Full Length Research Paper

Molecular characterization of *rpoB* gene encoding the RNA polymerase β subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from south India

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Polymerase chain reaction (PCR) mediated direct DNA sequencing was evaluated for rapid detection of Rifampicin resistance (RMP[r]) of *Mycobacterium tuberculosis.* After amplification of the *rpoB* gene, the product was sequenced using ABI 310 Genetic Analyzer and the rifampicin resistance in *M. tuberculosis* were assessed using the sequence pattern. Among the 100 isolates, 83 isolates were rifampicin sensitive, exhibited a wild-type pattern on PCR mediated direct sequencing, and remaining 17 isolates were resistant. All the RMP[r] isolates were multidrug resistant, that is, resistant to at least Rifampicin and isoniazid. The mutation rate at the most effected codon 526 in the 81 bp rifampin resistance-determining region (RRDR) of *rpoB* gene was determined to be 35.29% in isolates from South India. In addition, the mutation rate at the most affected codon 451 was in the out side of the 81bp RRDR region of *rpoB* gene. A synonym mutation was detected (Thr→Thr) at codon 526 in one isolate, which was inside the active site (codons 507-533) of RMP binding in the *rpoB* gene region. The sequencing analysis for genotypic evaluation of Rifampicin resistance in highly sensitive assay provides a practical alternative to *in vitro* testing in *M. tuberculosis*.

Key words: Rifampin resistance, *Mycobacterium tuberculosis*, *rpoB* gene, sequencing, mutation.

INTRODUCTION

The worldwide increase in the incidence of drug resistant strains of *Mycobacterium tuberculosis* has highlighted the need for faster and more accurate detection of resistance to rifampin (RMP), one of the most important anti-tuberculosis drugs (Parsons et al., 1997). First-line antituberculosis treatment often fails in patients with rifampin-monoresistant or multidrug-resistant tuberculosis (TB) (Mitchison and Nunn, 1986). Inappropriate treatment can result in the development of resistance to additional antibiotics (Fischl et al., 1992; Goble et al., 1993) and increase mortality (Pablos-Mendez et al., 1996). Because *M. tuberculosis* grows extremely slow (Harris et al., 2000; Palacios et al., 1999) conventional susceptibility testing can require many weeks to be completed (Kent and Kubica, 1985). There is thus an urgent need to develop a rapid, simple and accurate assay to assess drug

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Abbreviations: RMP, Rifampin; RMP[r], resistance to rifampin; **rpoB,** ribonucleic acid polymerase; **PCR,** polymerase chain reaction; TB, tuberculosis; **RRDR,** rifampin resistance-determining region; **SDS,** sodium dodecyl sulfate; **CTAB,** cetyl trimethylammonium bromide; **BLAST,** basic local alignment search tool; **NCBI,** National Center for Biotechnology Information; **RMP[s],** rifampicin sensitive; **MIC,** minimum inhibitory concentration; **LJ,** Lowenstein-Jensen.

resistance in *M. tuberculosis* (Heifets and Cangelosi, 1999). Rifampin resistance is an excellent marker for multidrug resistant TB because all of these strains are resistant to rifampin. Therefore, a screening assay does not need to test susceptibility to all antituberculosis drugs (Telenti et al., 1993). Rifampin resistance is particularly amenable to detection by rapid genotypic assays because 95% of all rifampin-resistant strains contain mutations localized in an 81-bp region of the bacterial RNA polymerase gene, *rpoB*, which encodes the active site of the enzyme (Musser, 1995; Riska et al., 2000). Moreover, all mutations that occur in this region result in rifampin resistance. By contrast, all rifampin susceptible *M. tuberculosis* isolates have the same wild-type nucleotide sequence in this region (Sreevatsan et al., 1997). Thus, it is only necessary to detect a mutation in the *rpoB* core region to know that the bacilli are rifampin resistant.

