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ORIGINAL ARTICLE

Emerging Trend of Mutation Profile of *rpoB* Gene in MDR Tuberculosis, North India

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Abstract The present study was conducted on North Indian population to observe rpoB gene mutation profile in multidrug resistant *Mycobacterium tuberculosis*. This was an observational study. 30 cases of MDR-TB proven by culture and drug sensitivity were selected. DNA sequencing of 81 bp (codon 507–533) long RRDR of *Mycobacterium tuberculosis* was done to detect the sites of mutation. Out of 30 cases, 24 showed a single mutation in the RRDR region of rpoB gene in which 16 (53.33 %) showed mutation in codon 531(TCG→TTG), 5 cases (16.66 %) showed mutation in codon 526(CAC→TAC), mutation in codon 516(GAC→GTC, AAC) was present in 3 cases (10 %). It was also observed that mutation in more than one codon was present in 4 cases (13.33 %), which included deletion at codon 509(AGC→–GC), mutation at 513(CAA→CTA), 516, 526, 529(CGA→CTA) and 531. No mutation was detected in RRDR in 2 cases (6.66 %). Our finding of 13.33 % cases with multiple sites of mutation in RRDR region is in contrast to earlier studies done in North India which showed single mutation detected in RRDR of rpoB gene that highlights the emerging change in the trend of mutation profile of rpoB gene in rifampicin resistant *Mycobacterium tuberculosis*.

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Introduction

Tuberculosis (TB) is a major cause of death despite being a curable infectious disease. One-third of the world's population is infected with TB bacilli, i.e. have latent TB which is clinically expressed in the later life in about 10 % of them [1]. This global TB epidemic like situation is further aggravated by co-infection with HIV and emergence of drug-resistant tuberculosis [2]. A particularly dangerous form of drug-resistant TB is multidrug-resistant TB (MDR-TB), which is defined by WHO as disease caused by TB bacilli which is resistant to isoniazid and rifampicin [3]. Global incidence of tuberculosis in 2010 was 6.1 million of which India account for 1.5 million (25 %). Globally of the TB patients notified in 2010, an estimated 290,000 had multidrug resistant TB (MDR-TB), but only 53,000 (18 %) were detected as MDR-TB. In India estimated cases of MDR TB was 64,000 (44,000–84,000) out of it only 2,967 were diagnosed and treated [4]. So there is a huge gap between estimated and diagnosed case of MDR-TB. The emergence of resistance to drugs used to treat tuberculosis (TB), and particularly multidrug-resistant TB (MDR-TB), has become a significant public health problem in a number of countries and an obstacle to effective TB control [4].

Diagnosis of MDR-TB cannot be made clinically; it is purely based on laboratory testing. At present culture and drug sensitivity testing (CDST) is the gold standard test for diagnosis of MDR-TB. Culture can take up to 6 weeks before a definitive result can be obtained and another 2–4 weeks are required for drug susceptibility testing. So there is a need for rapid tests which can provide results within days and thus

enable prompt and appropriate treatment, decrease morbidity and mortality, and interrupt transmission.

Rifampicin is the most potent anti tubercular drug and is used in all the regimes of anti tubercular treatment (ATT). Rifampicin inhibits Mycobacterial DNA dependent RNA polymerase by binding with its β subunit. The β subunit of RNA polymerase is coded by the *rpoB* gene. Mutations in *rpoB* gene indicate resistance to rifampicin. More than 95 % of all mutations are located in a 81 bp (1,294–1,375 bp) core region (Rifampicin Resistance Determining Region, RRDR) of the *rpoB* gene between codons 507–533 [5–7]. Since majority of Rifampicin resistant strains showed an association with resistance to Isoniazid (INH) as well, determination of Rifampicin resistance serves as a surrogate marker for multidrug resistance TB [8].

