Rapid detection of MDR–*Mycobacterium tuberculosis* using modified PCR–SSCP from clinical Specimens

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**ABSTRACT**

**Objective:** To design a rapid test to detect the rifampin (RIF) and isoniazid (INH) resistant mutant based on polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP) technique that analyzes the *katG*, *rpoB* genes.

**Methods:** Biochemical test as well as IS6110 targeting PCR revealed 103 clinical samples were tuberculosis. To determine the susceptibility of isolates to anti TB drugs, the proportional method was used. Mutations presented within the amplified products of the *katG*, *rpoB* genes were evaluated by SSCP.

**Results:** Using proportional method, 12 (11.6%) and 9 (8.7%) isolates were resistant respectively to INH and RIF and 9 (8.7%) isolates showed resistance to both drug multi–drug resistant tuberculosis. Three (2.9%) multi–drug resistant tuberculosis and two INH resistant isolates were detected by the PCR–SSCP and sequencing. The sensitivity and specificity of PCR–SSCP for multi-drug resistant isolates were 33% and 100%, respectively.

**Conclusions:** Complete agreement between SSCP and sequencing can indicate that resistance–associated mutations have occurred in other genes except our considered genes.

**KEYWORDS**


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**1. Introduction**

TB has been known as a causative agent of the global morbidity and mortality during the history[1-2]. TB is still an important public health problem that occurs in numerous countries regardless of their economic and social situation[3-5]. The emergence of multi–drug resistant tuberculosis (MDR–TB) is one of the most hazardous events that threaten the public health. Of 250 000 individuals with the multidrug resistant TB, only 12% were diagnosed in 2009[6], and also 8.7 million people infected with *Mycobacterium tuberculosis* (*M. tuberculosis*) and 1.4 million deaths in 2011[4]. The increase in emergence of MDR–TB leads to raise the mortality, treatment failure rates and the period of transmissibility of the infection[1]. Generally, the MDR–TB includes the strains resistant to at least isoniazid (INH) and rifampin (RIF), the more efficient first–line drugs against TB[1,3,7]. The occurrence of mutations within several known genes is responsible for the MDR–phenotype. Discrepant mutations of the *katG*, *inhA*, *ahpC*, *kasA* genes involved in INH resistance. This drug is administered as a pro–drug and products of the described genes are capable...
to convert it to an active form. For instance, catalase–
prooxidase (encoded by katG) produces free radicals and
leading to activate INH. Furthermore, enoyl–acyl carrier
protein reductase and keto acyl–acyl carrier protein
synthase are other two important enzymes involved in
the INH activation in the target cells that are encoded by the
inhA and kasA genes, respectively. Mutations in these genes
lead to perturb mycolic acid biosynthesis. Overall, 50%–90%
of reported mutations in MDR–TB cases have occurred in the
catG gene, especially Ser315Thr[8]. Moreover, 20%–30% of
INH resistant cases have mutations in the promoter region of
the inhA gene.

The rpoB gene encodes the β–subunit of the RNA
polymerase[9]. Several studies demonstrated that mutations
in this gene cause resistance to RIF. Most prevalent
mutations have been reported within the core region of the
rpoB with the length of 81 bp. The type of these mutations
are deletions, insertions and missense mutations that
present in 95%–96% of the rifampin–resistant cases[10].
More than 35 mutations have been found in the core region,
however, missense mutations at codon 516, 526 or 531 are
most prevalent[11].

To limit the spread of MDR–TB, it is necessary to design an
efficient method for monitoring the level of drug resistance.
Common conventional methods used in clinical laboratory
can determine resistant isolates by the drug susceptibility
testing in the phenotypic manner. These methods are time
consuming because M. tuberculosis requires at least 6–8
weeks to grow and yield visible colonies on culture medium
containing antibiotics. Recently, the increase of our
knowledge about the genetic mechanisms of the resistance
allows establishing molecular methods for detection of drug
resistant cases. However, most of them focused on single
or/and single drug. Few studies performed by Mokrousov et
al.[12], Marine et al.[13], and Yang et al.[14] designed methods
that were able to assess the resistant to both INH and RIF,
but most of them are expensive and demand more technical
and equipment requirements.