Delayed detection, identification, and susceptibility testing of drug-resistant isolates and failure to appropriately isolate contagious patients and to begin adequate chemotherapy have all been identified as predisposing factors of nosocomial and extra nosocomial transmission of drug-resistant *M. tuberculosis* (Frieden et al., 1996). Molecular bases of drug resistance have been identified for all of the main antitubercular drugs, and drug resistance results from changes in several target genes, some of which are still undefined. These factors highlight the need to implement the rapid detection of drug resistance, for better management of patients as well as for control of the outbreaks and prevention of future nosocomial drug-resistant tuberculosis transmission. Recent major advances have been made in unraveling the molecular basis of *M. tuberculosis* resistance to isoniazid, streptomycin, quinolones and rifampicin, offering the prospect of a genotypic approach for the detection of resistance. The mechanism of resistance to RMP (RMP[r]) has been amply documented, in most *M. tuberculosis* strains, RMP[r] is attributed to point mutations and small insertions or deletions in a limited region of the gene encoding for the subunit of the RNA polymerase *(rpoB)*. Based on this data, Telenti et al. (1993) developed a screening method using the polymerase chain reaction (PCR) and single strand confirmation polymorphism analysis. Williams et al. (1994) used a PCR and heteroduplex formation to detect RMP[r] in cultures. These two authors, as well as Kapur et al. (1995) used direct sequencing of PCR products to detect or confirm the mutations. Here we report on the use of a fast and easy to perform sequencing method that allows the simultaneous detection of *M. tuberculosis* and its resistance to RMP directly in clinical specimens.

Rifampicin has proven to be an effective anti-tuberculosis agent and its use has greatly shortened the duration of chemotherapy for the treatment of TB. Rifampicin resistance heralds higher rates of treatment failure and death for the patient and a poor outcome if the isolate is also resistant to isoniazid (Goble et al., 1993). Rifampin is one of the major antituberculosis drugs (Bass et al., 1994), and the mechanism of rifampin resistance was the first characterized mechanism of drug resistance in *M. tuberculosis* (Telenti et al., 1993). The *rpoB* gene encodes the b-subunit of the RNA polymerase. Rifampin specifically interacts with prokaryotic RNA polymerase to inhibit transcription, which leads to and causes cell death, and specific mutations in *rpoB* produce drug resistance and diminishing rifampin binding affinity for the RNA polymerase (Jin and Gross, 1989). Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, have been shown to be strongly associated with rifampin-resistant phenotypes in multiple study populations (Huang et al., 2002; Mani et al., 2001; Spindola de Miranda et al., 2001). *rpoB* mutations are more likely segregated in an 81-bp region called the rifampin resistance-determining region (RRDR). Because up to 90% of rifampin resistant strains carry RRDR mutations within codons 516, 526, and 531, these mutational "hotspots" are being used to rapidly identify rifampin-resistant isolates (Kocagoz et al., 2005; Tang et al., 2005).

This paper represents the results of a study of 17 rifampicin resistant *M. tuberculosis* strains that were isolated in South India between Jan 2006 to Nov 2008. This study was designed to establish the molecular features of rifampicin drug-resistant isolates of *M. tuberculosis* in a population with high prevalence of treatment failure cases of the Government Hospital for Chest Disease at Puducherry, India. Those followed at a single clinical setting where a specific control program for the prevention of nosocomial transmission of tuberculosis is implemented. The goals of the present study are to evaluate the drug resistance profiles of these isolates and to analyze a region within the isolates' *rpoB* gene that had been associated with rifampin resistance.

MATERIALS AND METHODS

Bacterial samples

A total of 100 clinical strains of *M. tuberculosis* were isolated from sputum samples of treatment failure cases hospitalized at State TB Training and Demonstration Centre (Intermediate Reference Laboratory), Government Hospital for Chest Diseases, Puducherry, South India, during the period from Jan 2006 to Nov 2008. All specimens were processed immediately and aliquots of the decontaminated specimens were kept at -20°C.

