American Journal of Microbiological Research, 2014, Vol. 2, No. 3, 80-85
Available online at http://pubs.sciepub.com/ajmr/2/3/1

DOI:10.12691/ajmr-2-3-1

Characterization of *rpsL* Gene Mutations in Streptomycin-Resistant *Mycobacterium tuberculosis* Isolates

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Received March 17, 2014; Revised March 26, 2014; Accepted April 11, 2014

Abstract The worldwide emergence and spread of drug - resistant strains of *Mycobacterium tuberculosis* has adverse effects on tuberculosis (TB) control programs. The goal of this paper is to describe the advances made in the understanding of the molecular basis of *M. tuberculosis* resistance to streptomycin and to discuss the potential of molecular methods in early diagnosis of streptomycin resistant TB.Molecular methods such as DNA sequencing, polymerase chain reaction have been used to identify/detect mutations in *rpsL* gene-encoding proteins.Of the 77 SM resistant isolates, 22 (28.6%) exhibited mutation at codon 43 Lys→Arg, 11 (14.3%) isolates exhibited mutation at codon 83 Arg→Gln, 9(%) isolates exhibited mutation at codon 95 Tyr→His,whereas remaining 23 (40.8%) SM resistant isolates showed no mutation in *rpsL* gene. Among the 77 SM resistant isolates, 31(40.2%) were SM mono resistant and 46(59.8%) strains were poly resistant. All the 23 SM susceptible isolates as well as reference strain *M.tuberculosis* H37Rv exhibited wild type sequences of *rpsL* gene. Molecular methods to detect the most frequent mutations in the gene encoding functions that are targets for streptomycin drug have provided encouraging results for early diagnosis of resistance nature.

Keywords: *streptomycin resistance, Mycobacterium tuberculosis, Pulmonary tuberculosis, South-India*

Cite This Article: Usharani Brammacharry, and Muthuraj Muthaiah, "Characterization of *rpsL* Gene Mutations in Streptomycin-Resistant *Mycobacterium tuberculosis* Isolates." *American Journal of Microbiological Research*, vol. 2, no. 3 (2014): 80-85. doi: 10.12691/ajmr-2-3-1.

1. Introduction

Mycobacterium tuberculosis, the causative agent of human tuberculosis (TB) infects one of every three people in the world. In Asia, it infects one of every two people. Every year, about two million people in the world die of TB and eight million new cases occur. TB is the leading cause of mortality due to a single infectious agent. It kills 250,000 children every year and accounts for 26% of all avoidable adult deaths worldwide (Dye C et al., 1999). Considering the magnitude of the problem, the World Health Organization (WHO) declared TB as a Global Emergency. The above situation has emerged despite the fact that TB is usually a curable disease. The threat of TB is assuming alarming proportions with the dramatic worldwide increase in isolation of *M.tuberculosis* strains resistant to commonly used (first-line) anti-TB d rugs. This phenomenon is occurring in both immuno-competent and immuno-compromised patients (Cohn DL et al., 1997). With the increase in human immunodeficiency virus (HIV) epidemic, and the high risk of HIV-infected individuals to develop TB, the prevalence of MDR-TB is almost certain to rise in most countries of the world.

M.tuberculosis is a slow growing microorganisms,and isolation, identification and drug susceptibility testing can therefore take several weeks.In recent years, molecular methods have been developed for rapid detection,species identification and drug susceptibility testing of Mycobacterium. Mutations in several genes and genomic regions of *M.tuberculosis* are involved in the occurrence of resistance to isoniazid, rifampicin, streptomycin, Pyrazinamide and ethambutol, which are critical components of the first line drug for tuberculosis (Elif Ozturk C et al., 2005). The target for SM resistance is the ribosomal proteins. Mutations in genes *rpsL* and *rrs*, which are involved in the synthesis of these proteins, have been shown to be responsible for 70% of SM-resistant strains (Springer B et al., 2002). The drug resistance of *M. tuberculosis* to SM is related to *rpsL* gene mutation, with mutations in codon 43 being the most common cause (Huang HN et al., 2003). The genetic basis of antibiotic resistance in clinical drug resistant *M.tuberculosis* isolates has been widely studied and is generally believed to be caused by point mutations in drug target genes within the *M.tuberculosis* genome including *katG* for INH, *rpoB* for RIF and *rpsL* for SM. The most frequent mutation patterens of INH,RIF and SM resistant strains occurred at codon 315 (55-90%) of kat G;codon 531 (40-60) and codon 526 (10-30%) within the RIF resistance determining region (RRDR,codons 507 to 533) of rpoB and codon 43 (47-79%) of *rpsL*(Yuan-Chuan Wang et al.,2009).

