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Original Article

Characterization of Mutations in the Rpob and Katg Gene of *Mycobacterium Tuberculosis* Isolates From Pasteur Institute of Tehran

Hamid Tebyanian[1], Hamze farhadian[2], Mohamad Reza Imen Shahidi[2*], Mehdi Behdani[3]

[1]*Young Researchers and Elites Club, North Tehran Branch, Islamic Azad University, Tehran, Iran*
[2]*Department of Microbiology, Kerman Branch, Islamic Azad University, Kerman, Iran*
[3]*Biotechnology Research Center, venom & Biotherapeutics Molecules Lab., Pasteur Institute of Iran, Tehran, Iran*

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ABSTRACT

Objective: The Rifampicin resistance and susceptibility of *Mycobacterium tuberculosis* are caused by mutations in the 81-base pair region of the rpoB gene encoding the b-subunit of RNA polymerase. **Methods:** Isoniazid resistance of *M. tuberculosis* is related to mutations in inha , oxyR and ahpC genes which 30 to 90 percent of Isoniazid resistance is occurred in 3015 codons of katg gene. The rpoB and katG sequences of 30 isolates were analyzed to identify the mutations and compare the mutations with their related susceptibilities. **Results:** In this research, we investigated the location and type of rpoB and katG mutations in *Mycobacterium tuberculosis* which had been achieved from Pasteur Institute of Tehran. PCR Amplification and DNA sequencing methods were performed. In this assay, from 507 to 537 codons and 315 codons of rpoB and katG genes were sequenced and also mutations were analyzed, respectively.

1.INTRODUCTION

Multidrug resistance in *Mycobacterium tuberculosis* is a global burden (Lin et al. 2013). Tuberculosis (TB) is considered to be multidrug resistant (MDR) when its causal agent, *Mycobacterium tuberculosis*, is resistant to at least Rifampicin (RIF) and Isoniazid (INH), which are the main first line drugs used to treat TB. Resistance to these drugs requires the administration of less effective, more toxic, and more costly drugs. The molecular mechanisms of resistance have been determined for many anti-tuberculosis agents. It has been shown that *M. tuberculosis* isolates essentially become resistant through mutations that occur spontaneously at low frequency in chromosomal genes. In clinical practice, drug resistance occurs as a result of inadequate therapeutic regimens or patient failure to comply with treatment. INH is one of the main anti-TB drugs; mutations in the katG gene are related to INH resistance, with katG S315T mutation being the most common in INH-resistant strains. Many studies have shown that RIF-resistance is due to mutations mostly within a defined region of 81 bp of the rpoB gene, which encodes the b-subunit of the RNA polymerase. Mutations in this region of the gene have been found in approximately 96% of the RIF-resistant M. tuberculosis strains that were recovered globally (Spies et al. 2013). Another first-line drug used in the treatment of TB is streptomycin (STR, #9). Many STR-resistant

*Corresponding Author: Mohamad Reza Imen Shahidi, Department of Microbiology, Kerman Branch, Islamic Azad University, Kerman, Iran(mohammadrezaimenshahidi@gmail.com)

