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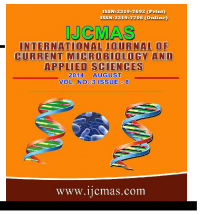

Original Research Article

Characterization of *katG* and *rpoB* gene mutations in Multi Drug Resistant *Mycobacterium tuberculosis* clinical isolates

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Keywords

Tuberculosis, Isoniazid, Rifampicin, Drug resistance, PCR-SSCP, *katG*, *rpoB*

A B S T R A C T

Rapid molecular diagnosis of tuberculosis (TB) in clinical specimens facilitates prompt detection of *Mycobacterium tuberculosis* infection, provides precise treatment regimen to control TB infection at early. Totally 25 Multi Drug resistant *M. tuberculosis* (MDR-TB) clinical specimens were collected and subjected to Proportional Sensitivity Test and BACTEC MGIT 960^{TM} methods. PCR amplification of *katG* and *rpoB* genes, PCR-Single Strand Conformation Polymorphism (PCR-SSCP) and automated DNA sequencing methods were performed to analyse the point mutations responsible for MDR-TB. High level drug resistance was observed at 10 µg/mL and 64 µg/mL for INH and RIF drugs respectively. S315T (68%) mutation was found predominant followed by a novel mutation at codon G297P (16%) in *katG* gene and S531L (72%) followed by H526 (16%) in rpoB gene among MDR-TB isolates. PCR-SSCP bands produced identical mobility shift and difference in banding pattern with reference to the presence of point mutations in respective genes. The efficacy of the each method was compared with DNA sequencing results and PCR-SSCP showed consistency with mutation detection. PCR-SSCP based mutation detection method was less expensive and could be used for diagnosis of MDR-TB.

Introduction

Presently, tuberculosis (TB) re-emergence and spread are of worldwide concern (Mokrousov *et al.*, 2002). The global spread of the disease is complicated by the ubiquitous appearance of drug-resistant strains, and particularly multidrug-resistant (MDR) strains defined as resistant to at least isoniazid (INH) and rifampicin (RMP) which comprises the backbone of antitubercular chemotherapy (Mokrousov *et al.*, 2002; Laurenzo and Mousa 2011). Resistance to INH and RIF is a key factor in determining the effectiveness of the currently recommended standard treatment regimens (Cavusoglu *et al.*, 2002; Edwards *et al.*, 2001). While RIF resistance is essentially mediated by the *rpoB* hot spot region mutations but, INH resistance is

apparently controlled by a more complex genetic system that involves several genes, namely, *katG*, *inhA*, *kasA*, and *ahpC* (Ramaswami and Musser 1998; Slayden and Barry 2000).

The *rpoB* gene encodes the ß-subunit of the RNA polymerase, and mutations in a specific 81 base pair region of the *rpoB* gene (amino acids 507–533) especially, codons 516, 526, and 531 are responsible for 97% of rifampicin-resistant strains (Ramaswamy and Musser, 1998).

Mutations in two genes account for the majority of INH-resistant strains: *katG*, the catalase peroxide that activates isoniazid, and *inhA*, the eventual target of activated isoniazid (Guo *et al.,* 2006). Several mutations in *katG* have been identified that affects peroxidase activity/efficiency. One mutation in particular, the *katG* Ser315Thr substitution mutation, causes the majority of isoniazid-resistance and is found in 50–90% of isoniazid-resistant strains (Wade and Zhang, 2004).

The elucidation of the mechanism of action of these drugs, which was accomplished recently, has led to the development of new rapid diagnostic methods. Prompt detection of TB drug resistance is essential for controlling the development and spread of MDR-TB as it facilitates the appropriate and timely delivery of anti-TB therapy reducing overall cost of treatment and transmission of resistant cases (Mokrousov *et al.,* 2002; Laurenzo and Mousa 2011).

In this study, we studied the mutations in *rpoB* and *katG* genes, responsible for the cause of multi drug resistance in hospitalized treatment failure or severely ill patients. We further extended the use of single - stranded conformational polymorphism (SSCP) technique to evaluate the effect of the diagnosis of most frequently observed mutations in RIF and INH-resistant clinical isolates. The results were compared with conventional drug susceptibility test and automated DNA sequencing services.