Sputum processing for AFB culture

Sputum in excess of 5 ml was discarded into the disinfectant bath. To each volume of remaining sputum, 2 volumes of 4% NaOH was added taking care to avoid contact between the specimen bottle rim and the NaOH flask. The bottles were shaken by hand for 1 minute. Then the bottles were placed in a rack on the shaking machine and were left to shake gently for 20 min. The specimens were removed from the shaker. The sputum bottles were centrifuged for 15 min at 4000 rpm to make sure that the centrifuge buckets were counter

balanced. After the bottles were removed from the centrifuge, the supernatant was carefully poured off into the disinfectant bath and the rim of each bottle was wiped with sterile filter paper. The bottles were filled with 20 ml of sterile distilled water, shaken by hand to mix the deposit and were centrifuged for 15 min at 4000 rpm. The supernatant was poured off as before and again the neck of the bottle was wiped with sterile filter paper. Finally, the sediment was inoculated with a 5 mm diameter loop onto the pre-sterilized and numbered Lowenstein-Jensen's slopes. The inoculated media was placed in the 37°C incubator (Davies et al., 2000; Morlock et al., 2000).

Drug susceptibility test-proportion method (stand and economic variant)

With a loop, a representative sample of approximately 4 - 5 mg is taken from the primary culture and placed in a McCartney bottle containing 1 ml sterile distilled water and 3 mm diameter of 6 glass beads. The bottle was vortexed for 20 - 30 s and the opacity of the bacterial suspension was then adjusted by the addition of distilled water to obtain a concentration of 1 mg/ml of tubercle bacilli by matching with McFarland standard No.1. After preparing the standard neat suspension, the dilution 10^{-2} dilution 10^{-4} were produced by discharging two loopfuls (24 SWG-3 mm Nichrome wire) of the bacterial suspension. The contents were mixed by shaking. Two slopes of medium without drug and one slope of medium with ethambutol drug (2 µg/ml) are inoculated with a loopful of each dilution. The slopes were incubated at 37°C and the proportion tests were read at the 28th and 42nd day (Hirano et al., 1998).

Mycobacterium DNA extraction

One loopful of culture was taken in 100 µl of sterile distilled water and was homogenized. The entire homogenized samples were treated with 50 µl of lysozyme (10 mg/ml) at 37°C for overnight incubation. 70 µl of 14% sodium dodecyl sulfate (SDS) and 6 µl of proteinase K (10 mg/ml) was added to precipitate the proteins and was incubated at 65°C for 15 min. 10 µl of 5M NaCl and 80 µl of cetyl trimethylammonium bromide (CTAB)/NaCl were added to remove the polysaccharides and unwanted residues and was incubated at 65°C for 10 min. 800 µl of phenol:chloroform: isoamylalcohol (25:24:1) mixture was added to remove the proteins from the preparation of nucleic acid. The chloroform denatures the proteins while isoamyl alcohol reduces foaming during extraction and facilitates the separation of the aqueous and organic phase. Centrifugation was carried out at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and 600 µl of isopropanol was added to precipitate the DNA and then incubated overnight at -20°C. Centrifugation was carried out again at 12000 rpm in 4°C for 10 min. The pellet was washed with 70% ethanol to remove any remaining solutes. The pellet was air-dried and was dissolved in 20µl of 1x TE buffer (Mani et al., 2001; Muthuraj et al., 2010).

PCR amplification for species identification

The isolated template DNA was amplified using IS6110 primer in an authorized thermal cycler (Eppendorf Gradient Cycler). This confirms the template DNA as *M. tuberculosis*. The PCR reaction was set up as follows using the primer for *Mycobacterium IS6110* amplification F 5'GTGAGGGCATCGAGGTGG 3' (10 pmol/µl) R 5'CGTAGGCGTCG GTCA CAAA 3' (10 pmol/µl) (Helen et al., 2003). The PCR cycling parameters were 94°C for 5 min; followed

by 40 cycles of 94°C for 1 min, 57°C for 1 min and 72 °C for 1 min; and a final extension of 72°C for 10 min. The PCR was then kept at hold at 4°C for 15 min. The amplified PCR product was withdrawn from the thermal cycler and run on a 2% agarose gel in TAE buffer.

