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Original Article

Detection of genomic mutations in *katG*, *inhA* and *rpoB* genes of *Mycobacterium tuberculosis* isolates using polymerase chain reaction and multiplex allele-specific polymerase chain reaction

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ABSTRACT

Objective: Isoniazid (INH) and rifampin (RIF) are the most effective first line antibiotics against *Mycobacterium tuberculosis*. Mutations in several genes determine resistance of *M. tuberculosis* to INH, with the most common gene target of *katG*, and resistance to RIF is due to mutation in *rpo*B gene. The aim of present study was to assess the mutations in the regions related to RIF and INH resistance.

Methods: We characterized 80 clinical isolates of confirmed *M. tuberculosis* to analyze the most commonly observed INH and RIF mutations. PCR analysis and sequencing were used to detect mutations related to RIF and INH resistance. The multiplex allele-specific-PCR (MAS-PCR) was performed as a comparative assay and for evaluation of this method.

Results: The sequencing of the 250-bp region of *katG* codon 315, revealed point mutations at 5 different codons in 13.7% of the *M. tuberculosis* isolates. The sequencing of the 270-bp central region of the *rpo*B gene revealed point mutations at 7 different codons in 12 (15%) of the *M. tuberculosis* isolates. The results obtained with MAS-PCR are in accordance with PCR-sequencing with high sensitivity and specificity for *katG*315, *inhA*15, and *rpo*B (531, 516, 526).

Conclusion: The results of this study suggested that molecular techniques can be used as a rapid tool for the identification of drug resistance in clinical isolates of *M. tuberculosis*. Both DNA sequencing and MAS-PCR yielded high sensitivity for the detection of RIF and INH mutations and detecting multi-drug resistant tuberculosis cases.

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Introduction

Drug-resistant tuberculosis (TB) is particularly alarming and an important threat to the control of the disease globally.[1] There is also much concern that the TB condition will aggravate with the growing human immunodeficiency virus (HIV) pandemic worldwide, as the host immune system can weaken and become susceptible to TB endogenous reactivation and exogenous re-infection.[2] Effective TB control is challenged by HIV infection and drug-resistant TB, a fatal combination. In the most recent studies on the anti-tuberculosis drug resistance surveillance, a global project has been published by the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD).[3] The ratio of multidrug resistance (MDR), denoting resistance to at least isoniazid (INH) and rifampin (RIF), in new cases ranged from 0 to 22.3%. The highest proportion of MDR-TB reported was 60% among previously treated cases. It has been estimated that 489,139 cases of MDR-TB emerged in 2006, and the global proportion of such resistance among all cases amounted to 4.8%.[3] The strategy of directly observed treatment short course (DOTS) is achieving substantial progress in the control of tuberculosis worldwide. However, MDR-TB has emerged as a new challenge, especially in developing countries. This is mainly due to lack of funding to support the treatment of MDR-TB with second line anti-TB drugs.[4] The emergence of MDR strains of *Mycobacterium tuberculosis* (MTB) poses a significant threat to the global control of tuberculosis. Worldwide surveillance has demonstrated that drug-resistant strains are now widespread and reaching alarmingly high-levels in certain countries.[5] MDR-TB is a potentially untreatable, transmissible disease associated with a high mortality.[6] Expeditious identification of antimicrobial susceptibility patterns of MTB is essential for the control of MDR-TB.

INH is the most widely used first-line anti-tuberculosis drug. Mutations in several genes determine resistance of MTB to INH, but the most common mutations are in codon 315 of *kat*G & *rpo*B gene mutations in *M. tuberculosis*. *kat*G accounts for 50-95% of INH-resistant clinical isolates. Resistance to INH can also occur by mutations in the promoter region of *inh*A operon, causing over-expression of *inh*A, or by mutations at the *inh*A active site, lowering the *inh*A affinity to the INH-NAD adduct.[7,8] Mutations in *inh*A or its promoter region are usually associated with low-level resistance and are less frequent than *kat*G mutations.[9,10] RIF is also an important first-line drug for the treatment of TB. The vast majority of RIF resistance is caused by mutations located in the 81-base pair (bp) region of the *rpo*B gene. Mutations at positions 531, 526 and 516 are among the most frequent mutations in RIF-resistant strains.[11] Most of the frequent point mutations associated with drug resistance involve the codons screened in the present study, as reported by previous investigations.[9,12,13] In this investigation, we have studied the prevalence of predominant mutations related to INH and RIF in TB patients in Ahvaz, Iran.

