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

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which the authors evaluated PCR-SSCP method which is capable to detect mutations within two genes, the *katG* and *rpoB* genes, simultaneously during 13 h. The results are interesting and suggest that this method is a appropriate technique for rapid detection of MDR-*M. tuberculosis* 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

Drug resistance, *Mycobacterium tuberculosis*, PCR-SSCP, Phenotypic method, *rpoB*, *katG*

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

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to convert it to an active form. For instance, catalase-proxidase (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 *katG* gene, especially Ser315Thr[8,9]. 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*, sepecimens 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 targetting 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/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′-CGG CGA TGA GCG 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 TTG 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 *katG*463 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 10× 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/L $MgCl_2$ 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 Daneshvari 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 anequal 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×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 0.5× 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 that they are known as MDR-TB. Three isolates were only resistant to INH. Table 1 illustrates the pattern of drug resistance 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.

Code	Phenotypic	Sequencing	SSCP	
			rpoB	*katG*
B5	Resistance to INH	AGC→ACC (Ser→Thr) Codon 315 *katG*	R	R
	Resistance to RIF	TCG→ TTG (Ser→Leu) Codon 531 *rpoB*		
Z21	Resistance to INH	AGC→ACC (Ser→Thr) Codon 315 *katG*	S	R
	Susceptible to RIF	NA		
E31	Resistance to INH	Negative	S	S
	Resistance to RIF	Negative		
E4	Resistance to INH	Negative	S	S
	Susceptible to RIF	NA		
E13	Resistance to INH	Negative	S	S
	Resistance to RIF	Negative		
E19	Resistance to INH	Negative	S	S
	Resistance to RIF	Negative		
K11	Resistance to INH	AGC→ACC (Ser→Thr) Codon 315 *katG*	R	R
	Resistance to RIF	TCG→ TTG (Ser→Leu) Codon 531 *rpoB*		
Z33	Resistance to INH	AGC→ACC (Ser→Thr) Codon 315 *katG*	S	R
	Susceptible to RIF	NA		
S1	Susceptible to INH	NA	R	S
	Susceptible to RIF	NA		
S3	Resistance to INH	Negative	S	S
	Resistance to RIF	Negative		
S5	Resistance to INH	AGC→ACC (Ser→Thr) Codon 315 *katG*	R	R
	Resistance to RIF	TCG→ TTG (Ser→Leu) Codon 531 *rpoB*		
E′4	Resistance to INH	Negative	S	S
	Resistance to RIF	Negative		
E17	Resistance to INH	Negative	S	S
	Resistance to RIF	Negative		

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

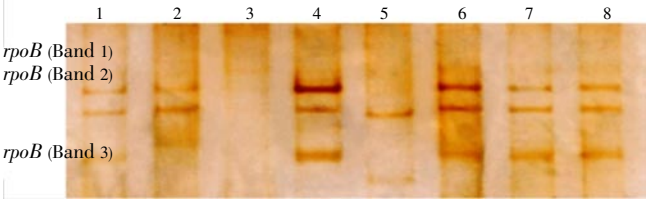

Figure 1. Gel electrophoresis of PCR-SSCP products of the *rpoB* gene. Lanes numbered 1, 4 show patterns of *rpoB* of H37Rv (without mutation), and *rpoB* of H37Rv (without mutation), respectively; lane 3 is a mixture of PCR without our considred amplicons as negative control for SSCP test; lane 2 is 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.

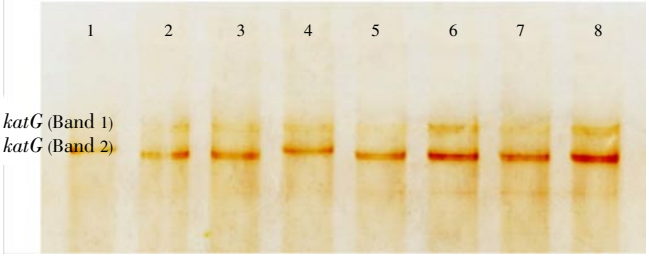

Figure 2. Gel electrophoresis of PCR-SSCP products of the katG gene. Lane 1 is a pattern of clinical sample without mutation; Lane 2 is M1 with mutation in band 2 of *katG*; Lanes numbered 3, 5, 6, 7 and 8 show the presence of mutation that have been represented in band 2; lane 4 is H37Rv (a negative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

Drug and genre	Number of resistance isolates	Number		Sensitivity		Specificity (%)
		SSCP	Seq.	SSCP (%)	Seq. (%)	
INH resistance	12	5	5	41.67	41.67	
NMIR	3	2	2	66.66	66.66	100.00
MIR	9	3	3	33.33	33.33	
INH Sus.	91	91	–	–	–	
RIF resistance	9	3	3	33.33	33.33	
MRR	9	3	3	33.33	100	98.94
RIF Sus.	94	93	–	–	–	
MDR	9	3	3	33.33	33.33	100.00
None MDR	94	94	–	–	–	

NMIR: 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* particularythe 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 arapid 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.*[23], the resistance to ofloxacin, rifampicin, ethambutol, 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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Comments

Background

M. tuberculosis is an important pathogen and an important public health problem that occurs in numerous countries. Currently, the strains with MDR-TB are a complicate problem in these countries. Therefore it is necessary to design an efficient method for monitoring the level of drug resistance. In this study, authors attempted to design a rapid and efficient molecular method based on PCR-SSCP that can detect all the mutations in the *katG* and *rpoB* genes which contribute in MDR-TB phenotype. In addition, the authors compared their findings to results from sequencing and conventional methods among clinical isolates.

Research frontiers

M. tuberculosis is an important pathogen and an important public health problem that occurs in numerous countries especially Iran. Currently, the strains with MDR-TB are a complicate problem in Iran. Therefore it is necessary to design an efficient and fast method for monitoring the level of drug resistance.

Related reports

Cheng et al. studied a new Multi-PCR-SSCP assay for simultaneous detection of INH and RIF resistance in *M. tuberculosis*. Study of efficient method for monitoring the level of drug resistance in *M. tuberculosis* is very few in Tehran, Iran.

Innovations and breakthroughs

In this study, the authors attempted to design a rapid and efficient molecular method based on PCR-SSCP that can detect all the mutations in the *katG* and *rpoB* genes which contribute in MDR-TB phenotype. In addition, the authors compared their findings to results from sequencing and conventional methods among clinical isolates in Iran.

Applications

Controlling and decreasing the rate of *M. tuberculosis* particularly the MDR-TB is a highlight subject that is aimed by public healthcare systems worldwide especially Iran. In this study, they 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.

Peer review

This is a good study in which the authors evaluated PCR-SSCP method which is capable to detect mutations within two genes, the *katG* and *rpoB* genes, simultaneously during 13 h. The results are interesting and suggest that this method is a appropriate technique for rapid detection of MDR-*M. tuberculosis* from clinical specimens.

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