The ethidium bromide stained gels were observed in a UV transilluminator and photographed using a Geldoc.

PCR amplification of *rpoB* gene

The isolated template DNA was amplified using *rpoB* primers (rpo95- 5' CCACCCAG GACGTGGAGGCGATCACAC, rpo397-5' CGTTTCGATGAACCCGAACGGGTTGAC) (Elif et al., 2005) in an authorized thermal cycler (Eppendorf Gradient Cycler). The PCR cycling parameters were 94°C for 5 min; followed by 40 cycles of 94°C for 1 min, 57 °C for 1 min and 72 °C for 1 min; and a final extension of 74°C for 10 min. The PCR was then kept at hold at 4°C for 15 min. The amplified PCR product was withdrawn from the thermal cycler and run on a 2% agarose gel in TAE buffer. The ethidium bromide stained gels were observed in a UV transilluminator and photographed using a Geldoc.

Agarose gel electrophoresis

The gel running tray was placed in a clean gel casting tray to form the gel uniformly and the comb was fixed at one end. 400 mg of agarose (2%) powder was added to 20 ml of 0.75x TAE and was boiled for few seconds to dissolve the agarose completely. Less than 1 µl of ethidium bromide (0.5 mg/ml) was added into the hand bearable heat 250-ml conical flask containing melted agarose gel and was poured into the gel running tray. 1 µl of gel loading dye was transferred into a 5 × 5 cm size parafilm. To it, 5 µl of polymerized DNA was added and mixed thoroughly. The whole volume aliquot of amplified sample with gel loading dye was loaded into a well of 2% agarose gel in 0.75x TAE buffer and was subjected to electrophoresis for 30 min at 100 volts. The gel was observed under UV transilluminator for specific DNA bands and was photographed. The DNA bands were identified according to the size by comparing with the molecular weight marker (100 bp DNA ladder) loaded in a separate lane.

Electropherogram analysis of PCR amplified products

DNA dye concentration and DNA gel matrix were allowed to equilibrate at room temperature. 25 µl of dye concentration was added to DNA gel matrix, vortexed and transferred to spin filter and centrifuged at 2240 g for 15 min. The gel dye was allowed to settle at room temperature for 30 min. A new DNA chip was placed on the chip priming station. 9 µl of gel dye mix was pipetted into the well marked as G and the chip priming station was closed. The plunger was pressed down until it is held by the chip for 60 s. After 5 s, the plunger was pulled back slowly to 1 ml position. The chip priming station was opened and 9 µl of gel dye was pipetted into the well marked G and 1 µl of ladder was added to the well labeled ladder. 5 µl of marker was pipetted into all 12 sample wells and in ladder well. 1 µl of sample was added into the well. The chip was placed in the Laser Induced Fluorescent instrument (Bioanalyzer-Agilent, 2100) and the results were interpreted (Lucy et al., 2007).

DNA sequencing

The amplified PCR product *rpoB* gene from clinical isolate strains were run on 2% agarose gel and the product was purified using using PCR purification kit (Invitrogen). The purified PCR product

Table 1. Comparison of *in-vitro* testing (on clinical isolates) with PCR mediated direct sequencing.

