Rapid Detection of Mutation in RRDR of *rpo B* Gene for Rifampicin Resistance in MDR-Pulmonary Tuberculosis by DNA Sequencing

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Abstract To detect the site of mutation in RRDR of *rpo B* gene for rifampicin resistance in MDR-TB by DNA sequencing. 50 MDR-TB patients were enrolled in our study after informed written consent. Mycobacterial DNA was extracted from sputum samples by Universal Sample Processing (USP) method and RRDR of *rpo B* gene was amplified by PCR using primers RP4T and RP8T and then sequenced by automated DNA sequencing. The nucleotide sequences of RRDR of *rpo B* gene were compared with the reference sequence. We observed three different types of mutation in the RRDR of *rpo B* gene. The frequency of mutation in codon 531 (TCG → TTG), 526 (CAC → TAC) and 516 (GAC → GTC) are 60, 26.6 and 6.6% respectively. Of the total cases studied, 6.6% cases, although resistant to rifampicin, did not show any mutation in the RRDR of *rpo B* gene. Codon 531 (TCG → TTG) is the most common site of mutation in RRDR of *rpo B* gene for rifampicin resistance in MDR-pulmonary tuberculosis

followed by codon 526 (CAC → TAC) and codon 516 (GAC → GTC).

Keywords Multidrug resistance · Tuberculosis · Rifampicin resistance · *rpo B* gene

Introduction

Tuberculosis (TB) is globally still a leading cause of adult mortality arising from a single infectious agent and 21% of the world's TB-infected population is in India [1]. Recently, the enormity of the problem has increased with the emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* and compounded with human immunodeficiency virus (HIV), MDR-TB is a virtual death sentence in this era. Thus the MDR-TB and XDR-TB are serious threats to the global tuberculosis control programme.

The magnitude of the problem is reflected by the fact that an estimated 490,000 new MDR-TB cases and 30,000 new XDR-TB cases are reported every year [2]. According to the 4th Global report, The WHO/IUATLD Global project on Anti-tuberculosis Drug resistance Surveillance (2002–2007), the estimates of MDR-TB among all TB cases in India and Worldwide are 4.9 and 4.8% respectively [3].

MDR-TB as defined by WHO is the resistance to both isoniazid and rifampicin. Rifampicin resistance is of particular epidemiological importance, since it represents a valuable surrogate marker for multidrug resistant (MDR) tuberculosis strains as more than 90% of rifampicin resistant isolates are also isoniazid resistant [4, 5]. Rifampicin interferes with transcription by binding to the beta subunit of DNA-dependent RNA polymerase. The genetic basis of

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rifampicin resistance in approximately 95% of the cases is due to mutations in an 81-bp Rifampicin Resistance Determining Region (RRDR) of the *rpo B* gene, corresponding to codons 507–533 (*Escherichia coli* numbering system), which codes for the beta subunit of the RNA polymerase of *M. tuberculosis*.

Thus, the alarming increase in morbidity and mortality of MDR-TB has highlighted the urgency for rapid molecular diagnostic methods because the conventional diagnostic methods (ZN staining, Culture and drug sensitivity testing) are limiting with respect to the time taken in reaching a definitive diagnosis.

Studies conducted in different geographical areas have reported variable prevalence of the mutations in *rpo B* gene for *M. tuberculosis* strains varies in different countries [6–9]. Thus, it is important to determine the sites of mutations leading to rifampicin resistance and their distribution prior to the introduction of molecular techniques for diagnosis and/or screening of MDR-TB cases.

To have a future screening program for MDR-TB cases, it is imperative to know the site and frequency of mutation in different geographical regions. Our study is one such endeavor for north India. In our study, we evaluated the application of DNA sequencing of RRDR of *rpo B* gene for prediction of rifampicin resistant *M. tuberculosis* in clinical samples.

Materials and Methods

The study was conducted jointly in the Departments of Biochemistry and Microbiology, Lady Hardinge Medical College and Associated Hospitals, New Delhi and Department of Chest clinic, Lok Nayak Hospital, New Delhi, India.

A total of 50 cases of MDR-TB were enrolled in our study after written informed consent. The study was conducted after approval from Institutional ethical committee. Patients of any age group or sex who were diagnosed to be MDR-TB by Culture (LJ medium) and drug sensitivity testing (DST).

All patients were subjected to detailed history and clinical examination. Routine investigations included biochemical and complete hemogram were done.

Early morning sputum samples were collected from patients and stored in sterile plastic vials and immediately recapped. The sputum samples for PCR were stored at -20°C until processed. They were processed in the Mycobacteriology laboratory, Department of Microbiology, Lady Hardinge Medical College, New Delhi. Self protection practices such as use of overalls, double mask, double gloves, footwear and the hood with UV laminar airflow were followed.