Materials and Methods

Bacterial Sample

Twenty five MDR-TB clinical strains of *M. tuberculosis* were recovered from sputum sample of hospitalized patient at State TB Training and Demonstration Centre (Intermediate Reference Laboratory) Government Hospital for Chest Diseases, Puducherry, India. Patients were admitted as per the physician advice and clinical symptoms of TB infection. Clinical strains were maintained in L.J medium for reproducibility of the results. Mycobacterial cell suspension was matched with McFarland 1.0 standard and used as inoculum for further studies.

Phenotypic drug susceptibility testing

Sputum sample was processed by the standard NaOH treatment method. Phenotypic drug susceptibility test-proportion method to INH, rifampicin, streptomycin, ethambutol and pyrazinamide was performed using conventional L.J (Muthuraj *et al.,* 2010) and BACTEC MGIT culture system (Becton Dickinson, USA) following the manufacturer's instructions.

Mycobacterial Genomic DNA Isolation

Mycobacterial genomic DNA was extracted using the method as described previously and the pellet was air-dried and was dissolved in 20 μL of 1× TE buffer (Mani *et al.,* 2003).

PCR amplification for species identification

PCR program was performed in an authorized thermal cycler (Eppendorf Gradient Cycler). IS*6110* element was amplified for species identification and *katG* and *rpoB* genes were amplified to appraise mutations by allele specific PCR in MDR-TB clinical isolates. The primers used in this study were summarized in table 1 and concentration at 10 pmol/µL (Kathirvel *et al.,* 2013). The PCR cycling parameters were 94°C for 5 minutes; followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute and 74°C for 1 minute; and a final extension of 74°C for 10 minutes. The PCR product was then kept at hold at 4°C for 15 minutes. The amplified PCR product was run on a 2% Agarose gel in TAE buffer and photographed using a Geldoc.

PCR-Single Strand Conformation Polymorphism (PCR-SSCP) Analysis

The protocols and reaction components utilized for the PCR-SSCP analyses have been described previously (Altschul *et al.,* 1997). The SSCP gel plate was sealed with 1% agarose. 15% PAGE gel was prepared (50X acrylamide mix-1.5ml, 5X TBE buffer-1ml, 10% APS-70 µl, Glycerol-0.50ml, ultrapure water (Milli Q)-4.4ml, TEMED-10 µl) and poured between the glass plates and allowed it to stand at least 15 minutes for polymerization. Pre-run was performed and 10 µl of PCR amplified products of corresponding genes and 5 µl of SSCP dye were taken in a PCR tube and mixed well. The tube was incubated at 94°C for 10 minutes and immediately flashes chilled ice for 10 minutes. 10 µl denatured sample was loaded each well and electrophoresis was performed at 100V for 2 hours using 1X TBE buffer. Silver staining was further performed to visualize the bands in the gels and documented.

DNA Sequencing

The amplified PCR products of *katG* and *rpoB* genes were further purified using PCR purification kit (Invitrogen). The purified PCR product was directly sequenced in an automated DNA Sequencer (IIT, Chennai, India). The nucleotide sequences obtained were analyzed using BLASTn and BLASTx bioinformatics tools available at National Centre for Biotechnology Information (NCBI) compared with wild type *M. tuberculosis* (H37Rv).

Result and Discussion

A 123-bp amplified fragment of IS*6110* element specific for MTB complex was clearly visualized on agarose gel electrophoresis in clinical isolates (Figure 1a). The observation of clear bands at 206-bp and 329-bp regions in agarose gel confirmed the amplified products of *katG* and *rpoB* genes of *M. tuberculosis* clinical isolate respectively (Figure 1b).

Totally 25 high drug resistant clinical isolates of *M. tuberculosis* were analysed from hospitalized treatment failure cases to find mutations in the *katG* and *rpoB* genes. *In vitro* susceptibility testing showed high level drug resistance in 15 strains (60%) MDR-TB clinical at 10 µg/mL against INH and 18 strains (72%) at 64 µg/mL against RIF drugs. The results of the sequence analysis of *katG* and *rpoB* genes from MDR-TB clinical isolate are presented in table 2. *katG315* codon mutation was found in 19 (76%) MDR-TB strains which showed higher degree of resistance against INH drug. *rpoB531* codon mutation was found in 18 (72%) MDR-TB strains which exhibited high level of drug resistance against RIF drug. We found nucleotide substitutions and insertions leading to change in the corresponding amino acids.

