 SM-resistant isolates (22%) and nucleotide 263 (A→G, Lys88Arg) in 3 SM-resistant isolates (9%).

Conclusions: The results suggest an association between *rpsL* mutation and SM-resistant strains of Beijing genotype. The existence of SM resistance in 25% of isolates without mutation in *rrs* and *rpsL* suggests the occurrence of further mechanisms associated with SM resistance in these isolates.

1. Introduction

Tuberculosis (TB) remains an important problem for public health worldwide, especially in developing countries [1]. The emergence of drug-resistant TB is a serious threat to TB control and is a major concern in several countries [2]. Most of the current drugs for the treatment of TB have been in use for over half a century. This, in combination with poor management, has made it possible for strains to develop resistance to one or all of the anti-TB drugs [3]. The global TB report by the World Health Organization (WHO) stated 5.4 million new TB cases and 0.7 million previously treated TB cases reported to the WHO during 2013, among which there were an estimated 300 000 cases of multidrug-resistant (MDR) TB [4]. According to the WHO, the estimated incidence rate of TB in Iran is 21 per 100 000 population [5]. A meta-analysis of drug resistance in Iran revealed that 23% of new cases and 65.6% of previously treated cases of TB were resistant to at least one drug [6].

Streptomycin (SM), an aminoglycoside antibiotic derived from *Streptomyces griseus*, was the first drug successfully used for the treatment of TB in the 1940s and it became the drug of choice for all forms of TB [7]. Following the introduction of other anti-TB drugs, SM was used as an important component of combination therapies for the disease. Nevertheless, high rates of SM resistance and the risk of toxicity led to a gradual decline in the use of SM. However,

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SM continues to be an integral part of chemotherapy for TB either in combination therapy or as the first-line agent for cases still susceptible to aminoglycosides [8].

The mechanism of action of SM in mycobacteria entails permanent binding to the ribosomal protein S12 and 16S rRNA, which are constituents of the 30S ribosomal subunit. Resistance to SM is mainly associated with mutations in the *rpsL* and *rrs* genes encoding ribosomal protein S12 and 16S rRNA, respectively, which are responsible for ca. 70% of SM resistance among *Mycobacterium tuberculosis* (MTB) strains. The *rpsL* gene encoding the S12 subunit protein has predominant mutations at codon 43 (A/G, Lys→Arg, Thr) and codon 88 (A/G/C, Lys→Gln, Arg, Thr). The most frequent mutations in the *rrs* gene encoding 16S rRNA are in loop 530, mostly A514C or C517T alleles, and in region 912 [9]. High-level SM resistance has often been linked with point mutations in *rrs* and *rpsL* genes [10]. Reports have suggested that an efflux system and mutations in the *gidB* gene, coding for a 7-methylguanosine methyltransferase specific for 16S rRNA, may also be involved in low-level SM resistance [11]. On the other hand, up to 50% of clinical SM-resistant isolates may present no mutation in any of these three genes and thus there is uncertainty about the resistance mechanism [12]. The type and frequency of SM resistance mutations vary depending on the population and geographical area. Because of the lack of data regarding the nature of SM resistance in Iran, and since mutations in the genes responsible for SM resistance have been poorly studied in Iran, the present study was designed with the aim of determining the type and frequency of *rpsL* and *rrs* mutations among SM-resistant isolates from major cities of Iran.

2. Materials and methods

2.1. Bacterial strains and sample collection

A total of 100 clinical monoresistant and MDR consecutive MTB isolates were collected from several TB reference laboratory centres (Ahvaz, Shiraz, Gorgan, Kermanshah, Mashhad and Qom) in Iran over a 4-year period from 2012 to 2015. The selected centres were among the most high-burden TB areas in the country.

2.2. Drug susceptibility testing (DST)

All 100 isolates were renewed by subculture on Lowenstein–Jensen medium. To ensure their true resistance pattern and to eliminate interventional factors such as possible cross-contamination, laboratory errors or the presence of mixed infection in patients, the isolates were subjected to phenotypic DST for isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and SM using the proportion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [13]. Susceptibility was defined as no or <1% growth on medium containing the critical concentration of drug for SM (4 mg/mL). Resistance was defined as growth in the number of colonies of >1% in the drug-containing medium compared with the control medium.