Material and methods

M. tuberculosis *isolates*

A total of 80 clinical MTB isolates were collected from the TB reference laboratory of Khuzestan, Iran, over one year period from February 2010 to February 2011. The isolates were selected from positive cultures on Lowenstein (LJ) medium after 4-6 weeks growth at 37°C.

DNA extraction

For DNA extraction from MTB colonies harvested from the surface of LJ medium, the simple boiling method was used.[14] In brief, a few colonies were dissolved in TE (Tris-EDTA) buffer and boiled at 100°C for 15 minutes with subsequent precipitation in a 1200 x g refrigerated centrifuge at 4°C for 3 min. The supernatant containing DNA was used as template for PCR amplification.

PCR assay

For preliminary detection of MTB, the PCR assay was performed using the primers based on the *IS6110* gene.[15] The primers used for PCR amplification and detection of mutations in *kat*G 315, *inh*A 15 and *rpo*B genes are listed in Table 1. The primers for detecting resistance in RIF were designed to amplify a 270-bp fragment of the *rpo*B gene, containing the 81-bp hypervariable region comprising positions with the most frequent mutations in RIF-resistant strains (*rpo*B codons 511, 513, 516, 522, 526, 531, and 533).[16,17]

The amplification reactions were performed in a final volume of 20 µL, containing 0.2 µg of genomic DNA, 20 pmol of each primer and 10 µL of AmpliTaq Gold master mix (ABI, USA). PCR was performed in an Eppendorf thermal cycler (Germany), using the following condition for amplification of the *IS6110* gene: initial denaturation at 95°C for 5 min, one cycle of 95°C for 20 s, 45°C for 360 s and 72°C for 120 s, followed by 30 cycles of 95°C for 20 s, 62°C for 60 s and 72°C for 180 s.

Table 1 - Primers used for the amplification of *IS6110*, *rpo*B, *kat*G, *inh*A and sequencing[15-17]

Genes primer sequence	Amplicon size (bp)	
IS6110	5'-CTCGTCCAGCGC-CGCTTCGG-3'	130
	5'-CCTGCGAGCGTAGGCGTCGG-3'	
*rpo*B	5-GGTCGGCATGTCGCGGATGG-3'	270
	5-GTATGCGACGGGTGCA-CGTC-3	
*kat*G315	5-GGCCC-CGAACCCGAGGCTGC-3	250
	5-AACGGGTCCGGG-ATGGTGCCG-3	
*inh*A15	5- CCGCCGATGAGAGCGGTGAGC-3	245
	5- CCACTGCTTTGCCGCCACCGC-3	

Amplification conditions for *kat*G 315 genes were initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 66°C for 15 s, and extension at 72°C for 15 s, and a final extension at 72°C for 5 min. The PCR amplification for the *rpo*B was in a similar manner except that the annealing temperature was lowered to 62°C.

Gene sequencing

PCR products were collected and sent for sequencing analysis at Bioneer Company, Korea.

Multiplex allele-specific PCR (MAS-PCR)

MAS-PCR method was used for simultaneous detection of the *kat*G gene codon 315 and *rpo*B gene codons 516, 526, and 531, and mabA-inhA15. In this assay, in each allele-specific primer, 3′ end was located to pair with the base of the related codons where most point mutations have been found to compare with the wild-type sequences of strain H37Rv. Thus, the wild-type allele-specific fragment was amplified when no mutation existed at a related codon. No allele-specific PCR product was generated when there was a mutation at the targeted codons.

