MOLECULAR CHARACTERISTICS OF RIFAMPIN RESISTANCE AMONG MYCOBACTERIUM TUBERCULOSIS STRAINS ISOLATED IN NORTHEAST OF IRAN

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ABSTRACT: Background: Emergence of drug-resistant tuberculosis complicated the Tuberculosis Control programs seriously. Rifampin is a marker for MDR-TB therefore, characterizing and designing suitable target for rapid and simple molecular detection of resistance against rifampin is an urgent need. Method: Based on proportional method, fifteen rifampin-resistant isolates of Mycobacterium tuberculosis were identified in northeast of Iran during July 2012 to May 2013. Phenotypic characterization and genotypic studies such as PCR, high-resolution melting analysis and sequencing were performed. Results: In our study, 12 resistant isolates (80%) contain a mutation in the rifampicin resistance determinant region (RRDR). The most common mutation was in codon 531 (GTC→GTT) followed by codon 526 (CCC→CCT) both results in amino acid changes. A new silent mutation was also observed in codon 528. Among these isolates, two strains had several silent mutations in rpoB gene. Conclusion: According to results of this study analysis of RRDR region of rpoB gene alone is not sufficient and suggested the entire rpoB gene of M. Tuberculosis should be sequenced.

Keywords: Mycobacterium Tuberculosis Complex, MDR-TB, RpoB Gene, HRMA.

INTRODUCTION
The increasing emergence of drug-resistant Mycobacterium tuberculosis creates a problem in control and treatment of tuberculosis. The main strategy for controlling MDR-tuberculosis is early detection and use of an effective therapy to reduce transmission and spread of MDR-TB. So it is highly recommended to monitor drug-resistant in each isolate for early intervention.

One of the main drugs in treatment of tuberculosis is rifampin. Resistant against rifampin is often associated with resistance to isoniazid, hence rifampin is a good marker for multi-drug resistance (MDR) (1). The major mechanism of resistance against rifampin is mutations in the gene coding drug target site. Mutations associated with resistance to rifampin mainly occur in the central 81 bp (codons 507 to 533) rifampin resistance-determining region (RRDR) of the rpoB gene encoding the b-subunit of the RNA polymerase. The most common mutations reported in this fragment of the rpoB gene are as follows: D516V, H526Y, H526D, and S531L (2-5). The changes in these codons are associated with high level resistance to rifampin in M. tuberculosis strains; however, other mutations in this region were also reported that its effect on resistance to rifampin has not been determined. As more than 95% of rifampin-resistant M. tuberculosis isolates have mutation in the central 81 bp of the rpoB gene (2, 3) thus, this region is an ideal target for rapid detection of resistance to rifampin by molecular techniques instead of using conventional drug susceptibility testing for rifampin that takes several weeks. There
are several molecular techniques for detection of mutation. The high-resolution melting analysis (HRMA) is a simple, rapid, low cost, closed-tube method for detection known and unknown mutations with sensitivity and specificity reported up 90% for rpoB gene (6-8). The aim of this study was to characterize the rifampin-resistant Mycobacterium tuberculosis strains isolated from samples referred to regional tuberculosis reference laboratory in the North East of Iran and determine the mutated codons in the RRDR of rpoB gene of the resistant to rifampin M. tuberculosis isolates which help to develop useful molecular tools for rapid identification of drug resistance in M. tuberculosis.

MATERIALS AND METHODS

Sample collection
In this cross-sectional study from July 2012 to May 2013, all specimens referred to the Regional Tuberculosis Reference Laboratory in northeast of Iran that have positive cultures were included. This study was approved by the Ethics Committee of the Mashhad University of Medical Sciences. A total of 93 strains of 100 isolates which included were identified as M. tuberculosis complex (MTBC) by Ziehl-Neelsen staining, speed of growth and colony morphology, biochemical (niacin) and phenotypic methods and PCR of IS6110 gene (9, 10).

Drug Susceptibility Testing
Drug susceptibilities against isoniazid, rifampicin, ethambutol and streptomycin were determined by the standard proportional method using Löwenstein-Jensen (L-J) medium. For this purpose several fresh colonies dissolved in sterile deionized water until a bacterial density corresponding to 1 McFarland turbidity standards would be obtained. Two dilutions of this suspension, 10-2 and 10-4, were prepared. Then 200 μl of each dilution separately inoculated to one slope of medium without drug and each drug containing tubes rifampin (40 μg/ml), isoniazid (0.2 μg/ml), ethambutol (2 μg/ml) and streptomycin (4 μg/ml) then were incubated at 37°C for 28 days. After 28 days visible colonies was interpreted as bacterial growth, if there is no colonies Löwenstein-Jensen medium incubated for two more weeks and results were reported after 42 days and critical ratio were recorded for each drug (11).