Table.1 Oligonucleotide-primers used in the PCR amplification protocol

Target	Oligonucleotide	Product length (bp)
IS*6110*	F5' GTGAGGGCATCGAGGTGG 3'	123
	R 5 CGTAGGCGTCGGTCACAAA 3	
rpoB	F 5' CCACCCAGGACGTGGAGGCGATCACAC 3'	329
	R 5' CGTTTCGATGAACCCGAACGGGTTGAC 3'	
katG	5' GAAACAGCGGCGCTGATCGT 3'	206
	5' GTTGTCCCATTTCGTCGGGG 3'	

Figure.1 Example of PCR amplification of target genes

Figure A: Example of the amplified product IS*6110* element at 123-bp. Lane 1- 100-bp ladder, lane 2- positive control of *M. tuberculosis* H37Rv and lane 3-amplified product of MDR-TB clinical isolate.

Figure B: Example of the amplified products of target genes as in lane 1-100-bp ladder, lane 2-*rpoB* product at 331-bp and lane 3-*katG* gene products at 206-bp regions.

A B

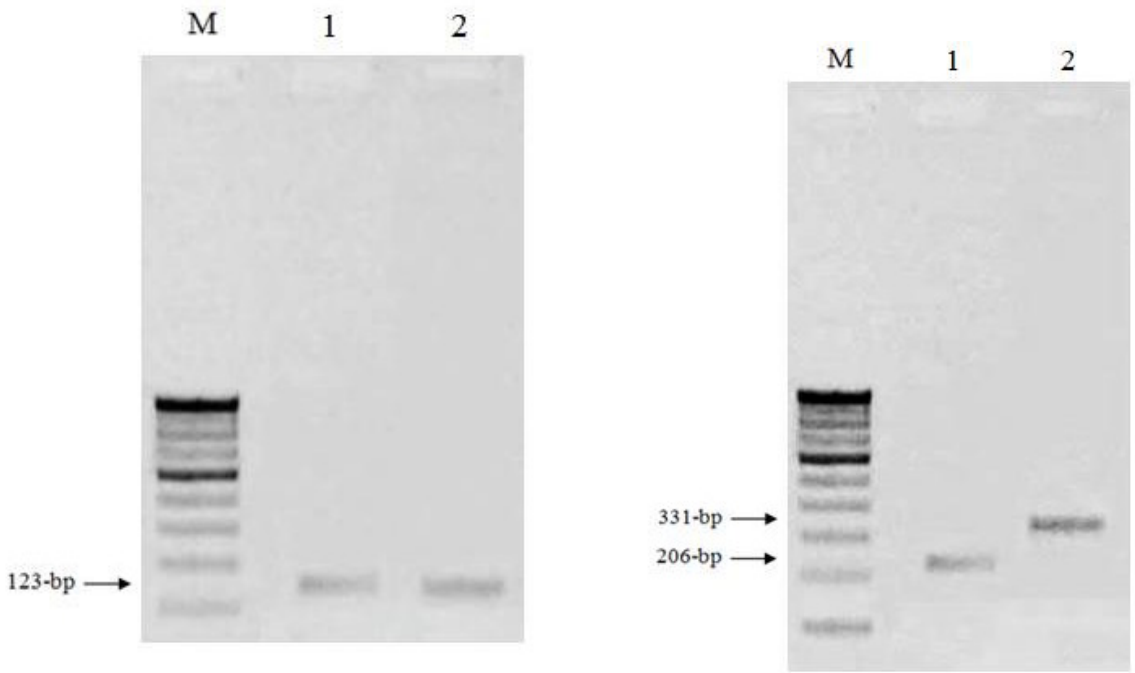


Table.2 Drug Resistance profile of *M. tuberculosis* clinical isolates

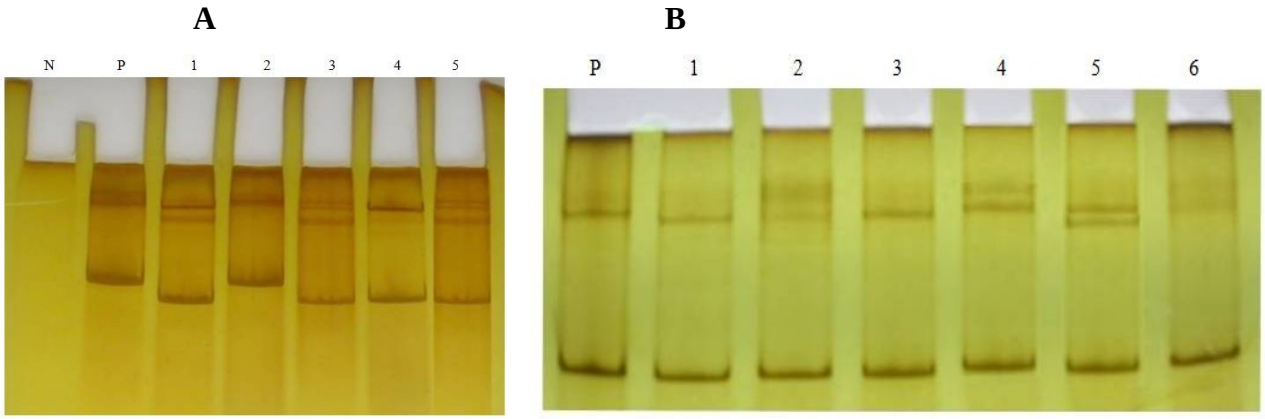
Drugs	No. of strains
H + E + R + S	3 (12%)
H + E + R	2 (8%)
H + R + S	4 (16%)
I + R	16 (64%)
Total	25 (100%)

Table.3 Mutations of *katG* and *rpoB* in MDR-TB clinical isolates

Target Gene	Nucleotide position	Amino acid change	No. of Strains
katG	315	Ser→Thr	17 (68%)
	315	Ser→Asn	3 (12%)
	295	Gln→Pro	2 (8%)
	315+295	Ser→Thr, Gln→Pro	2 (8%)
	287	Glu→Val	1 (4%)
rpoB	521+531	Glu→Asp, Ser→Leu	4 (16%)
	531	Ser→Leu	14 (56%)
	526	His→Tyr	4 (16%)
	516	Asp→Tyr	3 (12%)

Figure.2 Example of PCR-SSCP pattern analysis of MDR-TB clinical isolates

Figure A: Example of PCR-SSCP pattern of *katG* gene from MDR-TB isolates. N- Negative control, P-Positive control wild type H37Rv, Lanes numbered as 1 to 5 – showed *katG* amplified product of MDR-TB clinical isolates.
Figure B: Example of PCR-SSCP pattern of *rpoB* gene from MDR-TB isolates. P-Positive control wild type H37Rv, Lanes numbered as 1 to 6 – showed *rpoB* gene product of MDR-TB clinical isolates.
In both figures the bands showing the variation in their mobility pattern owing to the presence of mutations in the amplified region.