In the present study, we attempted to design a rapid and
efficient molecular method based on polymerase chain
reaction–single strand conformation polymorphism (PCR–
SSCP) that can detect all the mutations in the katG and rpoB
genes which contribute in MDR–TB phenotype. In addition,
we compared our findings to results from sequencing and
conventional methods among clinical isolates.

2. Materials and methods

2.1. Sample collection

In this study, clinical specimens from suspected patients
were collected from the Baqiyatallah Hospital in north of
Tehran, Iran during 2009–2011. To recognize specimens
containing M. tuberculosis, specimens were processed[15]
and were cultured on the Lowenstein–Jensen medium.
The suspected mycobacterial colonies were checked for niacin
production, nitrate reductase, resistance to thiophen–
2–carboxylic acid hydrazide and catalase tests. Isolates
eventually were assayed by PCR targeting IS6110[16].

2.2. Drug–susceptibility testing

The proportional method was used to assess the
susceptibility of isolates to INH (0.2 µg/mL) and RIF (4 µg/
mg/mL)[17]. The results were interpreted according to WHO
guidelines[18].

2.3. DNA extraction

Genomic DNA of M. tuberculosis isolates was extracted
using phenol–chloroform method[1]. The concentration DNA
was measured at 260 nm using Nanodrop (Thermo Scientific,
USA).

2.4. PCR condition

Primers described by Cheng et al., were used to amplify
the rpoB and katG genes as follow: forward 5′–GGG CCA
TGA GGG TTA CAC–3′ and reverse 5′–CGT CCT TGG CGG TGT
ATT–3′ for katG (Accession number X68081) with 458 bp
PCR product, also forward 5′–CAG ACG TGG ATC AAC ATC
CG–3′ and reverse 5′–TAC GGC GTT TCG ATG AAC–3′ for rpoB
(Accession number L27989) with 305 bp PCR product[1].
The position of katG463 known as natural polymorphism,
therefore this position is not including in amplifying
region[19–21]. The PCR were optimized for the amplification
of the rpoB and katG genes. The PCR reactions were done
in a final volume of 25 µL containing 2.5 µL of 10X PCR
buffer, 1 µL of 2.5 mmol/L of each deoxy–ribonucleoside
triphosphate, 1 µL of 10 pmol/L of each desired reverse
and forward primer, 1.5 and 3.5 µL of 50 mmol/mL MgCl2,
respectively for rpoB and katG, 1 IU of Taq polymerase
(CinnaClon Co., Iran), and 2–5 ng of DNA template. The
thermocycler program of PCR reactions was performing with
an initial denaturation step of 5 min at 95 °C; 40 cycles of 30
seconds at 95 °C, 30 seconds at an appropriate temperature,
and 45 seconds at 72 °C, and a final extension step of 7 min
at 72 °C.

The annealing temperatures for rpoB and katG were
optimized at 61 °C and 60 °C, respectively. The amplicon
of each reaction was tested by electrophoresis on 1.5%
agarose gel and visualized under UV illumination after
staining with ethidium bromide (0.5 mg/mL). For further
confirmation, relative bands from all PCR products were
amplified with the Pfu polymeraseand sequenced (Gene
Fanavaran Co., Iran. M1 and M2 strains were prepared from tuberculosis center of the Masih Daneshvar Hospital as MDR–TB strains and used as a mutant control. H37Rv, a susceptible strain, was used as a wild type control.