strains have mutations in the rpsL and rrs genes that encode the ribosomal protein S12 and 16S ribosomal RNA, respectively. Acquiring drug resistance in bacteria often carries a biological cost because antibiotics generally target essential, highly conserved genes. Although drug-resistant strains often suffer an initial reduction in fitness, they continue to evolve by acquiring one or more secondary-site mutations that can improve or even restore the fitness over time (Borrel et al. 2009; Spies et al. 2013). Furthermore, although the acquisition of drug resistance determinants is often associated with a loss in fitness, some strains with low- or no-cost mutations have been reported. For example, the mutation in codon 43 of the rpsL gene,8 the mutation katG S315T,9 and the mutation rpoB S531L were described as having a relative fitness greater than or equal to 1.0, which is considered to be the fitness of the susceptible *M. tuberculosis* strain (Billington et al. 1999; Spies et al. 2013). Some mutations have implications in virulence. For example, the katG mutation S315T has a high degree of INH resistance, retains enzyme activity (Pym et al. 2002) and is the only katG mutation associated with successful transmission (Gagneux et al. 2006). According to a report from World Health Organization, there were an estimated 8.8 million incident cases of tuberculosis (TB) (range, 8.5 millione9.2 million) globally in 2010, 1.1 million deaths (range, 0.9 millione1.2 million) among human immunodeficiency (HIV)-negative cases of TB, and an additional 0.35 million deaths (range, 0.32 millione0.39 million) among people who were HIV positive. There were an estimated 12.0 million prevalent cases (range, 11.0 millione14.0 million) of TB in 2010. In 2010, there were an estimated 650,000 cases of multidrugresistant TB (MDR-TB) among the world's 12.0 million prevalent cases of TB. Among the estimated 8.8 million new and recurrent cases of TB in 2010, 5.7 million were diagnosed and reported to national TB control programs; among notified cases, there were an estimated 290,000 cases of MDR-TB, of which only 53,000 patients (18%) were reported to have been diagnosed with the condition and enrolled on appropriate treatment (Lin et al. 2013). For the ancient disease TB as with other "neglected diseases" for which the Pharmaceutical Industry has low expectance in terms of revenues, since these are mostly widespread in underdeveloped countries, an exciting drug development pathway is open by targeting genetic make-up in susceptibility. Forty years have passed with no introduction into the market of new anti-TB drug classes, in spite of a huge diversity of attempts, providing identification of new targets, and the discovery of novel agents with novel mode of action (MoA). In current Directly Observed Therapy (DOT) programs, Rifabutin (RFB) plays a fundamental role in the treatment of patients with active TB. Although Rifampicin (RFP) and isoniazid (INH) are by far the most effective anti-TB agents, RFP and RFB are crucial for ensuring success of short-course (6-month) chemotherapy (King et al. 2010; Figueiredo et al. 2012). Namely, patients with INH-resistant TB can respond well to 6- month treatment without INH (on a regimen of RFP, pyrazinamide, and ethambutol), but patients with RFP-resistant TB do not respond well to short-course chemotherapy without RFP or RFB. Without RFP or RFB, most anti-TB regimens require at least 18 months of treatment, and every attempt should be made to use RFP or RFB in initial anti-TB treatment. In addition, anti-TB drug regimens for patients infected with the human immunodeficiency virus (HIV) pose a special challenge when certain antiretroviral medications are also indicated. RFP should not be taken together with most protease inhibitors or non-nucleoside reverse transcriptase inhibitors. RFB, however, often has to be used in these patients (Burman et al. 2001; Figueiredo et al. 2012). Nevertheless, a 2010 Cochrane Review concluded that the replacement of RFP by RFB for first-line treatment of TB is not supported by the current evidence, and also that HIV positive people with TB, the group most likely to benefit from the RFB use, are still under-represented in trials to date, and further trials in this group would be useful. In general, the major problem in the clinical use of RFB and other rifamycins (Rifs), is that their versatility and efficiency are limited by the rapid emergence of resistant strains, mostly as a consequence of mutations that occur on the active side of its molecular target: the ribonucleic acid polymerase enzyme (RNAP). To overcome this problem, it is necessary to understand the detailed MoA and the bacterial resistance to these drugs at the molecular level. A key breakthrough was the recent determination of the crystal structures of several inhibitors with the RNAP (Davies et al. 2010; Figueiredo et al. 2012). In this study, we showed that Characterization of mutations in the rpoB and katG gene of *Mycobacterium tuberculosis* isolated from Pasteur Institute of Tehran.

2. MATERIALS AND METHODS

2.1. Mycobacterium tuberculosis isolates

Bacteriologically (cultured in Lo¨wenstein- Jensen or MGIT medium) and biochemically (nitrate and niacin tests) of *M. tuberculosis* were identified in Pasteur Institute of Tehran (Huang et al. 2009).

2.2. Drug susceptibility testing (DST)

Test methods, media, and drug concentrations were used as previously described Growth on a control medium was compared to growth on a drug-containing medium in order to determine susceptibility or resistance. The DST results were classified as resistant or susceptible. Tests were validated by the susceptibility of *M. tuberculosis H37Rv*, included in the same test. Drug susceptibility testing (DST) to first-line drugs INH (0.25 and 1.0 µg/ml) and RIF (20.0 and 40.0 µg/ml) was performed in Pasteur Institute of Tehran. (Huang et al. 2009; Feuerriegel et al. 2012).