PCR mediated direct sequencing	Number	*In-vitro* sensitivity tests on LJ slants	
		RMP sensitive smear (+) ve	RMP resistant smear (+) ve
Resistant	17	06(35%)	11(65%)
Sensitive	83	83(100%)	00
Total	100	89 (89%)	11(11%)

was directly sequenced in an automated DNA Sequencer at Bioserve in Bangalore. The nucleotide sequence obtained was analyzed using Basic Local Alignment Search Tool (BLAST)n Bioinformatics tool available at National Center for Biotechnology Information (NCBI) (Altschul et al., 1997) to know the specificity of PCR amplification and to identify the nucleotide variation. The sequence was further subjected for BLASTx to know the amino acid changes in comparison with the wild type *M. tuberculosis* (H$_{37}$Rv).

RESULTS AND DISCUSSION

The isolated mycobacterial DNA was subjected to PCR amplification using species specific primers, targeting the insertion sequence IS6110 (Mtb 5'G TGAGGGCATCGA GGTGG 3') (Mtb 5'CGTAGGCGTCGGTCACAAA 3') to conform the *M. tuberculosis*. A clear band was formed in 2% agarose gel at 123 bp region confirming the presence of *M. tuberculosis* (Figure 3). The templates of *M. tuberculosis* clinical isolate and H37Rv wild type strain were amplified using *rpoB* primer (rpo95- 5' CCACCCAGGACGTGGA GGCGATCACAC, rpo397-5' CGTTTCGATGAAC CCGAACGGGTTGAC). A clear band was formed in 2% agarose gel at 329 bp region, confirming the amplification of *rpoB* region of *M. tuberculosis* (Figure 4). The PCR products were analyzed on Bio analyzer to purity and specificity of the amplified products and electropherogram analysis of PCR amplified *rpoB* gene confirmed the molecular size (329 bp) of the products (Figure 5).

Among the 100 samples, the *in-vitro* drug sensitivity testing on Lowenstein-Jensen (LJ) slants identified 89 rifampicin sensitive (RMP^s) and 11 RMP^r strains (Table 1); the minimum inhibitory concentration (MIC) on LJ for 11 strains was > 40 µg/ml. The six isolates had mutations (substitution) in 329 bp regions of the *rpoB* gene, that is, outside the 81 bp RRDR regions among the 89 rifampicin sensitive isolates. Moreover, all RMP^r strains were multidrug resistant, that is, resistant to at least RMP and isoniazid. All 100 samples were smear positive (3+ smear grade 67, 2+ smear grade 18, 1+ smear grade 12, scanty smear grade 3) as shown in all 17 rifampicin resistant strains were 3+ smear grade. The 83 *M. tuberculosis* isolates were identified as RMP^s, since they exhibited a wild-type pattern on PCR mediated direct sequencing. The PCR mediated direct sequencing revealed the presence of mutations in 17 isolates and was consequently identified as RMP^r. The amino acids affected and the corresponding amino-acid substitutions

of each clinical isolates are presented in Figures 1 and 2.

The activity of RMP involves binding to β subunit of ribonucleic acid (RNA) polymerase, resulting in the inhibition of transcription initiation (Ramaswamy and Musser, 1998). RMP resistance in *M. tuberculosis* complex strains emerges as the results of point mutations or small deletions or insertions in a limited region of the gene encoding for the *rpoB* gene (codons 507-533) encoding 27 amino acids (Somoskovi et al., 2001 ;Ahmad et al., 2001). Mutations of the RMP-resistant *M. tuberculosis* isolates are frequently located in an 81-bp core region (the rifampin resistance determining region [RRDR]) of the *rpoB* gene in up to 95 - 98% of RMP-resistant strains (Simon and Listiawan, 2003; Hillemann et al., 2005). Up to the present, more than 35 different mutations have been described in this region (Ahmad et al., 2001) and a mutation found in a resistant strain is generally accepted to be responsible for the resistant phenotype (Rossau et al., 1997; Gonul Aslan et al.,2008). In the present study, all the 17 (100%) isolates had mutation in RRDR of the *rpoB* gene. Seventeen RMP-resistant strains were found to have a mutation in the analyzed *rpoB* gene fragment. We detected the different patterns of mutations (Figure 1 and 2) and all of them included single nucleotide changes. The mutation rate at the most effected codon 526 in the 81 bp RRDR region of *rpoB* gene was determined to be 35.29% (Table 2) in isolates from south India. In addition, the mutation rate at the most affected codon 451 in the out side of the 81bp RRDR region of the *rpoB* gene. In the current study, mutations in 17 isolates at the outside of the 81 bp RRDR region of *rpoB* region listed in Figure 1 might represent novel mutations which were not reported previously. These codon are outside of the 81-bp RRDR (Ramaswamy et al., 1998), and in this respect, this amino acid substitution may cause resistance to RMP by causing conformational changes in the β subunit of RNA polymerase encoding *rpoB* rather than direct inhibition of the RMP binding site. Furthermore, we detected a synonym mutation (Thr→Thr) at codon 526 in one isolate. This codon site is inside the active site (codons 507-533) of RMP binding in the *rpoB* gene region (Ramaswamy and Musser, 1998); therefore this nucleotide substitution (ACG→ACA) may cause a mutation that disturbs the RMP binding.