### 2.5. Single strand conformational polymorphism testing (SSCP)

Amplified products from described PCR were mixed with unequal volume of formamide loading dye (97% formamide, 0.05% bromphenol blue and 20 mmol/L ethylene diamine tetraacetic acid. After denaturing of these mixtures at 95 °C for 6 min for rpoB and 75 °C for 2 min for katG, the products were loaded onto 8 cm х 7 cm gel containing 10.5% acrylamide:bisacrylamid (29:1) and 10% glycerol and electrophoresis were carried out at 100 V for 4 h for rpoB amplicon and 8 h for the katG amplicon in TBE 0.5X at room temperature. Consequently, the gel was stained by the silver staining method as described by Bassam et al.[22].

### 3. Results

#### 3.1. Drug susceptibility testing

By using specific biochemical test and PCR assay, 103 samples containing M. tuberculosis were isolated. Results obtained from drug susceptibility testing showed that 12 (11.6%) isolates were resistant to at least one antibiotic, INH or RIF. Furthermore, 9 (8.7%) isolates out of 12 showed the resistance properties to both INH and RIF simultaneously. Susceptibility of isolates under study.

#### 3.2. PCR–SSCP results

To amplify the rpoB and katG genes, we designed PCR, respectively. PCR condition for rpoB and katG were optimized for detecting of clinical isolates. The amplified fragments for the rpoB and katG genes are 305 bp and 458 bp, respectively. To determine the presence of known and unknown mutations within the mentioned genes, SSCP was applied. Figures 1 and 2 show examples of results from PCR–SSCP isolates. Overall, 6 (5.82%) isolates had a mutation at least in one gene compared to the H37Rv strain (Table 1). Among these isolates, 3 (2.9%) isolates were categorized in the MDR–TB group because of having mutations simultaneously in the rpoB and katG. In addition, mutations within the rpoB (resistant to RIF), katG (resistant to INH) genes were observed in one and two isolates, respectively. All the MDR–TB and two of INH resistant isolates were also identified by drug susceptibility testing.

### Table 1

The comparison of results obtained from three methods, the drug susceptibility testing, the sequencing and PCR–SSCP, performed in this study.

<table>
<thead>
<tr>
<th>Code</th>
<th>Phenotypic</th>
<th>Sequencing</th>
<th>SSCP rpoB</th>
<th>katG</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5</td>
<td>Resistance to INH: AGC-&gt;ACC; (Ser-&gt;Thr) Codon 315</td>
<td>315</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Z21</td>
<td>Resistance to INH: AGC-&gt;ACC; (Ser-&gt;Thr) Codon 315</td>
<td>315</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>E31</td>
<td>Susceptible to RIF: NA</td>
<td>NA</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>E4</td>
<td>Resistance to INH: Negative</td>
<td>Negative</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>E13</td>
<td>Resistance to RIF: NA</td>
<td>NA</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>E19</td>
<td>Resistance to INH: Negative</td>
<td>Negative</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>K11</td>
<td>Resistance to INH: AGC-&gt;ACC; (Ser-&gt;Thr) Codon 315</td>
<td>315</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Z33</td>
<td>Resistance to INH: AGC-&gt;ACC; (Ser-&gt;Thr) Codon 315</td>
<td>315</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>S1</td>
<td>Susceptible to RIF: NA</td>
<td>NA</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>S3</td>
<td>Resistance to INH: Negative</td>
<td>Negative</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>S5</td>
<td>Resistance to INH: AGC-&gt;ACC; (Ser-&gt;Thr) Codon 315</td>
<td>315</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>E4</td>
<td>Resistance to INH: Negative</td>
<td>Negative</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>E17</td>
<td>Resistance to INH: NA</td>
<td>NA</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>E19</td>
<td>Resistance to RIF: NA</td>
<td>Negative</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

All of isolates were detected resistance by at least one of the three methods. NA: not assay.

#### Figure 1. Gel electrophoresis of PCR–SSCP products of the rpoB gene. Lanes numbered 1, 4 show patterns of rpoB of H37Rv (without mutation), and rpoB of H37Rv (without mutation), respectively; lane 3 is a mixture of PCR without our considred amplicons as negative control for SSCP test; lane 2 is a pattern of the M1 strain as a MDR sample; lane 5 shows a mutation in each three band of rpoB; lanes 6, 7 and 8 are clinical samples without mutation.