2.3. DNA sequencing of the katG and rpoB genes

Mutations in katG and rpoB were analyzed by PCR amplification, and then were sequenced with the respective oligonucleotide primers: RF-F (5'-TCGGCGAGCCCATCACGTCG-3'); RF-R (5'-GCGTACACCGACAGCGAGCC-3') and KatG-F (5'-GTCACACTTTCGGTAAGAC-3'); KatG-R (5'-TTGTCGCTACGGAACG-3′) (Huang et al. 2009). PCRs were performed as follows: 33 cycles at 96°C for 1 min; annealing for 1 min at 64°C for rpoB, 55°C for katG and elongation at 72°C for 1 min. Then, the PCR products had been sent to Bioneer company for sequencing and the sequence data were assembled and edited by using Sequencing Analysis software (version 5.2.0; Applied Biosystems) (Huang et al. 2009; Lin et al. 2013). All strains were sequenced in the predominant resistance determining regions (RDR) of katG (codon 315) and rpoB (codon 507–537 according to E. coli numbering) (Feuerriegel et al. 2012).

3. RESULTS

In this research, Rifampicin and Isoniazid resistance and susceptibility of *M. tuberculosis* had been tested by drug testing which 12 and 4 samples of *M. tuberculosis* were resistant and susceptibility to Rifampicin in drug testing; respectively. Nine samples were resistant to Rifampicin and three samples were susceptibility to Rifampicin in PCR test. In addition, three samples had not been sequenced because of low volume of DNA. 10 and 4 samples of *Mycobacterium tuberculosis* were resistant and susceptibility to Isoniazid in drug testing; respectively. Six samples were resistant to Isoniazid and three samples were susceptibility to Isoniazid in PCR test and also, four samples had not been sequenced because of low volume of DNA. Finally, Rifampicin samples showed that one and two mutations were occurred in Rifampicin resistance and susceptibility samples; respectively. The results were shown in table 1 and 2. Isoniazid samples sequencing showed that there were not any mutations in 315 codons region. The sequencing of these samples had been performed and the results were shown in figure 1 and 2.

Table (1):

Mutations in a Rifampicin resistance sample

1607G>T	1479G>T	1349C>G
1608T>C	1485G>T	1324T>C
1610G>C	1499C>T	1320C>G
1625A>C	1508>C	1335C>T
1643C>G	1513>C	1339A>C
1665A>C	1519A>G	1341C>G
1675T>C	1527G>C	1349C>G
1677C>G	1528C>T	1409C>G
1680G>C	1541G>T	1412C>G
1682G>3	1547G>C	1418T>C
	1550C>C	1421G>C
	1582G>C	1224C>G
	1583A>T	1430G>A
	1584G>C	1438A>G
	1585C>G	1464G>C
	1588A>G	1467G>C
		1476G>T

Table (2):

Mutations in two Rifampicin susceptibility samples

7m-1	39m-2
1674C>T	1461T>G
	1659C>T

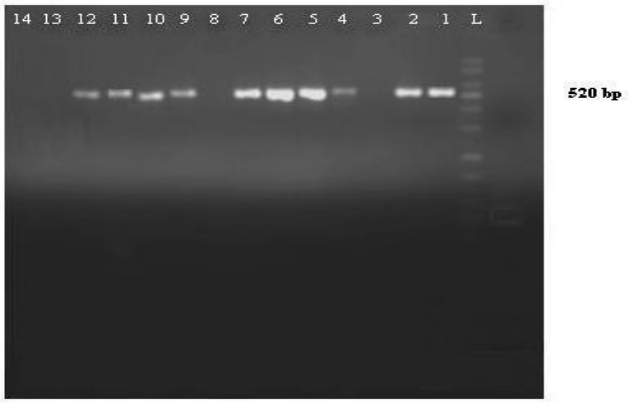

Figure (1): The rpoB gene of Rifampicin resistance of *M. tuberculosis* samples were extracted from samples: L; DNA size marker Ladder, 1; Positive control (*M. tuberculosis* sp.H37Rv), 2 to 13; *M. tuberculosis* samples, 14; Negative control.

