Brief Report

Rapid Detection of Streptomycin-Resistant *Mycobacterium tuberculosis* by *rpsL*-Restriction Fragment Length Polymorphism

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Abstract

Background: Molecular methods for the detection of drug-resistant tuberculosis (DR-TB) are potentially more rapid than conventional culture-based drug susceptibility testing, facilitating the commencement of appropriate treatment for patients with DR-TB. The aim of this study was to evaluate and develop polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays for the detection of mutations within *rpsL*, and for the determination of streptomycin (STR) resistance in *Mycobacterium tuberculosis*. **Materials and Methods:** Clinical specimens were collected from individuals with suspected TB referred to the TB Center of Isfahan, from which 205 *M. tuberclosis* were isolated and identified by conventional phenotypic methods. The minimum inhibitory concentration of STR for all isolates was determined using the proportion method and 10 isolates were recognized as STR resistant *M. tuberculosis*. The effect of genetic alterations in the *rpsL* gene for these resistant isolates were investigated by PCR-RFLP method. **Results:** Three (30%) isolates showed point mutation at codon 43 by RLFP analysis. **Conclusion:** Our results suggest that RFLP analysis of the *rpsL* gene is useful for the rapid prediction of STR resistant strains of *M. tuberculosis*.

Keywords: *Mycobacterium tuberculosis, resistance, restriction fragment length polymorphism, rpsL gene, streptomycin*

Introduction

Tuberculosis (TB) remains a burden global, and this burden is enhanced by drug-resistant (DR) TB. Failures to diagnose and remedy patients who suffer from DR TB have led to nosocomial prevalence, enhanced mortality, and resistance to additional anti-TB drugs.[1-3] Streptomycin (STR), an aminocyclitol glycoside antibiotic, was the first drug used to treat TB.[4,5]

Although STR is no longer regarded as the first-line drug in the US, it is still an alternative first-line anti-TB drug recommended by the World Health Organization.[6,7] STR is prescribed particularly when primary resistance to other first-line drugs (rifampicin, isoniazid, and pyrazinamide) is suspected.[8] Due to the increase of STR resistance prompt an urgent need for STR resistance detection available to all patients.

The mode of action of STR is binding to the ribosomal protein S12 and the 16S rRNA gene, which are two components of the 30S subunit of the bacterial ribosome.[9,10] STR inhibits protein synthesis by disrupting the relationship between these components.[9,10] STR resistance in *Mycobacterium tuberculosis* strains is associated with mutations in genes encoding these two constituents: the *rpsL* gene (encoding the ribosomal protein S12) and the *rrs* gene (encoding 16S rRNA).[10,11]

Conventional cultivation-based diagnostic procedures for the detection of STR resistance are less sensitive and time-consuming.[12,13] Rapid molecular diagnostic assays are introduced to detect STR resistance, such as direct DNA sequencing of polymerase chain reaction (PCR) products,[14,15] single-strand conformation polymorphism analysis,[16] reverse dot-blot hybridization assay,[17,18] restriction fragment length polymorphism (RFLP) method,[19] as well as DNA arrays.[20] Such molecular methods have brought critical improvements to drug-susceptibility testing (DST).

Since, the rapid availability of results of DST may have an important impact on

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therapeutic, we evaluated the RFLP analysis to detect mutation in *rpsL* in STR-resistant *M. tuberculosis* isolates.

Materials and Methods

Clinical isolates

Specimens were collected from individuals with suspected TB referred to the TB Center of Isfahan from 2014 to 2015. Specimens were decontaminated using N-acetyl-L-cysteine-NAOH and cultured on Lo¨wensteineJensen (L-J) medium.[21] The isolates were identified as *M. tuberculosis* by primary conventional standard phenotypic methods including: colony morphology, acid-fast staining, nitrate reduction, and niacin tests. The reference strain used in this work was *M. tuberculosis* H37Rv (ATCC 27294).

First-line DST was carried out with the conventional proportion method on L-J medium. Medium was incorporated with the STR (4 μg/ml). A control plate was also inoculated with the undiluted suspension. The drug susceptibility test results were recorded after 3 weeks incubation at 37°C in the presence of 5%–10% CO_2. The proportion of resistant organisms in the inoculum was calculated by comparing the number of colonies growing on the drug-free medium with the number growing on drug-containing medium. If 1% of the inoculum was found to grow in the presence of the critical concentration used, the isolate was regarded as DR.[17]

DNA extraction and polymerase chain reaction

The DNA of STR-resistant clinical isolates was prepared 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 min to inactivate bacteria and release DNA. Then, DNA extraction was performed by the lysozyme/proteinase K cetyl-trimethyl ammonium bromide method.[22] Extracted DNA was dissolved in TE buffer, its concentration was measured by spectrophotometry using a Nanodrop (Biowave II), and stored at −20°C until use.