The nature of the *katG* and *rpoB* gene mutations includes addition, deletion and missense mutations in the coding region of antibiotic resistance region and their amino acids variations, causing nonsense peptides leads to antibiotic resistance.

The distribution of *katG* and *rpoB* gene mutations is dispersed along the gene precisely; we found novel mutations in the *katG* and *rpoB* genes that set apart from mutational hot spots of respective antibiotic resistance genes (Table 2). Mutations were further defined by PCR-SSCP analysis. The antibiotic resistance strains having mutations in their corresponding genes showed mobility shift in migration pattern and the difference in bands in the gels when compared with wild type allele products (Figure 2). Susceptible and wild type strains produced identical banding patterns in gel.

TB disease remains a global tragedy regardless of the advanced usage of preventive measures and treatment regimen (Zhang *et al.,* 2005). Occurrences of mutations in corresponding antibiotic genes are responsible for the development of drug resistance in clinical settings (Leon *et al.,* 2005) makes the tubercle bacilli unsusceptible to available anti-TB drugs. Hence, the disease management in TB patients is ineffective and difficult to treat proper; leads to prolonged therapy.

Therefore, the emergences of MDR-TB in clinical settings render the inability of treatment effectiveness and control programs and complicate both TB treatment and prevent strategies (David *et al.,* 1995). The rationale behind the treatment failure and deadly clinical outcome in TB patients is due to RIF-resistance (David *et al.,* 1995). RIF exhibits excellent sterilizing activity in late period of semi-dormant TB bacilli. However, INH mono-resistance is common in isolates but RIF mono-resistance is rare in clinical settings. As an alternative RIF-

resistance occur most commonly along with INH-resistance (Ohno *et al.,* 1996).