In this report, we describe a simple PCR approach to detect the most frequently found mutation in SM resistant *M.tuberculosis*, a substitution in the gene *rpsL*, directly from sputum samples. We evaluated here the relationship between genetic alterations in *rpsL* and MICs of streptomycin in a large number of *M. tuberculosis* strains isolated from Pondicherry patients. We used direct DNA sequence analysis for their ability to detect mutations in *rpsL* in streptomycin resistant *M. tuberculosis* strains.The purpose of the study described here was (i) to characterize the level of resistance associated with the different molecular mechanisms and (ii) to investigate whether a permeability barrier contributes to drug resistance in streptomycin resistant isolates with wild-type *rpsL*.

2. Materials and Methods

A total of 100 clinical strains of *M.tuberculosis* were isolated from sputum samples of treatment failure cases hospitalized at Government Hospital for Chest Diseases, Puducherry and processed the samples in department of Microbiology at State TB Training and Demonstration Centre (Intermediate Reference Laboratory) South India, during the period from January 2007 to November 2010. All specimens were processed immediately and aliquots of the decontaminated specimens were kept at −20°C

2.1. Sputum Processing for AFB Culture

To each volume of sputum, 2 volumes of 4% NaOH were added. 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 minutes. The specimens were removed from the shaker. The sputum bottles were centrifuged for 15 minutes at 4000 rpm. After the bottles were removed from the centrifuge, the supernatant was carefully poured off into the disinfectant bath. The bottles were filled with 20 mL of sterile distilled water, shaken by hand to mix the deposit and were centrifuged for 15 minutes at 4000 rpm.The supernatant was poured off and 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 (Muthuraj Muthaiah et al., 2010).

2.2. 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 seconds and the opacity of the bacterial suspension was then adjusted by the addition of distilled water to obtain a concentration of 1mg /mL of tubercle bacilli by matching with McFarland standard No.1. After preparing the standard neat suspension, the dilution 10^{-2} and 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 two slopes of L.J media are used as controls for the test, and each one slope of medium with streptomycin (4 µg/mL), isoniazid (0.2 µg/mL),Rifampicin,(40µg/mL),ethambutal (2µg/mL) and Pyrazinamide (100µg/mL) are inoculated with a loopful of each dilution. The slopes were incubated at 37°C and the proportion tests were read at 28 days and again at 42 days (Hirano K. et al., 1998).

2.3. Mycobacterium DNA Extraction

One loopful of culture was homogenized in 100 µL of sterile distilled water. The entire homogenized samples were treated with 50 µL of lysozyme (10 mg/mL) at 37°C for overnight incubation. 70 µL of 14% SDS and 6 µL of Proteinase K (10 mg/mL) was added and incubated at 65°C for 15 minutes. 10 µL of 5 M NaCl and 80 µL of CTAB/NaCl were added and was incubated at 65°C for 10 minutes. 800 µL of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture was added and centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and 600 µL of Isopropanol was added to precipitate the DNA and incubated overnight at −20°C.Centrifuged at 12,000 rpm in 4°C for 10 minutes. The pellet was washed with 70% ethanol to remove any remaining solutes. The pellet was air-dried and was dissolved in 20 µL of 1× TE buffer (Mani C et al., 2003).

2.4. PCR Amplification for Species Identification

The isolated template DNA was amplified using IS6110 primer in an authorized thermal cycler (Eppendorf Gradient Cycler).This confirmed that the template DNA of the clinical isolates was *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'CGTAGGCG TCGGTCACAAA 3' (10 pmol/µL) (Fletcher, HA et al., 2005). 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 72°C for 1 minute; and a final extension of 72°C for 10 minutes. The PCR was then kept at hold at 4°C for 15 minutes. The amplified PCR product was withdrawn from 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.