Mycobacterium DNA Extraction
Two loopful of colonies was homogenized in 400 μl of TE buffer and heated at 80°C for 20 minutes to kill bacteria. Digestion was performed by lysozyme, protein kinase K and SDS %10. Then 100 μl of sodium chloride (NaCl) 5 mM and 100 μl of prewarmed N-Cetyl-N-3-trimethyl ammonium bromide (CTAB) was added and mixed thoroughly to get a milky solution, then was incubated in 65°C for 10 minutes to remove the polysaccharides. For purification of nucleic acids 750 μl of phenol: chloroform: isoamylalcohol (25:24:1) mixture was added, vortex thoroughly and separate the phases by centrifugation 13 000 g for 5 min. Aqueous phase was transferred to new microtubes and 100 μl of sodium chloride (NaCl) 5 mM was added, mixed and was kept in 4°C. For precipitation the DNA was precipitated and mixed with 1 ml of cold 70% ethanol and centrifuged at 13000 rpm for 15 min. The supernatant was removed then 1 ml of cold 70% ethanol added to each sample, up and down several times to wash the DNA. For DNA precipitation the DNA was precipitated then centrifuged at 13000 rpm for 10 minutes. The DNA Collect in a suitable amount of 1x TE buffer (20 to 80 ml depending on the size of the precipitated DNA) was dissolved and was kept in -20°C (12, 13).

PCR Amplification for Species Identification

1 μl of DNA of Mycobacterium was added to PCR reaction. 137bp fragment of IS6110 gene was amplified by specific primers. Each 20 μl of the reaction mixture containing: 1X buffer, 2.5 mM MgCl2, 200nM dNTPs mixture, 10 pmol/μl of each primer, 1u/μl Taq polymerase. Amplification was performed with the following program: initial denaturation for 5 min at 94°C then 35 cycles of 94°C for 45s, 63°C for 45s and 72°C for 45s; and a final extension of 72°C for 10 min. Following electrophoresis, PCR products was observed on %1.5 agarose gel stained with ethidium bromide.

Real-Time PCR And High-Resolution Melting (HRM) Of rpoB Gene

SYBER Green Real Time PCR Master Mix (ParsTous) was used for Real-Time PCR. Each 10 μl of 2X Master Mix contains: 1U Hotstar Taq polymerase, 5mM MgCl2, 2mM dNTPs mixture and SYBER Green I 2X. PCR
reactions were performed on the Rotor-GeneTM 6000 (Corbett Research Pty Ltd, Australia) with the following program: initial denaturation for 10 min at 94°C then 40 cycles of 94°C for 30s, 66°C for 30s and 72 °C for 40s; and a final extension of 72°C for 5 min. Each 20 μl of reaction mixture containing: 1X Master Mix, 200 nM of forward primer 5'-CAGGACGTGGAGGCGATCA-3', 200 nM of reverse primer 5'-CGACAGCGAGCCGATCAGA-3' and 5 μl of DNA template and water. A negative control containing sterile distilled water and from Mycobacterium tuberculosis H37Rv as reference strain was included in the experiment. After completion of the reaction, we define of one step hold 30 seconds at 60 °C, to make sure for re-association of DNA. We were considered the temperature range 89-79 °C for high-resolution melting (HRM) analyze and temperature increase of 0.1 °C was defined in each of 2 seconds hold. The HRM curve was analyzed using the Rotor-Gene 2.0.2.4 software.

**DNA sequencing**

The 285-bp fragments of the rpoB gene were sequenced and were purified by Bioneer, Korean Biotechnology Company. Sequencing samples were analyzed for mutations using the software Mega 5.

**Statistical analysis**

Data were analyzed using the chi-squared test for qualitative variables (gender, place of residence, history-TB, nationality) and the T-test for quantitative variables (age, time interval between the diagnosis of the started treatment) using the SPSS software, version 16.0. The level of significance for all statistical analyses was P<0.05.

**RESULTS**

A total of 93 of TB-patients, %35.5 were women and %64.5 were men in the age range 17-94 years and other patient data are summarized in Table 1. (Information about a number of patients was not available).

### Table 1. Information of TB-patients

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>33</td>
<td>60</td>
<td>93</td>
</tr>
<tr>
<td>Mean age</td>
<td>51.9</td>
<td>51.3</td>
<td>-</td>
</tr>
<tr>
<td>Place of Residence</td>
<td>Rural</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Urban</td>
<td>24</td>
<td>37</td>
</tr>
<tr>
<td>Nationality</td>
<td>Iranian</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Non-Iranian</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>History-TB</td>
<td>10</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>Prisoner</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

60% of patients were new cases and 40% of them were treated that theirs therapeutic Status is summarized in Table 2.