Mycobacterial DNA were extracted from sputum sample by using Universal Sample Processing (USP) method [10, 11].

The PCR assay of the processed clinical sputum sample, targeting the *rpo B* gene, was done using a 100 μl reaction mixture. The preparation of the master mixture was carried out in a hood with a laminar airflow taking all necessary precautions to prevent contamination and cross infection.

The primers [6, 12] used for the assay were forward primer RP4T and reverse primer RP8T.

RP4T (5'-GAGGCGATCACACCGCAGACGT-3')

RP8T (5'-GATGTTGGGCCCCCTCAGGGGTT-3')

The master mixture was prepared by using 25.1 μl of nuclease free water, 60 μl (1X) of buffer, 2.4 μl (1.5 mM) of MgCl_2 , 1.0 μl (250 μM) of dNTPs, 0.5 μl (0.25 μM) of both RP4T and RP8T, 0.5 μl (2.5 U) of Taq Polymerase to make a total of 90 μl .

10 μl of sample DNA template was added to 90 μl of the master mixture to make a 100 μl reaction mixture in an eppendorf. The reaction mixtures were put in the thermal cycler (PTC BIORAD) for DNA amplification.

The program of RP4T and RP8T PCR includes initial denaturation ($94^{\circ}\text{C} \times 10'$), cycle denaturation ($94^{\circ}\text{C} \times 1'$), cycle annealing ($65^{\circ}\text{C} \times 1'$), cycle extension ($72^{\circ}\text{C} \times 1'$) for 40 cycles and final extension ($72^{\circ}\text{C} \times 10'$).

A 255 bp fragment of *rpo B* gene was obtained that includes the 81 bp core region (RRDR). The amplicons were electrophoresed on 2% agarose gel containing ethidium bromide and viewed under ultraviolet light in a gel documentation system (Alpha DigiDoc, Alpha Innotech Corporation).

The amplified products were sequenced to locate the exact site of mutation. DNA sequencing was done in automated DNA sequencer. The DNA sequencing [6, 12] PCR was done by using forward primer RP5T and reverse primer RP13T to know the exact nucleotide sequence of RRDR of *rpo B* gene.

RP5T (5'-GGTGGTTCGCCGCGATCAAGGAG-3')

RP13T (5'-CAGCCCGGCACGCTCACGTGAC-3')

The nucleotide sequence data was compared with the reference sequence of the RRDR (of *rpo B* gene) of *M. tuberculosis*.

Results

In our study, we observed three different types of mutation in the RRDR of *rpo B* gene. The frequency of mutation in codon 531 (TCG $\rightarrow$ TTG), 526 (CAC $\rightarrow$ TAC) and 516 (GAC $\rightarrow$ GTC) are 60, 26.6 and 6.6% respectively. Of the total cases studied, 6.6% cases, although resistant to

rifampicin, did not show any mutation in the RRDR of *rpo B* gene.

The reference DNA sequence of RRDR (81 bp or codon 507–533) of *rpo B* gene is as follows [6]

507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533
GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
Gly	Thr	Ser	Gln	Leu	Ser	Gln	Phe	Met	Asp	Gln	Asn	Asn	Pro	Leu	Ser	Gly	Leu	Thr	His	Lys	Arg	Arg	Leu	Ser	Ala	Leu

Discussion

Multi Drug Resistant-Tuberculosis (MDR-TB) is not a clinical diagnosis. It depends on the Culture and Drug Sensitivity Testing (DST) for confirmation which is considered the ‘gold standard’ test for identification of rifampicin resistance which requires 6–8 weeks for a definitive diagnosis.

Rifampicin, the most potent anti-tubercular drug, is bactericidal to the *M. tuberculosis* as it inhibits mycobacterial DNA dependent RNA polymerase by binding with its β subunit which is coded by the *rpo B* gene. Mutations in *rpo B* gene indicate resistance to rifampicin and are associated with resistance to other drugs, most notably isoniazid.

Rifampicin resistance as reported by previous researchers is due to mutation in the Rifampicin Resistance Determining Region (RRDR) of *rpo B* gene. They reported the most frequent mutations in RRDR of *rpo B* gene are in codon 531 followed by codon 526 and codon 516 [13–17]. However, some of the previous researchers [17] also reported codon 526 as the most common site of mutation leading to rifampicin resistance in MDR-TB cases.

In the present study, we have performed DNA sequencing targeting the RRDR of *rpo B* gene in rifampicin resistant samples (DST proven) and observed that codon 531 (TCG → TTG), 526 (CAC → TAC) and 516 (GAC → GTC) of RRDR of *rpo B* gene are the most frequent site of mutation leading to rifampicin resistance. The frequency of mutation

observed in our study in codon 531, 526 and 516 are 60, 26.6 and 6.6% respectively (Table 1).