In conclusion, the present study supplies important data on the frequency and different kinds of mutations occurring at various loci related to RMP resistance in clinical

395	396	397	398	399	400	401	402	403	404	405	413
Arg	Glu	Arg	Met	Thr	Thr	Gln	Asp	Val	Glu	Ala	Asn
AGA	GAA	AGA	ATG	ACC	ACC	CAA	GAC	GTA	GAA	GCC	AAC
		GGA	GGA				GAG	CTC	GAC	GAC	CAC
		Gly	Gly	AGC			Glu	Leu	Asp	Asp	His
				Ser			GTC		GGA	CAC	AGC
				AGA			Val		Gly	His	Ser
				Arg						GTC	
										Val	

4.9 %(3)

422	423	430	431	432	433	434	435
Lys	Glu	Leu	Ser	Gln	Phe	Met	Asp
AAG	GAA	CTC	AGC	CAA	TTC	ATG	GAC

AAC	GGA	CGC		GAA		ATC	GAG
Asn	Gly	Arg		Gly		Ile	Glu
ATG	GTA					AAG	GGC
Met	Val					Lys	Gly
						AAC	GTC
						Asn	Val
						AGG	GCC
						Arg	Ala

6.6 %(4) 6.6 %(4)

451	452	453	454	455	456	457	458	459	468	475	478	492
Ala	Leu	Gly	Pro	Gly	Gly	Leu	Ser	Arg	Asp	Gly	Cys	Gly
GCA	CTA	GGA	CCA	GGC	GGA	CTC	AGC	CGC	GAT	GGA	TGC	GGT
GAC					GTA	CAC	ATC	CTC	GAA	GTA	AGC	GCT
Asp					Val	His	Ile	Leu	Glu	Val	Ser	Ala
GAA												
Glu												
GGA												
Gly												
GTA												
Val												
CCA												
Pro												
TCA												
Ser												
CAA												
Gln												

11.48 %(7)

Figure 1. Mutations in the outside of 81-bp RRDR of *rpoB* in Rif^r M. tuberculosis isolates. The mutated codons with corresponding amino acids are indicated underneath the original sequence. The frequencies of the four most frequent mutation positions are indicated below.

507	508	509	510	511	512	513	514	515	516	517	518	519
Glu	Thr	Pro	Tyr	Arg	Lys	Val	Val	Asp	Gly	Val	Val	Ser
GAA	ACC	CCC	TAC	CGC	AAG	GTC	GTA	GAT	GGC	GTC	GTG	AGC

TGC
Cys
CAC
His
CCC
Pro

4.9 %(3)