2.5. PCR Amplification of rpsL Gene

The isolated template DNA was amplified using rpsL primers. *rpsl* 1 (5'-GGCCGCAA CAGAACGT-3') & *rpsl* 2 (5'-GTTCACCAACTGGGTGAC-3') (Elif et al., 2005) in an authorized thermal cycler (Eppendorf Gradient Cycler). 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 72°C for 1 minute; and a final extension of 74°C for 10 minutes. The PCR was then kept at hold at 4°C for15 minutes. The amplified PCR product was withdrawn from thermal cycler and run on a 2% Agarose gel in TAE buffer. The ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc (Vasanthi N et al., 2010).

2.6. 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 0.75 × TAE and was boiled 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 Para film. 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 0.75 × TAE buffer and was subjected to electrophoresis for 30 minutes at 100 volts. The gel was observed under UV Transilluminator for specific DNA bands and was photographed. The DNA bands were identified according to size by comparing with the molecular weight marker (100 bp DNA ladder) loaded in a separate lane (Srinand Sreevatsan et al., 1996).

2.7. 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 minutes. The gel dye was allowed to settle at room temperature for 30 minutes. 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 seconds. After 5 seconds 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 (Muthuraj Muthaiah et al., 2010).

2.8. Automated DNA Sequencing of the rpsL Gene

The strategies used to amplify and sequence the entire open reading frame of *rpsL* and a 1,042-bp segment of *rrs* have already been described in detail (Srinand Sreevatsan et al., 1996).The oligonucleotide primers used to amplify *rpsL* were as follows: forward, 5'-GGCCGA CAAACAGAACGT-3'; reverse, 5'-GTTCACCAACTGGGTGAC-3'. An automated DNA sequencing instrument (model 373A; Applied Biosystems, Inc.,) was used to characterize the regions of *rpsL* studied. The nucleotide sequence obtained was analyzed using BLASTn Bioinformatics tool available at National Center for Biotechnology Information (Altschul SF et al., 2004) to know the specificity of PCR amplification and to identify the nucleotide variation. The sequence was further subjected for BLASTx to identify the amino acid changes in comparison with the wild type *M.tuberculosis* (H37Rv).

3. Results and Discussion

A clear PCR product band of 123 base pairs (bp) was observed on a 2% agarose gel confirming the *M. tuberculosis* (Figure 1).The observation of a clear band at the 548 bp region on 2% agarose gel confirmed the amplification of the pncA gene of *M. tuberculosis* (Figure 2). The PCR products were analyzed on a Bioanalyzer (Agilent 2100) to check the purity and specificity of the products. Electropherogram analysis of the PCR-amplified insertion sequence and *pncA* gene confirmed the molecular size (123 and 548 bp) of the products (Figure 3 & Figure 4).

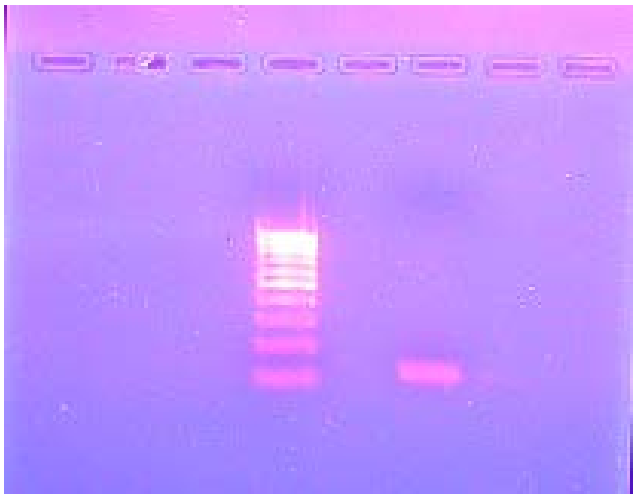

Figure 1. PCR Amplification for Species Identification. Lane 4: 100 bp DNA ladder, Lane 6:123 bp PCR amplified product