Bostanabad et al. [9] in 2008 demonstrated that mutations in the ‘hot spot’ region (RRDR) of *rpo B* gene leading to

rifampicin resistance with predominant nucleotide changes reported to be in codon 510, 523, 526 and 531. Study by Deepa et al. [18] in 2005 studied on five clinical isolates of rifampicin resistance reported presence of mutations in three cases of which two were at codon 531 (TCG → TTG). Meera et al. [17] in 2003 studied strains of rifampicin resistant *M. tuberculosis* by Line Probe Assay (LiPA). They reported that the codon 526 (CAC → TAC) to be the most frequent site of mutation in RRDR of *rpo B* gene. Mani et al. [5] in 2001 from Tuberculosis Research Centre (TRC), Chennai reported the presence of mutation in codon 531 (53%) and codon 526 (19%) in the RRDR of *rpo B* gene. Andreia et al. [19] in 2000 from Brazil reported that the codons most frequently affected were 531 (TCG → TTG), 526 (CAC → TAC) and 516 (GAC → GTC) with frequencies of 54, 21 and 7% respectively. Garcia et al. [6] in 2001 from Spain studied rifampicin resistant *M. tuberculosis* clinical isolates by PCR-ELISA method and reported mutations in codon 531 (48%), codon 526 (22%) of RRDR of *rpo B* gene.

In the present study, we found 6.6% of rifampicin resistant strains (DST proven) revealing no mutations in the RRDR of *rpo B* gene. This finding is in agreement with other reports indicating mutations outside the 81-bp segment (RRDR) of *rpo B* or additional molecular mechanisms that may be involved in rifampicin resistance of *M. tuberculosis*. Mechanisms such as a permeability barrier or membrane proteins acting as drug efflux pumps may also confer resistance to rifampicin [7, 8, 20]. In our study, we did not attempt to characterize any gene other than RRDR of *rpo B* and a second formal possibility to account for the lack of detected *rpo B* mutations in these rifampicin-resistant cases is that changes might have occurred in one or more genes whose products participate in antibiotic permeability or metabolism [21].

Bostanabad et al. [9] in 2008 reported that 5 rifampicin resistant isolates do not show any mutations in RRDR of *rpo B* gene. Mani et al. [5] in 2001 from Tuberculosis Research Centre (TRC), Chennai reported that 13.6% of mutations occurs outside RRDR of *rpo B* gene.

Table 1 Mutations in RRDR of *rpo B* gene

Mutation in codon ^a	Change in nucleotide sequence	Change in amino acid sequence	Frequency (%)
531	TCG → TTG	Ser → Leu	60
526	CAC → TAC	His → Tyr	26.6
516	GAC → GTC	Asp → Val	6.6
No mutation detected	–	–	6.6

^a Codon numbers correspond to the *Escherichia coli* numbering system for the RNA polymerase β subunit

Bobadilla-del-Valle et al. [7]. 2001 studied rifampicin resistant *M. tuberculosis* isolates and reported that 2% of rifampicin resistant *M. tuberculosis* isolates reveals mutation outside the RRDR of *rpo B* gene. Hirano et al. [8] 1999 reported that 5.6% of rifampicin resistant *M. tuberculosis* isolates reveals mutation outside the RRDR of *rpo B* gene.

The most important advantage of our DNA sequencing method is the time between sampling and availability of the test results for clinical decision making. In general, conventional method of culture and rifampicin susceptibility testing will take 6–8 weeks for a definitive diagnosis. Accordingly, clinical decisions based on the results of the test cannot be made before 45–60 days.

In comparison, the results of rifampicin susceptibility of *M. tuberculosis* based on genetic alteration in the RRDR of *rpo B* gene are available within 3–5 days. This period is based on 1–2 days for DNA extraction from clinical sample followed by PCR and 2–3 days for amplification and sequencing of RRDR of *rpo B* gene.

To conclude, early detection of MDR-pulmonary TB cases is of immense clinical importance. DNA sequencing is a rapid, conclusive and more advantageous over conventional drug susceptibility testing (DST). It can determine the exact site and frequency of mutation in RRDR of *rpo B* gene leading to rifampicin resistance in different geographical areas. This DNA sequencing can serve two fold function. First it can provide an accurate and rapid prediction of rifampicin resistant *M. tuberculosis* to be clinically useful and secondly it can serve as useful data in developing a screening protocol for detection of MDR-TB in future.