The amount of each pair of primers in MAS-PCR was balanced to achieve acceptable amplification of all target regions. For each MAS-PCR reaction, a standard 35 µL final reaction was used. Each final volume included 8 primers of [*rpo*B516 (20 pmol in 2 µL), *rpo*B526 (10 pmol in 1 µL), *rpo*B531 (30 pmol in 3 µL), RIRm (reverse primer for *rpo*B531,516,526) (50 pmol in 5 µL), *kat*G OF (10 pmol in 1 µL), *kat*G5R (10 pmol in 1 µL), *inh*AP-15 (10 pmol in 1 µL), and *inh*APF (10 pmol in 1 µL)], 2 µL of genomic DNA (40ng) and 13 µL of AmpliTaq Gold master mix (ABI) (Table 2).[18] The cycling parameters included an initial denaturation at 96°C for 3 min, 27 cycles of 95°C for 50 s, 64°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis on 2.5% agarose gel and visualized with gel documentation.

Table 2 - Primers used in MAS-PCR for detection of INH and RIF resistance mutations[14]

Target allele-specific primers		Amplicon size (bp)
*kat*G 315 (5R)	5'-ATACGACCTCGATGCCGC	292
*kat*G 315 (OF)	5'-GCAGATGGGGCTGATCTACG	
*rpo*B 516	5'-CAGCTGAGCCAATTCATGGA	218
*rpo*B 531	5'-CACAAGCGCCGACTGTC	170
*rpo*B 526	5'-CTGTCGGGGTTGACCCA	185
RIRm	5'-TTGACCCGCGGCGTACAC	
*inh*A P15	5'-GCGCGGTCAGTTCCACA	270
*inh*A PF2	5'-CACCCCGACAACCTATCG	

Results

Results of PCR analysis

In the PCR assay using *IS6110* for confirmation of the isolates, all were positive and the results of amplification are presented in Fig. 1. Amplification of RRDR of *rpo*B gene yielded 270 bp products compared to DNA size marker in agarose gel electrophoresis (Fig. 2). *inh*A regulator sequence amplification of INH yielded 245 bp PCR products (Fig. 3), and *kat*G codon 315 amplification of INH yielded 250 bp PCR products (Fig. 2).

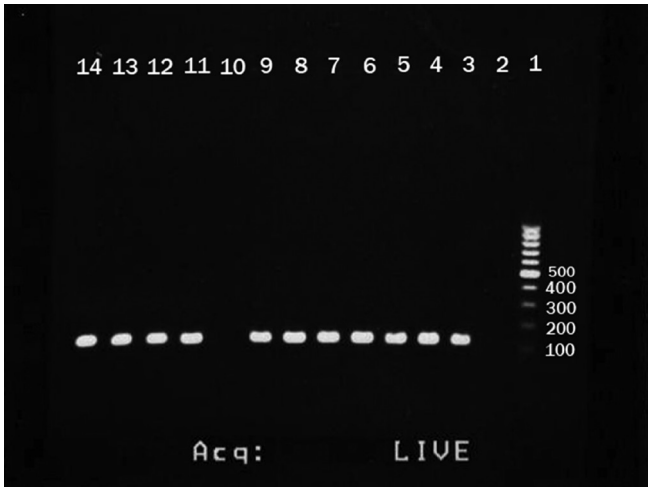

Fig. 1 - Agarose gel electrophoresis of PCR assays for the identification of (a) *IS6110* gene. 1, DNA size marker ; 2, negative control; 3-9 and 11-13, positive samples (130bp); 14, positive control.

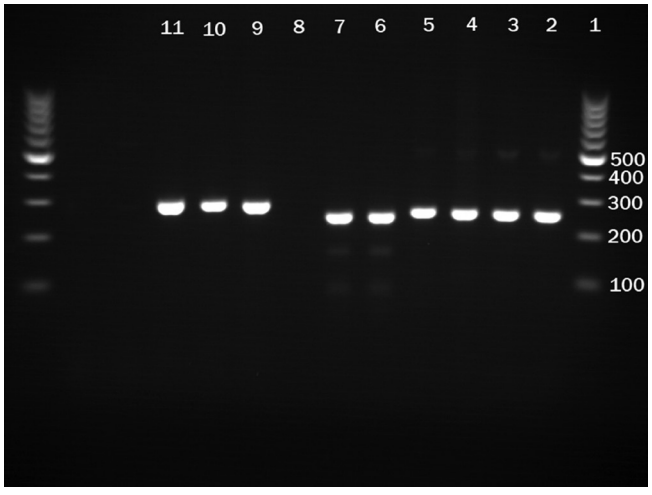

Fig. 2 - Agarose gel electrophoresis of PCR assays for the identification of (a) *kat*G codon315- *inh*A- *rpo*B. 1, DNA size marker; 2-5, *kat*G codon315 (250bp); 6 and 7, *inh*A (245bp); 8, negative control; 9-11, rpoB (270bp).