#### Figure 2. Gel electrophoresis of PCR–SSCP products of the katG gene. Lane 1 is a pattern of clinical sample without mutation; Lane 2 is M1 with mutation in band 2 of katG; Lanes numbered 3, 5, 6, 7 and 8 show the presence of mutation that have been represented in band 2; lane 4 is H37Rv (a negative control without mutation).

### 3.3. DNA sequencing

To confirm the findings from SSCP, 12 (11.6%) resistant
isolates separated using the drug susceptibility test, DNA sequencing method was done. Three (2.9%) MDR–TB and two INH resistant isolates were detected by this method and also by both other methods.

All the mutations emerged within the rpoB and katG genes were Ser531Leu and Ser315Thr, respectively (Table 1).

3.4. Specificity and sensitivity

To determine the specificity and sensitivity of our methods, the drug susceptibility testing was utilized as an standard method. Sensitivity defined as probability of mutation pattern if the resistances have been shown by phenotypic drug susceptibility testing[1]. Specificity defined as probability of wild pattern if the susceptibility has been shown by phenotypic drug susceptibility testing[1]. From nine MDR isolates, only three were recognized by the PCR–SSCP, therefore the sensitivity of our method was 33.33%. Five of 12 INH resistant strains showed mutation by PCR–SSCP and the sensitivity was measured as 41.67%. Also three of nine RIF resistant strains detected by PCR–SSCP and sensitivity was measured as 33.33%. Since the results from sequencing test were similar to findings from PCR–SSCP, sensitivity of both tests were equal. The specificity of PCR–SSCP and sequencing for MDR, INH resistant and RIF resistant strains were 100%, 100%, and 98.94%, respectively. Table 2 shows the sensitivity and specificity of discrepant resistant groups of isolates in details.

Table 2
The sensitivity and specificity of the PCR–SSCP and DNA sequencing for detection of drug resistance strains.

<table>
<thead>
<tr>
<th>Drug and gene</th>
<th>Number of resistance isolates</th>
<th>Number</th>
<th>Sensitivity (SSCP)</th>
<th>Number</th>
<th>Sensitivity (Seq.)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH resistance</td>
<td>12</td>
<td>5</td>
<td>41.67</td>
<td>5</td>
<td>41.67</td>
<td>100.00</td>
</tr>
<tr>
<td>XNIR</td>
<td>3</td>
<td>2</td>
<td>66.66</td>
<td>2</td>
<td>66.66</td>
<td>100.00</td>
</tr>
<tr>
<td>MIR</td>
<td>9</td>
<td>3</td>
<td>33.33</td>
<td>3</td>
<td>33.33</td>
<td>100.00</td>
</tr>
<tr>
<td>INH Sus.</td>
<td>91</td>
<td>91</td>
<td>-</td>
<td>91</td>
<td>-</td>
<td>100.00</td>
</tr>
<tr>
<td>RIF resistance</td>
<td>9</td>
<td>3</td>
<td>33.33</td>
<td>3</td>
<td>33.33</td>
<td>98.94</td>
</tr>
<tr>
<td>RIF Sus.</td>
<td>94</td>
<td>93</td>
<td>-</td>
<td>93</td>
<td>-</td>
<td>100.00</td>
</tr>
<tr>
<td>MDR</td>
<td>9</td>
<td>3</td>
<td>33.33</td>
<td>3</td>
<td>33.33</td>
<td>100.00</td>
</tr>
<tr>
<td>None MDR</td>
<td>94</td>
<td>94</td>
<td>-</td>
<td>94</td>
<td>-</td>
<td>100.00</td>
</tr>
</tbody>
</table>

NMR: non MDR INH resistance; MIR, MRR: MDR INH resistance and MDR RIF resistance respectively; INH Sus, RIF Sus: susceptible to INH and RIF respectively; Seq.: DNA sequencing.