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References

1. TB India 2008. RNTCP Status report 2008. p. 13.
2. TB/HIV facts 2008. WHO report 2008. pp. 1–2.
3. Anti-tuberculosis drug resistance in the World, 4th global report, The WHO/IUATLD Global Project on Anti-Tuberculosis drug resistance surveillance (2002–2007). Annexure X. pp. 1–4.
4. Drobniewski FA, Wilson SM. The rapid diagnosis of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*—a molecular story. *J Med Microbiol*. 1998;47:189–96.
5. Mani C, Selvakumar N, Narayanan S, Narayanan PR. Mutations in the *rpo B* gene of multidrug resistant *Mycobacterium tuberculosis* clinical isolates from India. *J Clin Microbiol*. 2001;10:2987–90.
6. Garcia L, Alonso-Sanz M, Rebollo MJ, Tercero JC, Chaves F. Mutations in the *rpo B* gene of rifampin-resistant *Mycobacterium tuberculosis* isolates in Spain and their rapid detection by PCR-enzyme-linked immunosorbent assay. *J Clin Microbiol*. 2001;39:1813–8.
7. Bobadilla-del-Valle M, Leon AP, Huertero CA, Alarcon GV, Maeda MK, Small PM, et al. *rpo B* gene mutations in rifampicin resistant *Mycobacterium tuberculosis* identified by polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP). *Emerg Infect Dis*. 2001;7:1010–3.
8. Hirano K, Abe C, Takahashi M. Mutations in the *rpoB* gene of rifampicin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. *J Clin Microbiol*. 1999;37:2663–6.
9. Bostanabad SZ, Noghanian M, Graviss AE, Reza A. Multiple mutations in the *rpo B* gene of *Mycobacterium tuberculosis* isolates correlate with high levels of resistance to rifampicin in patients with active pulmonary tuberculosis in Afghanistan border of Iran. *Afr J Microbiol Res*. 2008;2(4):95–102.
10. Chakravorty S, Tyagi JS. Novel multipurpose methodology for detection of mycobacteria in pulmonary and extrapulmonary specimens by smear microscopy, culture, and PCR. *J Clin Microbiol*. 2005;43:2697–702.
11. Chakravorty S, Dubeja M, Hanif M, Tyagi JS. Utility of universal sample processing methodology, combining smear microscopy, culture, and PCR, for diagnosis of pulmonary tuberculosis. *J Clin Microbiol*. 2005;43:2703–8.
12. Ohno H, Koga H, Kuroita T, Tomono K, Ogawa K, Yanagihara K, et al. Rapid prediction of rifampin susceptibility of *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med*. 1997;155:2057–63.
13. Herrera L, Jimenez S, Valverde A, Garci Aranda MA, Saez-Nieto JA. Molecular analysis of rifampicin-resistant *Mycobacterium tuberculosis* isolated in Spain (1996–2001). Description of new mutations in the *rpo B* gene and review of the literature. *Int J Antimicrob Agents*. 2001;21:403–8.
14. Fan XY, Hu ZY, Xu FH, Yan ZQ, Guo SQ, Li ZM. Rapid detection of *rpo B* gene mutation in rifampicin resistant *Mycobacterium tuberculosis* isolates in Shanghai by using the amplification refractory mutation system. *J Clin Microbiol*. 2003;41:993–7.
15. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*. *Tuber Lung Dis Update* 1998;79:3–29.
16. El-Haji HH, Salvatore AE, Tyagi S, Russel F, Alland D. Detection of rifampicin resistance in *Mycobacterium tuberculosis* in a single tube with molecular beacons. *J Clin Microbiol*. 2001;39:4131–7.
17. Sharma M, Sethi S, Mishra B, Sengupta C, Sharma SK. Rapid detection of mutations in *rpo B* gene of rifampicin resistant *Mycobacterium tuberculosis* strains by Line Probe assay. *Ind J Med Res*. 2003;117:76–80.
18. Deepa P, Therese KL, Madhavan HN. Detection and characterization of mutations in rifampicin resistant *Mycobacterium tuberculosis* clinical isolates by DNA sequencing. *Ind J Tuberc*. 2005;52(3):132–6.
19. Andreia RM, Lucia M, Rossetti R, Ribeiro MO, Zaha A. Mutations in *rpo B* gene of multi drug resistant *Mycobacterium tuberculosis* isolates from Brazil. *J Clin Microbiol*. 2000;38:3119–22.
20. Ohno H, Koga H, Kohno S, Tashiro T, Hara K. Relationship between rifampin MICs for and *rpoB* mutations of *Mycobacterium tuberculosis* strains isolated in Japan. *Antimicrob Agents Chemother*. 1996;40:1053–6.
21. Kapur V, Li LL, Iordanescu S, Hamrick MR, Wanger A. Characterization by automated DNA sequencing of mutations in the gene (*rpo B*) encoding the RNA polymerase β subunit in rifampicin-resistant *Mycobacterium tuberculosis* strains from New York city and Texas. *J Clin Microbiol*. 1994;4:1095–8.