

## Correspondence

### **Association of mutations in *rpsL* gene with high degree of streptomycin resistance in clinical isolates of *Mycobacterium tuberculosis* in India**

Sir,

Streptomycin (SM) has been used in the treatment of tuberculosis (TB) for more than 50 years and is an important part of current treatment regimens for category II pulmonary TB cases. Due to its usage over a period of time and even as a monotherapy, resistance to streptomycin has increased. Globally, the range of initial and acquired streptomycin resistance has been reported to be 0-51.5 and 0-73.1 per cent, respectively during the period 1999-2002<sup>1</sup>. Understanding the molecular mechanism(s) of SM resistance can help develop new generation rapid molecular diagnostics. Presence of mutations in *rpsL* gene and *rrs* sequences has been reported in SM resistant isolates in varying frequencies ranging from 47-60 per cent<sup>2-4</sup>.

It is well known that determination of SM resistance is time consuming by conventional methods<sup>5</sup>, and there is a need to search for alternative techniques for determining SM resistance. The present study was carried out to determine the susceptibility levels of *Mycobacterium tuberculosis* isolates to streptomycin, to identify mutation(s) in *rpsL* gene, and to analyse relationship between these mutations and streptomycin minimum inhibitory concentration (MIC) levels. We evaluated a PCR- restriction fragment length polymorphism (RFLP) assay of *rpsL* gene for rapid, simple and cost-effective detection of mutation<sup>3</sup> and confirmed these results by sequencing of *rpsL* gene.

A total of 69 biochemically well-characterized *M. tuberculosis* isolates<sup>6</sup> from previously treated pulmonary TB patients deposited in Mycobacterial Repository Center at National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, along with reference strain *M. tuberculosis* H37Rv (TMC-102, NIH, USA) were used in this study. The area-wise distribution of isolates was as follows: Agra (n=26),

Cochin (n=1), Haridwar (n=2), Jaipur (n=22), Kanpur (n=6), Chennai (n=1), New Delhi (n=8), Port Blair (n=1), Varanasi (n=2). The susceptibility testing against SM (Sigma Chem. Co., USA) was performed on the Löwenstein-Jensen (LJ) medium by the resistance ratio method<sup>5,7</sup>. The LJ medium was prepared at our Institute using analytical grade reagents (Hi-Media Laboratories Pvt. Ltd, Mumbai, India). A standard suspension of bacilli (4 mg/ml, *i.e.*, approximately 10<sup>8</sup> bacilli/ml) was used for drug susceptibility testing against SM on LJ medium. The resistance ratio for an isolate was calculated by dividing the MIC of test isolate by MIC of reference susceptible strain *M. tuberculosis* H37Rv. An isolate was identified as SM resistant if resistance ratio of  $\geq 8$  was obtained<sup>5,7</sup>. Of the 69 isolates, 49 were found to be SM resistant and remaining 20 were SM susceptible. Isolates found resistant were further tested at 32, 64, 128, 256, 512, 1024 and 2048 mg/l concentration of SM in order to determine their MIC values.

DNA from the log phase growth of isolates were extracted by the procedure of van Embden *et al*<sup>8</sup> using lysozyme, proteinase-K (Bangalore Genei Pvt. Ltd., India), deproteinization with chloroform-isoamyl alcohol and precipitation with isopropanol. A 360 bp fragment encoding part of *rpsL* gene was amplified in Gene Amp PCR System 9700 (Applied Biosystems, USA) for PCR-RFLP analysis<sup>3</sup>. The above amplicons were restricted with restriction enzyme *MboII*, (Fermentas Life Sciences, USA) and generated into two fragments of 138 and 222 bp in susceptible wild type isolates and reference strain *M. tuberculosis* H37Rv. On the other hand, the 360 bp fragment remains undigested in resistant isolates with mutation at 43 codon. This method thus detects only the codon 43 mutation of *rpsL* gene<sup>3</sup>.

A 505 bp fragment containing the entire *rpsL* gene was amplified and sequenced<sup>9</sup>. The PCR reagents

were obtained from Bangalore Genei Pvt. Ltd., India, and primers were got synthesized from Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India. The 505 bp band containing *rpsL* gene was excised from agarose gel slices and DNA was extracted using QIAEX<sup>R</sup> Gel Extraction kit (QIAGEN, USA). The sequencing reaction was performed with Big Dye Terminator mix v3.1 (Applied Biosystems, Foster City, CA, USA) using same primers as for amplification<sup>9</sup>. The sequencing of the amplicon was carried out using the ABI PRISM 310 DNA sequencer (Applied Biosystems, USA). Sequences generated were compared with wild type *rpsL* gene sequences of SM susceptible reference strain *M. tuberculosis* H37Rv using MegAlign program of DNASTAR software (Madison, WI, USA).