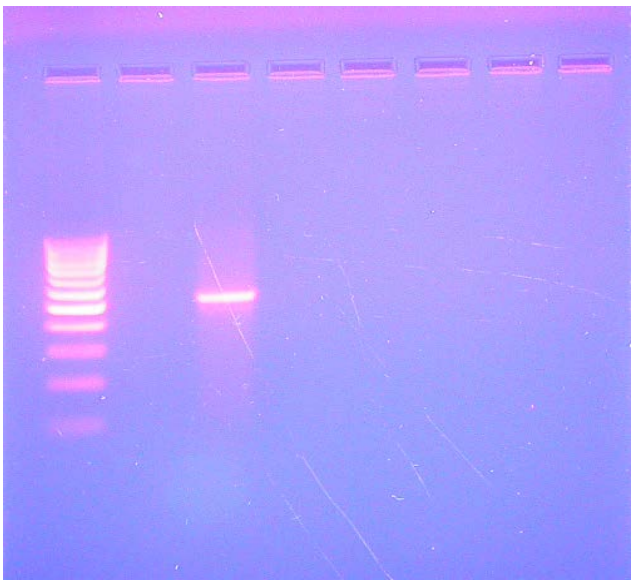

Figure 2. PCR Amplification of *rpsL*. Lane 1: 100 bp DNA ladder, Lane 3: 548 bp PCR, Amplified *rpsL* gene product

The present study is the first investigation involving molecular characterization of streptomycin drug-resistant *M tuberculosis* clinical isolates from Puducherry, South India. In this study, 77 streptomycin drug-resistant isolates and 23 susceptible isolates were assessed by DNA direct sequencing. Streptomycin interacts with the 16S rRNA and S12 ribosomal protein (*rrs* and *rpsL*) (Escalante et al., 1998; Finken et al., 1993; Sreevatsan et al., 1996; Abbadi et al., 2001) inducing ribosomal changes, which cause misreading of the mRNA and inhibition of protein synthesis. Although streptomycin is a recommended anti-TB drug, is it less effective against *M. tuberculosis* than INH and RIF. Point mutations in streptomycin resistant

isolates have been reported in *rpsL* genes in 65–67% of STR resistant isolates (Ramaswamy et al.,1998).Mutations in the *rpsL* gene at codon 43 (AAG-AGG/ACG) (Lys-Arg/Thr) and codon 88 (AAGAGG/CAG) (Lys-Arg/Gln) are associated with streptomycin resistance. MIC analysis of streptomycin resistant isolates indicates that amino acid replacements in the *rpsL* genes correlate with a high level of resistance (Cooksey et al., 1996; Meier et al., 1996).