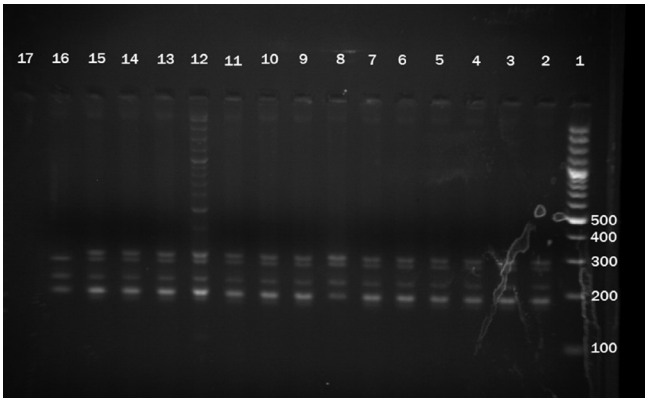

Fig. 3 - Results of MAS-PCR shown by 2.5 % agarose gel electrophoresis. The 292-bp band: the *kat*G codon 315-specific PCR product; the 218-bp band: *rpo*B codon 516-specific PCR product; the 270-bp band: the 15- promoter region of *mab*A-*inh*A–specific PCR product; the 185-bp band: *rpo*B codon 526-specific PCR product; the 170-bp band: rpoB codon 531-specific PCR product.

Results of DNA sequencing

The sequencing of the 250 bp region of *kat*G codon 315 revealed point mutations at 5 different codons in 13.7% of the 80 MTB isolates. The sequencing of the 270 bp central region of the *rpo*B gene revealed point mutations at 7 different codons in 12 (15%) of the 80 MTB isolates (Table 3). Among *rpo*B gene point mutations, in 2 isolates, mutation was observed in codon 531, causing TCG to TTG substitution, in 7 isolates mutation was observed in codon 527, causing AAG to AAC and in 3 isolates mutation was observed in codon 512, causing AGC to ACC substitution. One isolate had a mutation in codon 511 and one isolate had two mutations in codons 539 and 541 unexpectedly since these codons are out of the 81 bp region. No mutation was detected in the control RIF sensitive isolate (H37Rv). For INH, the most frequent mutation was observed in *kat*G codons 315 and 312, in which a change from AGC to ACC and GCG to GAG was observed, respectively. No mutation was observed in the *inh*A promotor region or in the INH sensitive control isolate (H37Rv).

Results of MAS-PCR

Out of 10 isolates that proved to have different mutations in *kat*G gene, 3 isolates which showed mutation in codon 315 by sequencing, were detected by MAS-PCR method. These were accounted for 30% of total *kat*G mutations. The results of PCR-nucleotide sequencing and MAS-PCR assay were identical for the *inh*A gene (Fig. 3).

Similarly, among 12 isolates that proved to have different mutations in *rpo*B gene, 2 isolates had a mutation in codon 531 which were also detected by MAS-PCR.

Discussion

By using PCR and subsequent sequencing and MAS-PCR, we were able to detect the mutations related to drug resistance to RIF and INH. Based on the obtained results, we found out that MAS-PCR is a preferred method for detecting drug resistance in MTB in shorter time compared with PCR.