Of the 49 SM resistant isolates, 27 (55.1%) exhibited mutation at codon 43 Lys→Arg and 2 (4%) isolates exhibited mutation at codon 88 Lys→Arg whereas remaining 20 (40.8%) SM resistant isolates showed no mutation in *rpsL* gene. All the 20 SM susceptible isolates as well as reference strain *M. tuberculosis* H37Rv exhibited wild type sequences of *rpsL* gene.

Mutations and their association with streptomycin MIC levels are described in the Table. All the 29 isolates with high degree of SM resistance (MIC ≥ 2048 mg/l) exhibited mutations in *rpsL* gene, while remaining 20 SM resistant isolates with low MIC values (MIC ≤ 128 mg/l) exhibited no mutation in the *rpsL* gene. All the 20 SM susceptible isolates were found to have wild type *rpsL* sequences. Among the 29 SM-resistant isolates having mutations in *rpsL*, 27 (93.1%) exhibited mutations at codon 43 by sequencing as well as PCR-RFLP method (Fig.). Two isolates (7%) exhibited mutation at codon 88 by sequencing method

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

SM (MIC mg/l)	Degree of resistance	Number of isolates with mutation detected in <i>rpsL</i> gene	Number of isolates with mutation detected in <i>rpsL</i> gene		No mutation
			Total AAG→AGG (Lys43Arg)	AAG→AGG (Lys88Arg)	
>2048 <sub>R</sub>	High	27	27 (55.10%)	Nil	Nil
2048 <sub>R</sub>	High	2	Nil	2 (4.08%)	Nil
128 <sub>R</sub>	Moderate	1	Nil	Nil	1 (2.04%)
64 <sub>R</sub>	Low	5	Nil	Nil	5 (10.20%)
32 <sub>R</sub>	Low	14	Nil	Nil	14 (28.57%)
≤16 <sub>S</sub>	Susceptible	20	Nil	Nil	20

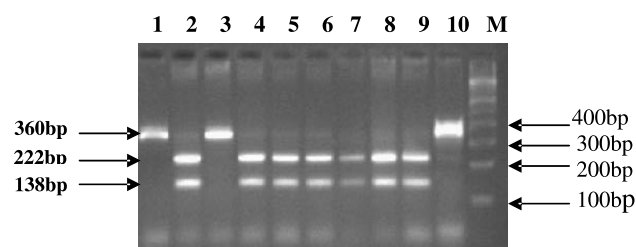
R, resistant; S, susceptible; percentages denote proportion among SM resistant isolates investigated in this series

but showed wild-type pattern by PCR-RFLP. This is as expected because the PCR-RFLP system used in this study detects mutation only at codon 43, not at codon 88. Remaining 20 SM resistant isolates as well as the 20 SM susceptible isolates exhibited no mutation in their *rpsL* gene by sequencing as well as PCR-RFLP. Thus, this PCR-RFLP assay appears to be a cost-effective approach for detection of high level SM resistance in Indian isolates, showing same sensitivity as observed in other parts of world<sup>3</sup>.

In our study, mutations were found at codons 43 and 88 in 59.2 per cent (29/49) of SM resistant isolates. Frequency of *rpsL* mutations has been also reported to be 47-60 per cent in SM resistant isolates in different studies<sup>2-4</sup>. The SM resistance associated mutations most commonly reported in the *rpsL* locus are Lys43Arg/Thr, or Lys88Arg/Thr<sup>2-4,10,11</sup>. A silent mutation (AAA→AAG, Lys→Lys) at codon 121 has been reported from India in our previous study<sup>9</sup>. The association of high degree of SM resistance (MIC 500-2048 mg/l) with mutations at codons 43 and 88 (Lys→Arg) has been reported previously also<sup>3,10,12</sup>. We conclude that mutations in *rpsL* gene at codons 43 and 88 could be useful markers for high degree of SM resistance (MIC ≥ 2048 mg/l).

Knowledge 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. However, as our isolates were not selected by a proper sampling method, the findings of this pilot study need to be validated in a statistically significant number of isolates.

The mutations in *rrs* gene, efflux pumps or other undiscovered mechanism(s) might be involved in the



**Fig.** Results of PCR-RFLP analysis of *rpsL* gene in *M. tuberculosis*. Lane 1, 3 with mutation Lys43Arg (MIC > 2048 mg/l); Lane 2 with mutation Lys88Arg (MIC 2048 mg/l); Lanes 4, 5, 6 no mutation at codon 43 in SM resistant isolates (MIC 32-128 mg/l); Lanes 7, 8 no mutation at codon 43 in wild susceptible isolates (MIC < 16 mg/l); Lane 9 no mutation in reference strain *M. tuberculosis* H37Rv; Lane 10 without *MobII* digestion; Lane M 100bp molecular weight marker.

remaining isolates which showed relatively lower levels of SM resistance in the present study. Mechanisms in isolates not having any mutations in *rpsL* gene need to be elucidated. Plasmid mediated SM resistance in *M. tuberculosis* has not been reported. Two possible resistance mechanisms have been reported to be theoretically conceivable: alteration of the putative drug target(s) or alterations in the permeability of the cell wall that lead to reduced drug uptake<sup>13,14</sup>. In a previous study by our group, efflux pump has been reported to be one of the resistance mechanisms in a rifampicin, isoniazid, and ofloxacin resistant strain of *M. tuberculosis*<sup>15</sup>. Further studies in a significant number of isolates are required to understand these mechanisms.