4. Discussion

Controlling and decrease the rate of M. tuberculosis particularly the MDR–TB is a highlight subject that is aimed by public healthcare systems worldwide. The classical diagnostic method based on culture and phenotypic drug susceptibility testing are common methods for detecting clinical samples containing M. tuberculosis. Although these methods have been approved as a gold standard, they require at least 6–8 weeks representing outcomes. Developing a rapid detection method is essential in this field. However, researchers designed several molecular methods for this concept, but most of them are expensive and need specific equipments that are unavailable in numerous countries. In the present study, we designed PCR–SSCP method which is capable to detect mutations within two genes, the katG and rpoB genes, simultaneously during 13 h. In addition, this method can be done in laboratories with a limited equipment and space.

To our knowledge, few studies attempted to detect RIF and INH resistant strains by using PCR–SSCP, simultaneously. At the first time, Cheng et al. developed the detection method based on PCR–SSCP[1]. In this study, known and unknown mutations created in the katG, inhA and rpoB genes which respectively involve in the RIF and INH resistance by using multiplex PCR–SSCP. Data from that showed that designed method is capable to screen more up to 80% of resistant clinical isolates. Its specificity for RIF and INH was 92% and 100%, respectively[1]. In another study performed by Chan et al., the resistance to ofloxacin, rifampicin, ethambotol, isoniazid and pyrazinamide, anti-mycobacterium drugs, was evaluated by SSCP/multiplex PCR amplimer conformation analysis. They identified 102 new mutations within the seven genes named the gyrA, rpoB, embB, katG, inhA, ahpC and pncA genes, which contribute in the resistance to five described drugs[23]. It should be noted that numerous resistant isolates showed no mutations in the interest genes in this study. The purified DNA obtained from culture of clinical samples was utilized in both studies mentioned above.

Recently, Grutzmacher et al. evaluated of resistance to five antibiotics described above by detecting mutations within the gyrA, rpoB, embB, katG, inhA, ahpC and pncA genes by the PCR–SSCP in clinical sputum samples[24]. In this study, only one sample that was resistant to ethambotol, represented the mutation within the embB gene by SSCP and other resistant samples detected by phenotypic diagnostic methods had wild–type patterns. In this study, the authors reported that PCR–SSCP wasn’t able to screen resistant strains compared to classical methods.

Here, our objective was to assess potency of PCR–SSCP as a rapid screening tool for determination of resistant M. tuberculosis strains. Despite of low sensitivity, all the mutations determined by sequencing method were identified by our PCR–SSCP method that it showed the accuracy of our methods. Higher frequency of resistant strains was recognized by the drug susceptibility testing. According to agreement between sequencing tests and SSCP test and significant difference between them and phenotypic drug susceptibility test, we can conclude that resistance–
associated mutations occurred in other genes except katG and considered region of rpoB which weren’t analyzed by our method. Coll et al.[25], Piatek et al.[26] and Tudo et al.[27] have reported high rate of resistance-associated mutations in genes except katG which are match with our result and confirm our findings. Since genes involved in drug resistance is a region specific and discrepant geographical regions have a different gene pattern, it is necessary to identify this pattern for each country to increase capability of this method. Furthermore, usage of DNA purified from colonies but not directly from clinical samples is another finiteness of our developed method that requires justifying. Considred to high specificity of our test, we can utilize this method together with gold standard methods including drug susceptibility testing. In addition to speed and accuracy, our method was optimized in room temperature and for 8 cm ×7 cm gel, therefore this is applicable in a broad range of laboratories even in poor economic region. According to agreement among sequencing, SSCP tests and significant difference with phenotypic drug susceptibility method, we conclude that resistance-associated mutations can occur in other genes except katG and considered region of rpoB. High rate of resistance-associated mutations in genes except katG have been reported which are match with our result and confirm our findings.

Conflict of interest statement
We declare that we have no conflict of interest.

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