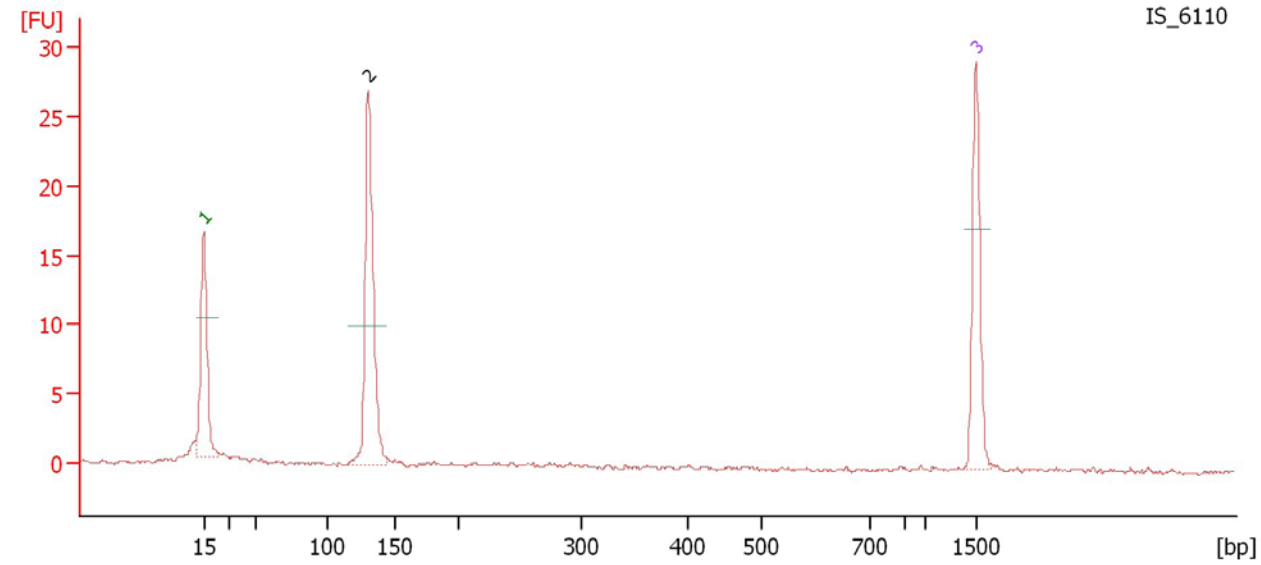

Figure 3. Electropherogram Analysis of PCR Amplified Products. Peak 1: Lower Marker (15 bp), Peak 2: PCR amplified product of insertion sequence gene (123 bp) and Peak 3: Upper marker (1500 bp)

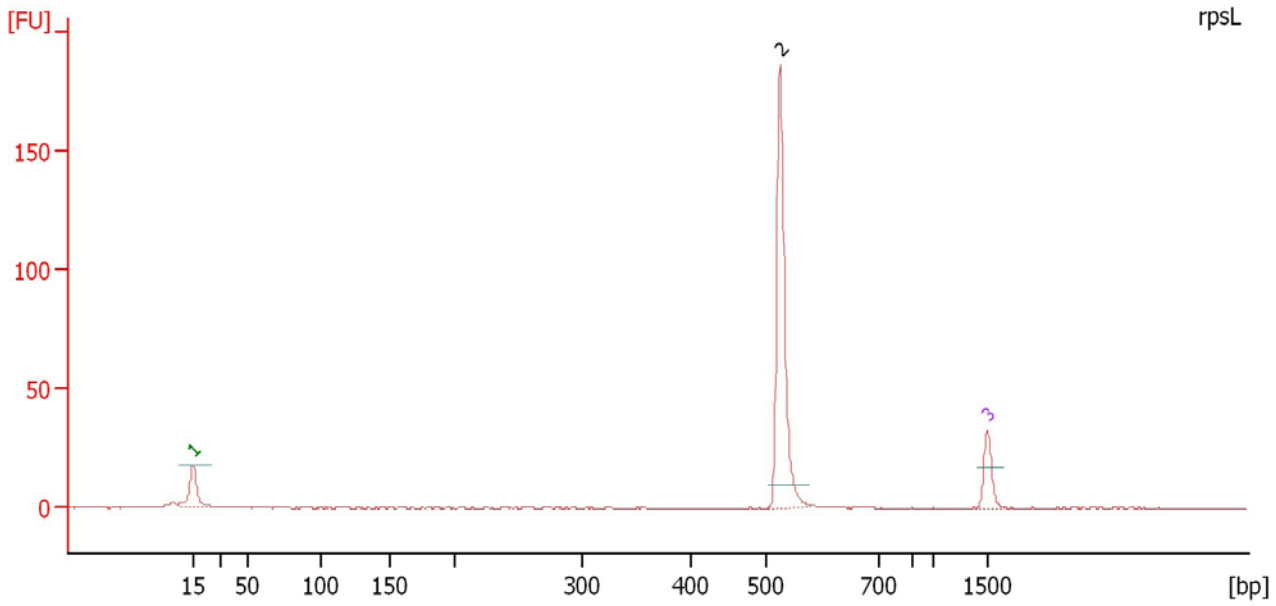

Figure 4. Electropherogram Analysis of PCR Amplified Products. Peak 1: Lower Marker (15 bp), Peak 2: PCR amplified product of *rpsL* gene (548 bp) and Peak 3: Upper marker (1500 bp)