**Ram Das, Pushpa Gupta  
Pushpendra Singh, D.S. Chauhan  
Kiran Katoch & V.M. Katoch\*<sup>+</sup>**

Department of Microbiology & Molecular Biology  
National JALMA Institute for Leprosy &  
Other Mycobacterial Diseases (ICMR)  
Tajganj, Agra 282 001, India

\*Present address:

Secretary, Department of Health Research  
Government of India & Director-General  
Indian Council of Medical Research  
V. Ramalingaswami Bhawan, Ansari Nagar  
New Delhi 110 029, India

<sup>+</sup>For correspondence:

vishwamohan\_katoch@yahoo.co.in  
jalma@sancharnet.in

### References

1. Paramasivan CN, Venkataraman P. Drug resistance in tuberculosis in India. *Indian J Med Res* 2004; 120 : 337-86.
2. Finken M, Kirschner P, Meier A, Wrede A, Bottger EC. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alteration of the ribosomal protein S12 gene and point mutations with a functional 16S ribosomal-RNA pseudoknot. *Mol Microbiol* 1993; 9 : 1239-46.
3. Fukuda M, Koga H, Ohno H, Yang B, Hirakata Y, Maesaki S, et al. Relationship between alteration of the *rpsL* gene and streptomycin susceptibility of *Mycobacterium tuberculosis* in Japan. *J Antimicrob Chemother* 1999; 43 : 281-4.
4. Gegia M, Mdivani N, Mendes RE, Li H, Akhalaia M, Han J, et al. Prevalence of and molecular basis for tuberculosis drug resistance in the Republic of Georgia: validation of a Qiaplex system for detection of drug resistance-related mutations. *Antimicrob Agents Chemother* 2008; 52 : 725-9.
5. Canetti G, Fox W, Khomenko A, Menon NK, Mitchison DA, Rist N, et al. Advances in techniques of testing mycobacterial drug sensitivity and the use of sensitivity test in tuberculosis control programs. *Bull World Health Organ* 1969; 74 : 23-7.
6. Vestal AL. In: *Procedure for the isolation and identification of mycobacteria*, US Department of Health, Education and Welfare Center for Disease Control and Prevention, Atlanta, Georgia; 1977. p. 32-89.
7. Paramasivan CN, Chandrasekaran V, Santha T, Sundersanam NM, Prabhakar R. Bacteriological investigations for short course chemotherapy under the tuberculosis programme in two districts of India. *Tuberc Lung Dis* 1993; 32 : 118-21.
8. van Embden JDA, Cane MD, Crawford JT, Dale JE, Eisenach KD, Jicquel B. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: Recommendations for standardized methodology. *J Clin Microbiol* 1993; 31 : 406-9.
9. Siddiqi N, Shamim M, Hussain H, Chaudhary RK, Ahmed N, Banerjee S, et al. Molecular characterization of multidrug resistant isolates of *Mycobacterium tuberculosis* from patients in north India. *Antimicrob Agents Chemother* 2002; 46 : 443-50.
10. Honore N, Cole ST. Streptomycin resistance in mycobacteria. *Antimicrob Agents Chemother* 1994; 38 : 238-42.
11. Meier A, Kirschner P, Bange FC, Vogel U, Bottger EC. Genetic alterations in streptomycin-resistant *Mycobacterium tuberculosis*: mapping of mutations conferring resistance. *Antimicrob Agent Chemother* 1994; 38 : 228-33.
12. Cooksey RC, Morlock GP, McQueen A, Glickman SE, Cranford JT. Characterization of streptomycin resistance mechanisms among *Mycobacterium tuberculosis* isolates from patients in New York City. *Antimicrob Agents Chemother* 1996; 40 : 1186-8.
13. Mitchison D. Drug resistance in mycobacteria. *Br Med Bull* 1985; 40 : 84-90.
14. Martin C, Ranes M, Glcquel B. Plasmids, antibiotic resistance, and mobile genetic elements in mycobacteria. In: MacFadden J, editor. *Molecular Biology of the Mycobacteria*. Guildford, United Kingdom: Surrey University Press; 1990. p. 121-38.
15. Siddiqi N, Das R, Pathak N, Banerjee S, Ahmed N, Katoch VM, et al. *Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a Tap-Like efflux pump. *Infection* 2004; 32 : 109-11.