### Table 2. Therapeutic Status of TB-patients

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Relapse</th>
<th>Treatment failure</th>
<th>Absent after treatment</th>
<th>MDR</th>
<th>XDR</th>
<th>Improved</th>
<th>During treatment</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>32</td>
<td>22</td>
<td>5</td>
</tr>
</tbody>
</table>

A total of 100 patients, only one of them had extra pulmonary and remaining patients were diagnosed with pulmonary tuberculosis.

**Drug Susceptibility Testing**

From 93 of M. tuberculosis isolates, 15 isolates were resistant to four drug rifampin, isoniazid, streptomycin and ethambutol, two isolates were resistant to singledrug (one to isoniazid and one to streptomycin) and the remaining of the isolates tested were susceptible to all four drugs.

**Statistical analysis**
In our study, a significant relationship between drug resistance with variables gender, place of residence, history-TB, nationality, age, time interval between the diagnosis of the started treatment not found.

**Real-Time PCR And High-Resolution Melting (HRM) of rpoB Gene**

We tested the assay for 15 drug-resistant M. tuberculosis isolates. With the exception of two isolated, 13 of drug-resistant M. tuberculosis isolates were different from reference strains (Table 3). HRMA curve profiles of rifampin-resistant M. tuberculosis isolates in the normalized graphs and the difference graphs.

**Table 3.** Compared Sequencing rpoB gene with HRMA profiles and resistance phenotype

<table>
<thead>
<tr>
<th>Isolate</th>
<th>HRMA</th>
<th>Resistance phenotype</th>
<th>Sequencing rpoB gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>2</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>3</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>4</td>
<td>WT</td>
<td>R</td>
<td>Non-Mutated</td>
</tr>
<tr>
<td>5</td>
<td>WT</td>
<td>R</td>
<td>Non-Mutated</td>
</tr>
<tr>
<td>6</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>7</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>8</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>9</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>10</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>11</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>12</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>13</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>14</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
<tr>
<td>15</td>
<td>V</td>
<td>R</td>
<td>Mutated</td>
</tr>
</tbody>
</table>

V=variant; WT=wild type; R=resistant

**Graphs:**
- **Difference graph**
- **Normalized graph**

Available online at [www.ijapbs.com](http://www.ijapbs.com)
Figure 1. Normalized and temperature-shifted difference plots for mutant discrimination by HRMA in rpoB gene

DNA Sequencing

In our study, 80% (12/15) isolates resistant to rifampin contain mutations in the 81 bp core of rifampicin resistance determinant region (RRDR), 20 percent (3/15) had the mutations outside this region and 20% (3/15) isolates had no mutations in the rpoB gene. A total of 8 single nucleotide mutation in codon 531 and 526, a double mutations in codon 528-550, a insertion between codon 485-486 and two of isolates had several silent mutations in rpoB gene at codons 507, 508, 509, 512, 516, 523, 524, 529 along changes in codon 552 led to change in the amino acid (Table 3). The most common mutation in the 81 bp of RRDR, 75% in codon 531 (GTC → GTT) followed by 12.5% in codon 526 (CCC → CCT) had occurred that resulted in amino acid changes. A silent mutation in new codon, codon 528, occurred in this region.

Table 4. Codons modified in rpoB gene of rifampin-resistant M. tuberculosis isolates identified by sequencing

<table>
<thead>
<tr>
<th>Codon</th>
<th>507</th>
<th>508</th>
<th>509</th>
<th>512</th>
<th>516</th>
<th>523</th>
<th>524</th>
<th>526</th>
<th>528</th>
<th>529</th>
<th>531</th>
<th>550</th>
<th>552</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGC</td>
<td>AC</td>
<td>C</td>
<td>AG</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>GGG/ A</td>
<td>AC</td>
<td>TG</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>His Tyr</td>
<td>No change</td>
<td>No change</td>
<td>Ser Leu</td>
<td>Val Ala</td>
<td>Pro Ser</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

In our study, 80% (12/15) isolates resistant to rifampin contain mutations in the 81 bp core of rifampicin resistance determinant region (RRDR), 20 percent (3/15) had the mutations outside this region and 20% (3/15) isolates had no mutations in the rpoB gene. In study of Minh and his colleagues in 2012 in Vietnam, mutations in this region occurred in 76% and 20.3% of mutations occurring outside this region and mutations in the three strains (4.05%) were not found (14). In the study of Mr. Li et al in 2012 in China 77.4% of isolates had a mutation in this gene (15). In the study of Sun et al in 2009 and Mr. Wang and his collaborators in China in 2013, 98% of strains had mutations in the rpoB gene (16, 17). In the study of Gupta and colleagues in 2013 in India, 93.16% of the isolates were resistant to rifampicin had mutations in the RRDR of rpoB gene, two strains in this region were not mutation (18). In the study of Doustar et al, Bahramd et al, Bostanabad et al in Iran, respectively 3.3%, 10.86%, 15% of rifampicin-resistance isolates in this region were not mutation (19-21). Our results are based on global data and cause of rifampicin-resistant strains without mutations in 81 bp of the region RRDR of rpoB gene probably is mutation in out of interest region of rpoB gene mutation associated with resistance has occurred in other genes.

