

# DETECTION OF ISONIAZID AND RIFAMPIN RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATED FROM TUBERCULOSIS PATIENTS USING CONVENTIONAL METHOD AND PCR

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## ABSTRACT:

**Objective:** To investigate the drug susceptibility patterns among TB isolates from patients in Ahvaz, Iran.

**Study:** Descriptive study.

**Place and duration of study:** TB reference laboratory, PHLS, Ahvaz, Iran from May 2001 to December 2001.

**Patients and methods:** A total of 100 sputum samples from patients suspected of having tuberculosis were collected for detection of *M. tuberculosis* (MTB). For identification of the isolates acid fast stain, cultural and biochemical techniques were used. The isolates were examined for INH and RIF resistance using conventional MIC method and PCR technique in the next step. MIC were done based on proportion method and PCR was performed according to manufacturer's instruction and by using specific INH (*Kat G*) and RIF (*rpo B*) resistant primers.

**Results:** Eighty samples were identified as MTB. Using susceptibility testing, 7 isolates were resistant to both INH and RIF by MIC method. In PCR technique, 5 and 6 out of 7 above mentioned strains showed resistant to INH and RIF respectively.

**Conclusion:** The prevalence of resistance to INH and RIF is high in the region of study. The conventional MIC method despite being time consuming is more sensitive for evaluation of drug resistance among TB isolates. However, PCR as a rapid and sensitive technique is recommended additionally to conventional method for having quicker results to start treatment and disease control management.

**KEY WORDS:** *Mycobacterium tuberculosis*, Isoniazid, Rifampin, Resistance, PCR

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## INTRODUCTION

The emergence of drug-resistant strains of *Mycobacterium tuberculosis* (MTB) is an increasing problem in developed and developing coun-

tries alike. Today rifampin (RIF) and isoniazid (INH) are important components of effective multidrug therapy and prophylaxis for *M. tuberculosis* infections. However, widespread use of these agents and failure of patients to complete prescribed treatment have led to the emergence of RIF- and INH-resistant strains.<sup>1,2</sup>

The detection of resistant *M. tuberculosis* strains is generally performed by conventional susceptibility assays which require the isolate to be cultured in the presence of the different drugs. This usually delays in the detection of resistance. Thus methods that guarantee the early detection of resistant *M. tuberculosis* strains are required in order to avoid delays in the initiation of effective therapies and to prevent transmission of multidrug-resistant (MDR) strains.

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The molecular basis of resistance to anti-TB drugs is now becoming clearer. More than 95% of RIF-resistant strains are associated with mutations within an 81-bp region of the *rpoB* gene.<sup>3</sup> Between 60 to 70% of the INH-resistant strains encode mutations in *KatG*.<sup>4</sup> These findings have led to the development of different genotypic approaches to the more rapid prediction of resistance in *M. tuberculosis*, especially to RIF and INH.<sup>5-7</sup> Our aim was to investigate the rough prevalence of resistance of MTB isolates to INH and RIF in the region and to apply PCR for the detection of resistant strains and compare the sensitivity of the technique to conventional MIC method.

## MATERIAL AND METHODS

**Detection of *M. tuberculosis*:** The study was performed on 100 sputum samples from different patients who were suspected of having tuberculosis based on clinical symptoms and radiographic evidence. The samples were taken before starting the anti-TB therapy for most of the patients. The isolates were identified as MTB by acid fast staining of direct smears prepared from sputum samples, culture in Lowenstein Jensen (LJ) medium and subsequent biochemical tests, including the niacin accumulation test, the nitrate reduction test, and heat-labile catalase test.<sup>8</sup>

**Susceptibility testing:** Rifampin and Isoniazid resistance testing were performed by the proportion method according to the CDC laboratory procedures manual.<sup>9</sup> In brief, few colonies were harvested from surface of LJ medium and suspended in 3ml sterile saline, mix thoroughly and the turbidity was adjusted to McFarland solution 1 (to yield  $3 \times 10^8$  cells/ml). One ml of the suspension were inoculated into separate LJ media containing 40µg, 20µg and 10 µg of RIF per ml and 1µg, 0.2µg and 0.1µg of INH per ml. The media were incubated for two weeks at 37°C and the isolate was considered resistant if there was more than 1% growth on the antibiotic containing medium compared with the growth on the drug-free medium.

**DNA extraction:** Few colonies were removed from the surface of LJ medium and suspended

Table-I. Oligonucleotide-primers used in the amplification protocol by PCR of the *M. tuberculosis* strains.