Table 3 - Distribution of mutations associated with RIF and INH resistance among MTB isolates

rpoB gene				rpoB gene			
AA (amino acid)		Nucleotide		AA (amino acid)		Nucleotide	
Isolate	Codon	Change	Change	Isolate	Codon	Change	Change
54	512	Ser→Thr	AGC→ACC	4	315	Ser→Thr	AGC→ACC
39	527	Lys→Asn	AAG→AAC	5	315	Ser→Thr	AGC→ACC
38	512	Ser→Thr	AGC→ACC	42	312	Ala→Glu	GCG→GAG
38	517	Gln→Lu	CAG→CCG	17	319	Ala→Val	GCG→GTG
37	531	Ser→Lu	TCG→TTG	18	328	Trp→Val	TGG→GTG
31	512	Ser→Thr	AGC→ACC	14	326	Thr→Pro	ACG→CCG
42	527	lys→Asn	AAG→AAC	13	12	Ala→Glu	GCG→GAG
13	527	lys→Asn	AAG→AAC	40	326	Thr→Gly	ACG→CCG
37	531	Ser→ Lue	TCG→TTG	13	315	Ser→Thr	AGC→ACC
37	511	Lue→Met	CTG→ATG	38	312	Ala→Glu	GCG→GAG
18	527	lys→Asn	AAG→AAC	59	319	Ala→Val	GCG→GTG
50	527	lys→Asn	AAG→AAC				
52	527	lys→Asn	AAG→AAC				

In this study, based on PCR and sequencing, the most common mutations related to drug resistance were demonstrated as RIF (15%) and INH (13.7%). The results were not in accordance with a previous report from Ahvaz[19] in which the author observed that the most frequent mutations were substitutions in codons 516, 526 or 531 of the *rpo*B gene, which were found in 25 (83.3%) isolates. Mutations in codon 531 of the *rpo*B gene were detected in 16 (53.3%) of 30 RIF resistant isolates.

A similar study from the middle-east reported INH resistance in their isolates with all mutations at codon 315 of the *kat*G gene. However, none of the reported mutations was AGC to ACC as found in our study.[20]

In the study of Yang et al.[21] on the simultaneous detection of *INH* and RIF in 174 clinical isolates of MTB in Turkey, distinct PCR banding patterns were observed for different mutation profiles and the correlation between MAS-PCR results and DNA sequencing was 99.4%. Other studies used multiplex PCR to detect the mutations in *rpo*B and *kat*G genes in 20 drug-resistant isolates of MTB from the Southeast of Mexico. Sequencing analysis showed 93% mutations in the *rpo*B gene; of which 47% exhibited a mutation at 531 (S→L). Fifty-eight percent of their isolates showed mutations in *kat*G; with 52% exhibiting a mutation at 315 (S→T).[22] Also, the usefulness of MAS-PCR in detecting mutations was reported in a study by Mokrousov et al.,[23] in which they were able to detect *rpo*B mutations at the rate of 82.8%. The reported mutations were in the *rpo*B 531, 526 and 516 codons. Similarly, in a study by Rathore et al.,[24] four mutations at *rpo*B codons 526, 531 and 12 were noticed in 16 RIF resistant isolates and 7 mutations were identified in INH resistant isolates in the *kat*G codon 315.

In a study on the detection of *kat*G and *inh*A gene mutations in MTB by multiplex allele-specific PCR, of 31 *INH*-resistant isolates, mutations in the *kat*G codon 315 were identified in 64.5% of the isolates by sequencing. From these, 19 were detected by MAS-PCR methods.[25]

Our findings showed that most of the resistance mutations to *INH* occurred in codon 315 and this was in agreement with other mentioned studies showing the major involvement of this codon in *INH* resistance all over the world. However, a variety of involved codons was reported for RIF resistance among MTB isolates in different studies. In our study, the main codon involved in RIF resistance was 527, while similar studies reported several other codons for RIF resistance.

In conclusion, although culture based phenotypic susceptibility test is the gold standard for detecting drug resistance in MTB and no molecular method can yet completely replace it, MAS-PCR provides a rapid screening tool for the majority of mutations occurring in genes related to *INH* and RIF antibiotics. However, the molecular assays only detected known mutations, which is the most important limitation in detection of drug resistance by such techniques. Based on the other studies, all mutations related to anti-TB drugs are not known yet. Since the prevalence of mutations may vary by geographic area, identification of a resistance-associated mutation can be instructive, but lack of a mutation in the target sequence must be interpreted with caution.

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Conflict of interest

All authors declare to have no conflict of interest.

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