In this study, 8 isolates with single nucleotide change in the RRDR region That the most common mutation were in codon 531 (75%), followed by codon 526 (12.5%) had occurred that led to amino acid changes. A silent mutation in new codon (12.5% at codon 528) had occurred. In a study Mr. Goncalves and colleagues in 2012 in Brazil, most mutations in codon 531 (67%), followed by codon 526 (23%), 516 (8%), 522 (1%) and 533 (1%) was reported (22). In studies of Minh et al, Wang et al and Gupta et al Similar to our studymost mutations were in codon 531, 526 and 516 respectively (14, 17, 18). The research was carried out by Mr. Miotto in 2009 in Italy, most of the mutations in codon 516 (43.8%), 526 (36.3%) and 531 (9.4%) had occurred (23). In study of Doustar and colleagues and Isfahani et al in Iran Similar to our study most mutations in codon 531 (53.3%), 526 (20%) and 516 (10%) was reported (21, 24) but in others studies were carried out by Bahramd et al on isolates of an area endemic for tuberculosis disease and Bostanabad in Iran was highest changed codon, codon 523 were reported (19, 20). In a study conducted by Ms. Khosravi et al in 2012 in Iran the most single nucleotide mutation
at codon 527 followed by 512, 531 and 511 occurred(25). Differences in the frequency and type of changed codon can be due to geographical differences or different genotype isolates are resistant to rifampin. In our study, one isolated with double mutation that involves a silent mutation in the codon 528, a mutation at codon 550, which led to a change in the amino acid at this codon. In one of the isolates between codons 485 and 486, a guanine nucleotide added and two of them had been several silent mutations in length of the rpoB gene at codons 507, 508, 509, 512, 516, 523, 524, 529 along change in codon 552 led to a change in the amino acid. In a study of Mr. Sun et al also changes in the two codon simultaneously were reported in 3 of 102 isolate resistant to rifampin (16). In a study conducted by Mr. Gupta and Associates in India 16.24% strains resistant to rifampin in two colon and 3.42% of them in three points were mutated also in this study, a deletion in the RRDR region and one at codon 522 was reported (18). The research was carried out by Mr. Wang in China, 23 single nucleotide change with 6 new mutation, 2-3 insertion 2 bp, 5 deletion, 20 mutations at two points and a three point mutations in rifampin-resistant isolates at codons 519-509 and 522, 523, 525, 526, 529, 531 and 553 of the rpoB gene was reported (17). In study of Doustdar et al., double mutations in codons 490-526 and 490-531 also has reported (21). In a study by Mr. Khosravi et al also reported double mutations in codons 539-541(25). The study's Bahrmand and colleagues, 76.09% of isolates had two to four mutations in the rpoB gene, 14.63% of isolates had single nucleotide change, 48.78% of isolates had the double mutation, 7.31% isolates triple mutation and 29.26% of isolates had quadruple mutations, codon modified in this study include 507, 508, 511, 516, 520, 523, 526, 527 and 531 were reported (19). In a study Bostanabad and colleagues, double mutations (34%), triple mutations (22%), quadruple mutations (3%) and 5 mutations (12%) have been reported also nucleotide deletion in codon the 510, 527, 520, 523 and 516 have been found and altered codon 509-507, 513-511, 516, 519 and 523 have been reported (20). According to the studies reviewed above mutation in the 81 bp of RRDR, nucleotide deletion, multiple mutation in the resistant isolates have been reported from different parts and the novel modified codon in and outside of this region be introduced to the world every day. In this study, HRMA method has been used for screening rifampin resistance by detection of mutations in the 81 bp of RRDR of the rpoB gene that the results matched with sequencing.

CONCLUSION
According to results of this study and studies done in this field in different regions, analysis of RRDR region of rpoB gene alone is not sufficient and suggested the entire rpoB gene of M. Tuberculosis Sequenced because of the mutations outside this region is reported and it should also be considered for the detection of resistance to rifampin. Furthermore, single nucleotide variation in genes is incorrect because multiple mutations associated with resistance is increasing. HRMA results matched with the sequences in our study, this technique can be used as a sensitive molecular techniques, accurate, simple, fast for detection of drug resistance in laboratories, especially in developing countries is routine.

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REFERENCES