GAC
Asp

GAC	TTC	GGG
Asp	Phe	Gly
GCC	ATC	GAG
Ala	Ile	Glu

520	521	522	523	524	525	526	527	528	529	530	531	532	533
Asp	Glu	Ile	Val	Tyr	Leu	Thr	Ala	Asp	Glu	Glu	Asp	Arg	His
GAC	GAG	ATC	GTT	TAC	CTA	ACG	GCA	GAC	GAA	GAG	GAC	CGC	CAC

GAC	GTC
Asp	Val

ATG
Met
ATG
Met
ACG
Thr
CAG
Gln
TGG
Trp
AAG
Lys

9.8% (6)

Figure 2. Mutations in the 81-bp RRDR region of *rpoB* in Rif[r] M. tuberculosis isolates. The mutated codons with corresponding amino acids are indicated underneath the original sequence. The frequencies of the four most frequent mutation positions are indicated below.
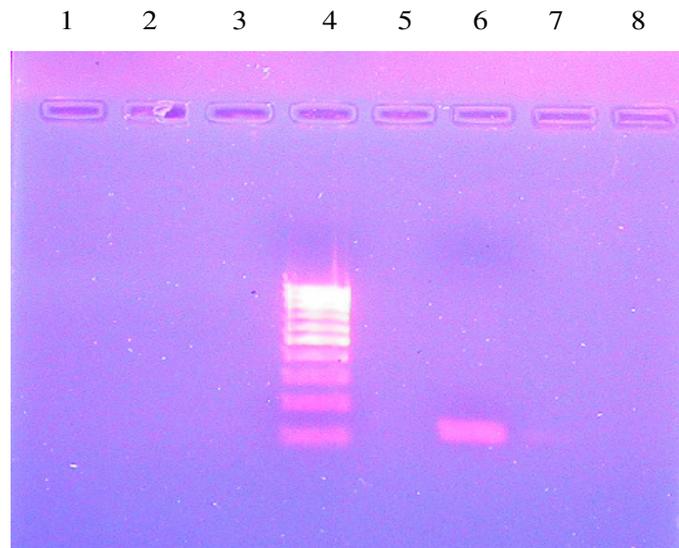
Figure 3. Isolated mycobacterial DNA analysis. Lane 4: 100 bp ladder and Lane 6: 123 bp products amplified with IS6110 primer.

Table 2. Frequency of mutations concerned with RMP resistance in RRDR region of *rpoB* gene detected by PCR mediated direct DNA sequencing.