2.3. DNA extraction and identification of isolates

Chromosomal DNA was extracted from bacterial cultures on Lowenstein–Jensen medium. A loopful of mycobacterial colonies was suspended in 500 μL of TE [Tris HCl–ethylene diamine tetra-acetic acid (EDTA)] buffer (pH 8.0) and was then boiled for 10 min. The suspension was centrifuged at 12 000 rpm for 10 min and the supernatant was stored at −20 °C and served later as PCR template [1]. For MTB complex (MTBC) identification, a 123-bp fragment of the IS*6110* gene was amplified by a set of specific primers (MTB1, 5′-CCTGCGAGCGTAGGCGTCGG-3′; and MTB2, 5′-CTCGTCCAGCGCCGCTTCGG-3′) according to a previously described procedure [14].

2.4. Region-of-difference (RD) PCR typing

One of the reliable techniques for differentiation of MTBC members is RD typing, which is a PCR-based method relying on the presence or absence of RDs. RD-9 PCR was used for identification of MTB from other members of the MTBC. PCR for RD analysis was performed as described previously [15].

2.5. MIRU-VNTR genotyping

SM-resistant isolates were subjected to genotyping using 12-locus-based mycobacterial interspersed repetitive units–variable-number tandem repeat (MIRU-VNTR) typing as initially published by Supply et al. [16]. The size of the amplicons was estimated by comparison with the control strain *M. tuberculosis* H37Rv and the size of ladder (100 bp and 50 bp) as previously described [17]. MIRU-VNTR genotyping data were transferred into a distance matrix on the website MIRU-VNTR*plus* (http://www.miru-vntrplus.org/MIRU/miruinfo.faces) using the default setting and were treated as categorical variables to determine the number of repetitions and for calculation of allelic diversity at each of the loci. The Hunter–Gaston discriminatory index (HGDI) was utilised for calculating MIRU-VNTR discriminatory power and allelic diversity (h) as described previously [18,19]. Based on the MIRU-VNTR profile of each isolate, a dendrogram based on 12 MIRU loci was constructed using UPGMA (unweighted pair-group method with arithmetic mean) after pairwise comparison of strains by calculating the Jaccard index through the NTSYS-pc version 2.00 software package (Exeter Software, Setauket, NY). Isolates with identical MIRU-VNTR genotypes were defined as belonging to the same cluster. The clustering rate was calculated using the following formula: clustering rate = $(nc - c)/n$, where nc is the total number of clustered isolates, c is the number of isolate clusters and n is the total number of isolates in the sample [20].

2.6. Amplification of rpsL and rrs genes

A 272-bp fragment of the *rpsL* gene, including codon 43 and codon 88, and a 552-bp fragment of the *rrs* gene considering the most common mutations (491, 513, 516 and 905) were amplified. The primers used were STR52R (5′-GTC AAG ACC GCG GCT CTG AA-3′) and STR34F (3′-TTC TTG ACA CCC TGC GTA TC-5′) for the *rpsL* gene and PR13F (5′-AAA CCT CTT TCA CCA TCG AC-3′) and PR30R (3′-CAG GTA AGG TTC TTC GCG TTG-5′) for the *rrs* gene [21,22]. PCR was performed in a final volume of 25 μL using 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 10 μM forward and reverse primers, 1 U of *Taq* polymerase, and 1 ng–1 μg of DNA template. Cycling was performed using a thermocycler (Eppendorf, Hamburg, Germany) with the following program: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 5 min. The amplified PCR products of these genes were purified using a Gene JET™ Gel Extraction Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The sequences of the products were determined using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the standard protocol of the supplier.

2.7. Analysis of sequence data

The obtained sequences for each gene were aligned separately and were compared with wild-type *rrs* and *rpsL* genes from MTB