With our proportional sensitivity study we found MDR-TB isolates showed higher level of resistance against INH and RIF drugs and clearly depicts that in a subset of MDR-TB clinical isolate, having mutations in the coding region of antibiotic genes (David *et al.,* 1995; Leon *et al.,* 2005; Rozwarski *et al.,* 1998). We found mutations in hot spots of *katG* and *rpoB* genes responsible for the anti-TB drug resistance due to presence of alterations/mutations in antibiotic altering enzyme (Cavusoglu *et al.,* 2002). In INH-resistant strains the most common point mutation was found at 315 regions (S315T, S315N) of *katG* gene is responsible for the resistant phenotype in clinical isolates. Apart from 315 mutations we found a couple of mutation as novel mutations which also part in high level drug resistance in clinical strains (Table 3). Owing to the presence of these common mutations, the MDR-TB strains showed high level-resistance against INH (MIC >10µg/ml) and RIF (MIC >64 µg/ml) (Zhang *et al.,* 2005; Yue *et al.,* 2003; Ohno *et al.,* 1996).

As a result of DNA sequencing we found mutations at S315T and novel mutations at E287V and G295P positions of *katG* gene. The amino acid variation occurs as Glu→Val (E→V), Gln→Pro (G→P) and Ser→Thr (S→T). Clinical isolates had 315 mutations demonstrated high level of resistance against INH drug (0.5µg-10µg). However, strains had S315N and Q295P mutations were susceptible at 1µg/mL of INH drug. Sensitive strains were used as control in this study to avoid the discrepancy of *katG*-PCR and PCR-SSCP results (Table 2).

In RIF-resistant strains, it was found that the most observed mutation at codon 531 (72%), followed by codon 526 (16%) and codon 516 (12%) in RRDR region of *rpoB* gene. Mutation at codons 531 and 526 was observed as a double mutation in 4 strains (16%) and occurrences of all the three mutations were observed only in 3 strains (12%). Our result was corroborated with several other studies showed that the occurrence of this frequent mutation at S531L in *rpoB* gene (Mani *et al.,* 2001; Caviusoglu *et al.,* 2002; Sajduda *et al.,* 2004). Eighteen MDR-TB strains (72%) showed high level of resistance against RIF drug (MIC> 32 to 64 µg/mL). However there is no correlation was found between the occurrence of mutation and the cause of high level of drug resistance against RIF drug.

In our PCR-SSCP study reveals the resistance pattern of MDR-TB strain clearly when compared with wild type allele. Both INH and RIF resistance strain having mutations in their respective genes showed identical mobility shifts during migration on gel reflects mutations even at single nucleotide level (Figure 2). The result of PCR-SSCP provides a better correlation with DNA sequencing studies and the presence of point mutations in the respective antibiotic genes. By analysing the banding pattern of denatured PCR amplified products of *katG* and *rpoB* genes, all susceptible strains showed identical pattern. But, on the other hand variations were observed in the banding pattern with reference to the number of mutations in it. Strains having single/common mutation showed only two bands but, strains having more than one mutation showed same number of bands in the gel. The resolution at single nucleotide in gel of the amplified products of *katG* and *rpoB* genes clearly demonstrated the drug resistance among the clinical isolates when compared to wild type and drug susceptible isolates.

The amino acid variations occur in the coding region of the gene products were completely analyzed by the retrieval of whole gene sequences of *katG* and *rpoB* genes. The existence of nucleotide and aminoacid mutations in the *katG* gene proves the hypothesis that a deletion of whole gene must be a rare event and there be partial expression of the catalase-peroxidase enzyme, which leads inactivation of prodrug INH (Rozwarski *et al.,* 1998; Sacchettini and Blanchard 1996). In case of *rpoB* gene we found the mutations within the RRDR region which represents the importance of the prevalence of mutation frequency in *rpoB* gene in MDR-TB pattern in clinical isolates.

To the conclusion, the PCR-SSCP and DNA sequencing assay performed to detect common mutations of *katG* and *rpoB* genes was simple and rapid molecular method to identify MDR-TB in clinical isolates. It is inexpensive and requires less equipment resource and can be implemented in routine clinical resources in laboratories in resource poor settings.

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