Target	Oligonucleotides	Position
<i>RpoB</i>	TR8 52 - GTGCACGTCGCGGACCTCCA	2492
	TR9 52 - TCGCCGCGATCAAGGAGT	2335
<i>KatG</i>	TB86 52 - GAAACAGCGGCGCTGATCGT	2759
	TB89 52 - GTTGTCCCATTTTCGTCGGGG	2967

in 100 µl of sterile double distilled water and subsequently 100µl of chloroform was added. After thorough mixing, the mixture was incubated at 80°C for 20 min. After centrifugation at 12000g for 1 min in a microcentrifuge, the supernatant was used for amplification.<sup>10</sup>

**Amplification:** The DNA preparation was amplified with the primers listed in Table-I. These primers were designed to amplify the regions between nucleotides 2335 and 2492 of the *rpoB* gene and nucleotides 2759 and 2967 of the *katG* gene.<sup>7</sup>

The composition of PCR mixture in 25µl final volume was: 1X PCR buffer, 1.5 mmol MgCl<sub>2</sub>, 0.2 mmol of each deoxynucleotide triphosphate, 0.5 µmol of each primer, 1 unit of Taq polymerase, 14µl of sterile distilled water and 5µl of DNA template. *Mycobacterium tuberculosis* strain H37Rv resistant to INH and RIF was used as positive standard control for each set of the reaction. The PCR kit and other reagents were purchased from Cinnagen Co. Tehran, Iran.

The reaction conditions were as described previously and it was 95°C for 45s followed by 35 cycles of 95°C for 15s, 55°C (annealing temperature for the *rpoB* gene) and 60°C (annealing temperature for the *katG* gene) for 15s and 72°C for 15s in a thermal cycler (Techgene).<sup>7</sup> The PCR products were loaded on 1.5% (w/vol) agarose gel with 0.5 mg ethidium bromide per ml. The gel was electrophoresed at 100v for at least 30 min and the DNA fragments were visualized by UV light.

## RESULTS

The total of 80 (80 %) of the specimens were culture positive for MTB. Among these, 35 were

Table-II. distribution of resistance to INH and RIF due to mutations in genes *rpoB* and *katG* among the isolates.

Drugs	Sensitive No. and %	Resistant No. and %
RIF	74(92.5)	6(7.5)
<i>rpoB</i>		
INH	75(93.75)	5(6.25)
<i>katG</i>		

isolated from females and 45 were belonged to male patients. The majority of patients were classified in age groups 20-30 and over 40 in both genders. In determining sensitivities, there was 73 (91.25%) isolates sensitive to both INH and RIF and 7 (8.75%) isolates showed resistance to all three concentrations of the drugs. Patients in age group 20-30 have shown the most predominant resistance in this study, of which 5 resistant cases were belonged to male patients.

PCR technique, revealed that 74 isolates were sensitive to RIF, 75 isolates sensitive to INH, 6 (7.5%) isolates resistant to RIF and 5 (6.25%) isolates showed resistance to INH, reflecting mutations in *rpoB* and *katG* genes respectively (Table-II).

Since a few of the patients had receive anti-TB therapy previously, the results were included in the resistance pattern in both treated and untreated groups. Comparison of sensitivity of both conventional and molecular methods in detecting resistant strains showed that on MIC, RIF and INH both had a resistance of 7 (8.75%) whereas on PCR the resistance was 6 (7.5%) and 5 (6.25%) for RIF and INH respectively.

## DISCUSSION

The present study was undertaken according to standard laboratory guidelines and showed high resistance of the MTB isolates to INH and RIF. Our findings revealed that 7 isolates out of 80 tested strains were resistant to both INH and RIF using MIC method. Based on reports from around the world the prevalence of MTB multi-drug resistant is estimated from 0% in Kenya to 40.6% in Dominican

Republic.<sup>11</sup> Reports from several countries provided by WHO, have indicated very low resistance to RIF compared to INH.<sup>12</sup> There is limited documented reports from the situation of TB drug resistance in Iran. In a study undertaken in 2001, the similar results were reported for INH from Eastern Iran with no resistance for RIF.<sup>13</sup> Using PCR could enable to detect 5 INH- and 6 RIF-resistant strains which was in agreement to other investigations,<sup>7,14,15</sup> reported that more than 95% of resistant strains to RIF and 60-70% of resistant strains to INH show mutations in *rpoB* and *katG* genes respectively.<sup>3,4, 7, 14,15</sup> So we may discuss that failure of this study to detect all resistant strains detected by MIC method, be due to mutation in other genes especially in INH resistant strains.<sup>16,17</sup> There are several other investigations concluded most of the resistant strains to RIF show mutation in *rpoB* gene and were in favor of our findings.<sup>18,19</sup>

Obviously, the number of MTB strains in present study was not sufficient enough to reach a firm conclusion about the exact percentage of INH and RIF resistance in this region of Iran. Besides, due to this and the short period of time of study, we cannot judge about the exact percentage of primary and secondary resistance which included both in present work, and is very important from epidemiology of the disease point of view in the region of study.

Despite that several rapid molecular and non-molecular techniques have been introduced by other workers for detection of susceptibilities of mycobacteria to anti-TB drugs,<sup>10, 12, 20,21</sup> it seems that we still need to retest all the isolates scored by such a technique as resistant to INH or RIF, by conventional MIC testing.

The present study demonstrated that the prevalence of INH and RIF resistance is very high in the region. However to find an exact prevalence of drug resistance more samples need to be tested. Technically, despite the fact that PCR is a rapid technique for detection of MTB resistant strains, but it is preferable to use both conventional and molecular methods to

have more precise information regarding the resistance pattern in a certain area.

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