Oligonucleotide primers used for PCR amplification gene were: rpsL-F 5′-ATGCCAACCATCCAGCAGCT-3′ and rpsL-R 5′-CTTAGCGCCGTAACGGCTGC-3′ for *rpsL* gene (360 bp).[23] PCR mixtures contained 10 μl of Hot Start Master Mix (Amplicon), 1 μM of each primer, 0.5 μl of template DNA and dd-water up to 20 μl reaction volume. Amplifications were performed in a T100™ Thermal Cycler (Biorad, Hercules, CA, USA) under the following amplification conditions: an initial step of 94°C for 5 min, followed by 30 cycles of 15 s at 94°C, annealing temperature at 60°C for 30 s, extension at 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 strain) controls were used in each set of PCR reactions.

Analysis of the *rpsL*

360-bp fragments of the *rpsL* gene from each of the STR-resistant DNA isolates were digested with *Mbo*II restriction endonuclease (Fermentas) for 1 h at 37°C and separated on 3% agarose gel.[23] As well as, fragments of the *rpsL* and *rrs* genes were analyzed by automatic nucleotide sequencing in other work by authors (unpublished).

Results

Of the 205 isolates examined, 10 isolates (4.8%, including MDR isolates) were phenotypically STR-resistant, and also resistant to other drugs. Among the STR-resistant isolates, resistance to isoniazid was found in 6 (60%), to ethambutol in 3 (30%), and to rifampin in 4 (40%) isolates. Finally, MDR-TB was identified in 4 (40%) of the isolates.

Results of the RFLP analysis of the *rpsL* gene are shown in Figure 1. Two types of RFLP pattern were observed. The 360 bp amplified products from the *rpsL* gene were digested by *Mbo*II into two fragments, of 130 bp and 230 bp, in 7 strains and in the H37Rv strain without mutation at codon 43. In the remaining 3 strains, which had a mutation at codon 43 and were resistant to STR, the 360 bp product was not digested by *Mbo*II.

Statistical analysis (Mc-Nemar) was employed to determine a *P* value. 10/205 (4/9%) isolates showed resistance by proportion method. By contrast, 3/205 (1/5%) isolates showed resistance by RLFP analysis. This difference was statistically significant ($P = 0.016$).

Discussion

To determine that different mechanisms by which drug resistance in *M. tuberculosis* develops, alterations in

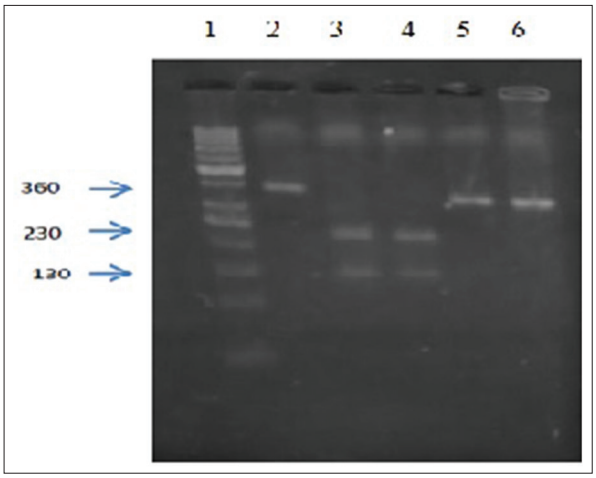

Figure 1: Digestion of the 360 bp fragment of the rpsL gene with endonuclease MboII. Lane 1, 50 bp DNA molecular weight marker; lane 2, strain with mutation at codon 43; lane 3, strain with mutation at codon 88; lane 4, resistant strain without mutation; lane 3 and 4 show a pattern similar to that of the control. Lane 5 and 6, both have a mutation at codon 43 which destroys the MboII restriction site, leaving the 360 bp fragment undigested similar to the lane 2

the mycobacterial genome should be studied and the correlation with *in vitro* susceptibility testing results should be evaluated. PCR-based methods are very beneficial since they can detect specific nucleotide changes in sequences present in low copy numbers. As well as, accurate information on drug susceptibility is essential for clinicians and should be available in a short period of time. In previous studies confirmed the suitability of various molecular methods for detect of changes in already known genes, for further application to clinical specimens of *M. tuberculosis*.[14-20,24]

In this study, the *rpsL* gene, known to be partially responsible for STR resistance, were analyzed. Our study showed that 30% (3 strains) of strains had mutation in codon 43. The results thoroughly were corresponded with the results of direct sequencing analysis of this gene in our other work. Our results also showed that RFLP analysis using *Mbo*II was useful in detecting certain point mutations at codon 43, however direct sequencing analysis is necessary for detailed informations on the nature of the point mutations at the codon 43 and other areas of gene. RFLP method is faster and cost-effective. Therefore, we advance a proposal that RFLP analysis is a useful screening test for detecting STR-resistant strains, particularly when assaeing a large number of clinical specimens.

Conclusion

Our results suggest that RFLP analysis of the *rpsL* gene is useful for the rapid prediction of STR resistant strains of *M. tuberculosis*.

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Conflicts of interest

There are no conflicts of interest.

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