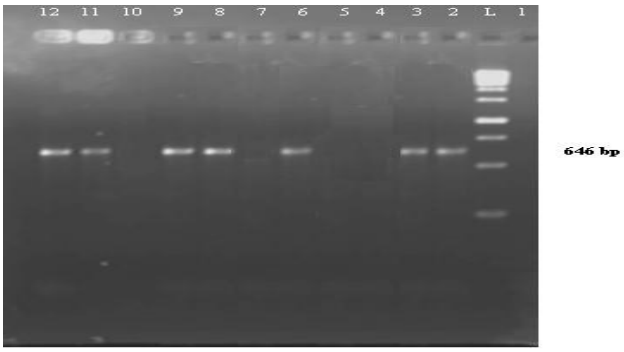

Figure (2): The katG gene of Isoniazid resistance of *M. tuberculosis* samples were extracted from samples: L; DNA size marker Ladder, 2; Positive control (*M. tuberculosis* sp.H37Rv), 3 to 12; *M. tuberculosis* samples, 1; Negative control.

4. DISCUSSION

The incidence of TB and RIF-resistant TB varies considerably among Middle Eastern countries. Nearly 60% of the total population of 2.3 million people in Kuwait comprises expatriate workers and their family members, mostly originating from TB endemic countries of South Asia, Southeast Asia and the Middle East (Ahmad et al. 2005). Every year thousands of expatriates leave and new ones arrive in Kuwait. Although all expatriates entering Kuwait are screened for TB (chest radiograph) and residence visas are granted only to those individuals who have had no prior exposure to active disease, nearly 80% of active TB cases and nearly all the MDR-TB cases occur in the expatriate population (Van Soolingen 2001; Abal et al. 2002; Ahmad et al. 2005). Ahmad and et al (2005) showed that most (18 of 19; 95%) RIF-resistant *M. tuberculosis* isolates were recovered from expatriate TB patients over a 3-year-period (1998–2000) in Kuwait and 18 of 19 (95%) could be rapidly identified as RIF resistant by LiPA. However, the results obtained with RIF-resistant *M. tuberculosis* strains isolated over the subsequent 4-yearperiod, as reported in this study, identified only 24 of 32 strains as RIF resistant even though the majority (30 of 32; 94%) of these isolates were still recovered from expatriate patients born outside of Kuwait(Ahmad et al. 2000; Ahmad et al. 2002; Ahmad et al. 2005). Spies and et al (2013), they collected 24 *M. tuberculosis* samples and then these samples had been characterized by PCR method. In their study, MDR *M. tuberculosis* strains, including STR resistance and the most common STR, RIF and INH drug resistance-conferring mutations, were selected. These mutations included the most frequently isolated strains that have been published in several studies. In this research, these samples had been prepared to drug testing in Pasteur Institute of Tehran which 12 and 10 samples were resistant to Rifampicin and Isoniazid; respectively. The percentage of efficient mutations in Isoniazid resistance has been reported by researchers, such as 91.7% in Petersburg, 62.2% in New York, 59.2% in South Korea, 34.6% in Madrid, 32% in Barcelona and 0% in Akvatvryal Gvynh which they had mutations in 315 codons region (Aktas et al.2005; Herrera et al.2004). In this research, there were not any mutations in 263 to 483 codons of katG after sequencing, in addition, efficient mutations possibly made Isoniazid resistance in other genes of katG. 95% of Rifampicin resistances of *M. tuberculosis* strains have mutations from 511 to 533 codons and the rest of them have mutations in 481, 505, 508 and 509 codons (Simon et al.1998) and in this assay, some mutations had been identified from 511 to 533 codons of rpoB gene. In this research, we tried to investigate on some regions of katG and rpoB gene which had the highest percentage of mutations. The results of this study showed that other mutations possibly caused resistance in *M. tuberculosis* samples which had been isolated from Iranian patients in Pasteur Institute of Tehran.

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