Of the 77 SM resistant isolates, 22 (28.6%) exhibited mutation at codon 43 Lys→Arg, 11 (14.3%) isolates exhibited mutation at codon 83 Arg→Gln, 9(11.7%) isolates exhibited mutation at codon 95 Tyr→His, 5(10 isolates-13%) isolates exhibited mutation at codon 48 Ala→Pro, codon 98 Ile→Thr, 4(16 isolates-20.8%) isolates each exhibited mutation at codon 53 Ala→Glu, codon 80 Val→Gly, codon 86 Arg→Pro, codon 92 Gly→Ala respectively and 3(9-isolates-11.7%) isolates each exhibited mutation at codon 74 Leu→Arg, codon 76 Gln→Leu, codon 90 Leu→His respectively, whereas remaining 23 (40.8%) SM resistant isolates showed no mutation in *rpsL* gene. All the 23 SM susceptible isolates as well as reference strain *M.tuberculosis* H37Rv exhibited wild type sequences of *rpsL* gene. Among the 77 SM resistant, 31 are SM mono resistant exhibited mutations at codon 43 Lys→Arg(17 strains), codon 83 Arg→Gln(5 strains), codon 95 Tyr→His(3 strains), codon 53 Ala→Glu(2 strains), codon 86 Arg→Pro(2 strains), codon 74 Leu→Arg(1 strain) and codon 90 Leu→His(1 strain).The remaining 46 isolates are poly resistant in addition to SM resistant. Among them 25 strains were resistant to INH, EMB, 15 strains were resistant RM, INH and 6 strains were resistant to EMB, PZA.

Mutations and their association with streptomycin MIC levels are described in the Table 2. All the 36 isolates with high degree of SM resistance (MIC ≥1024 mg/l) exhibited mutations in *rpsL* gene, while remaining 41 SM resistant isolates with low MIC values (MIC ≤ 128 mg/l) exhibited mutations in the *rps*L gene. All the 23 SM susceptible

isolates were found to have wild type *rpsL* sequences. Among the 77 SM-resistant isolates having mutations in *rps*L, 22 (28.6%) exhibited mutations at codon 43 and 11 isolates (14.3%) exhibited mutation at codon 83 by sequencing method. Point mutations producing high level streptomycin resistance occur in *rpsL* gene, mostly involving codon 43 and less frequently in codon 88(Suhail Ahmad et al., 2001) (Table 1). Low level of resistance to streptomycin may also occur due to cell permeability changes, production of amino glycoside modifying enzymes or alterations in other ribosomal molecules as some of the resistant isolates with MIC of 10 mg/ml have wild type *rpsL* genes. The study of *rpsL* mutations and their association with MIC levels of SM can help develop other new rapid methods like probes for detection of SM resistance directly from clinical samples. This understanding will be valuable for the development of rapid and reliable molecular approaches to the detection of drug-resistant *M. tuberculosis* in our restricted region.

Table 1. The prevalence and pattern of mutations in *rpsL* gene region among SM resistant isolate:

No. of strain	Codon	Nucleotide changes	Amino acid changes
22	43	AAG→AAC	Lys→Arg
5	48	GCA→CCA	Ala→Pro
4	53	GCA→GAA	Ala→Glu
3	74	CTA→CGA	Leu→Arg
3	76	CAG→CTG	Gln→Leu
4	80	GTA→GGA	Val→Gly
11	83	AGA→CAA	Arg→Gln
4	86	CGA→CCA	Arg→Pro
3	90	CTC→CAC	Leu→His
4	92	GGA→GCA	Gly→Ala
9	95	TAC→CAC	Tyr→His
5	98	ATA→ACA	Ile→Thr

Table 2. Results of association between MICs of streptomycin (SM) and mutations in the *rpsL* gene of *M. tuberculosis*

SM (MIC mg/l)	Degree of Resistant	Number of isolates with mutation detected in *rpsL* gene				No mutation
		Codon	43	83	95	
		Total	AAG→AGG	AGA→CAA	TAC→CAC	
$>2048_R$	High	20	20 (26%)	Nil	Nil	Nil
1024_R	High	16	02 (3%)	08 (10%)	03 (4%)	03 (4%)
128_R	Moderate	29	Nil	03 (4%)	05 (6%)	18(24%)
64_R	Low	07	Nil	Nil	01 (1%)	06(8%)
32_R	Low	05	Nil	Nil	Nil	Nil
$\leq 16_S$	Susceptible	23	Nil	Nil	Nil	Nil

R, resistant; S, susceptible; percentages denote proportion among SM resistant isolates investigated in this series

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