Location of mutation	Nucleotide changes	Amino acid changes	No. (%) of strains (n = 17)
511	CGC→TGC	Arg→Cys	3 (17.65%)
	CGC→CAC	Arg→His	
	CGC→CCC	Arg→Pro	
513	GTC→GAC	Val→Asp	1 (5.88%)
516	GGC→GAC	Gly→Asp	2 (11.76%)
	GGC→GCC	Gly→Ala	
517	GTC→TTC	Val→Phe	2 (11.76%)
	GTC→ATC	Val→Ile	
518	GTG→GGG	Val→Gly	1 (5.88%)
521	GAG→GAC	Glu→Asp	1 (5.88%)
522	ATC→GTC	Ile→Val	1 (5.88%)
526	ACG→AGC	Thr→Ser	6 (35.29%)
	ACG→ATG	Thr→Met	
	ACG→ACA	Thr→Thr	
	ACG→CAG	Thr→Gln	
	ACG→TGG	Thr→Trp	
	ACG→AAG	Thr→Lys	
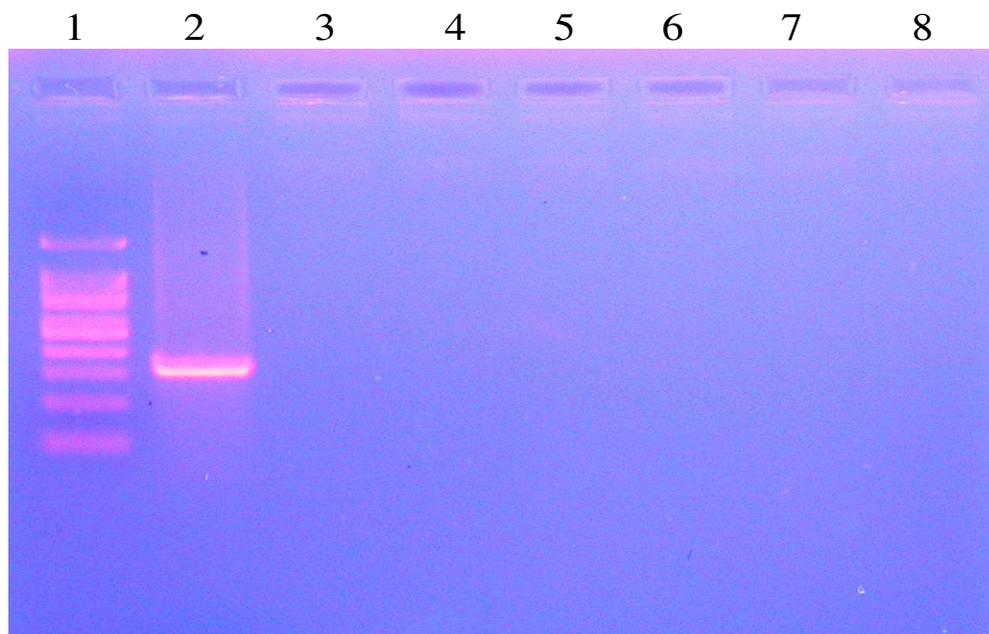
Figure 4. PCR amplified *rpoB* gene product. Lane 1: 100 bp ladder, Lane 2: 329bp.

isolates in our restricted region. We also detected new mutations which were not previously observed in codons 513,517 and 518. The sequence analysis technique that was used in this study is non radioactive and safer to perform than conventional radio active methods. Results were obtained in one working day and the technique was fairly economical, given the study's laboratory conditions. In this respect we think that this method   may be very useful   for revealing   prevalent mutations among *M. tuberculosis* complex isolates in our region.
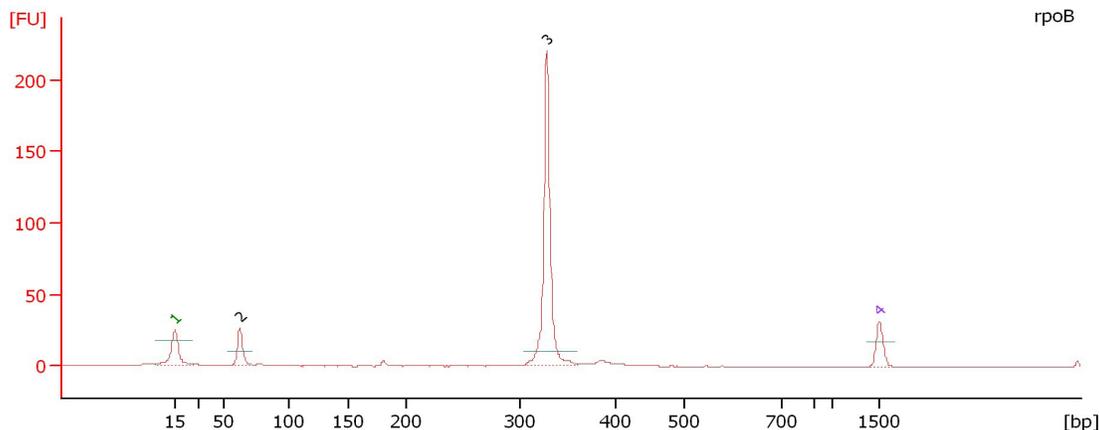
Figure 5. Electropherogram of PCR amplified *rpoB* gene (329 bp)   Peak 1: Lower Marker, Peak 3:   PCR product of *rpoB gene* and Peak 4: upper marker.

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