PCR amplification followed by DNA sequencing is the confirmatory technique to characterize mutations in the *rpoB* gene. The commonly reported mutation using DNA sequencing are at codon 531(T**C**G→T**T**G), 526(**C**AC→**T**AC) and 516(GA**C**→G**T**C) [8]. Studies conducted in different countries have reported variable prevalence of the mutations in *rpoB* gene for *M. tuberculosis* strains [7, 9–11]. Thus, determination of the sites of mutations leading to rifampicin resistance and their distribution according to geographical areas is important prior to the introduction of molecular techniques for diagnosis and/or screening of MDR-TB cases.

Various screening methods for detection of MDR-TB have been developed. Data from systematic reviews and meta-analyses to evaluate assay performance results against conventional DST methods have shown that line probe assays (LiPA) are highly sensitive and specific for the detection of rifampicin resistance, alone or in combination with isoniazid, on isolates of *M. tuberculosis* and on smear positive sputum specimens [12, 13]. Xpert MTB/RIF, a new rapid molecular test that has the potential to substantially improve and accelerate the diagnosis of TB and drug resistant TB, are presented. WHO has endorsed Xpert MTB/RIF in December 2010 [4]. Line Probe Assay and Xpert MTB/RIF are based on detection of mutation in RRDR region of rpoB gene for detection of rifampicin resistance [12–14].

Our primary aim was to study the mutation profile of *rpoB* gene in diagnosed MDR-TB patients by DNA sequencing. This will help in development of a screening protocol specific for that particular geographical area. Our study is one such endeavour for north India.

Materials and Methods

Study Subjects

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 after getting approval from the Institutional Ethical Committee. A total of 30 cases of MDR-TB were enrolled in our study after written informed consent. Cases included patients of MDR-TB of any age group or sex who were diagnosed by culture (LJ medium) and drug sensitivity testing (DST).

Study Design

This study was an observational type of study. Objective of the study was to detect the sites of mutation on *rpoB* gene of multidrug resistant *Mycobacterium tuberculosis.*

Sputum samples were collected and processed by Universal Sample Processing (USP) method [15, 16] to extract mycobacterial DNA. The extracted DNA was subjected to PCR using *rpoB* gene as target. Amplified product of PCR was then sequenced to detect the sites of mutation on *rpoB* gene of *Mycobacterium tuberculosis*.

Methods of Measurement

All patients were subjected to detailed history and clinical examination. Routine investigations including biochemical parameters and complete hemogram were done. For molecular study early morning sputum samples were collected from patients and stored in sterile plastic vials and immediately recapped. They were stored at −20 °C until analysed.

Mycobacterial DNA was extracted from sputum sample by using USP method [15, 16] in the Mycobacteriology laboratory of Microbiology department. Self protection practices such as use of N95 mask, double gloves, footwear and the hood with UV laminar airflow were followed.

The PCR assay of extracted DNA sample, targeting the *rpoB* gene, was done using a 100 µl reaction mixture. The preparation of the master mixture was done taking all necessary precautions to prevent contamination and cross infection. The primers [9, 17] used for the assay were forward primer RP4T and reverse primer RP8T.

RP4T-(5′-GAGGCGATCACACCGCAGACGT-3′)
RP8T-(5′-GATGTTGGGCCCCTCAGGGGTT-3′)

The 90 µl of master mixture was prepared by using 25.1 µl of nuclease free H_2O, 60 µl (1×) 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* DNA Polymerase. 10 µl of sample DNA template was added to 90 µl of the master mixture to make a 100 µl reaction mixture. The reaction mixtures were put in the thermal cycler (Peltier Thermal Cycler, PTC-100, BIORAD) for DNA amplification. The program of RP4T and RP8T PCR included initial denaturation (94 °C for 10 min), cycle

denaturation (94 °C for 1 min), cycle annealing (65 °C for 1 min), cycle extension (72 °C for 1 min) for 40 cycles and final extension (72 °C for 10 min).

A 255 bp fragment of *rpoB* gene was obtained which included 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 outsourced (Base Clear Group, Nederland), was done in automated DNA sequencer using fluorescent dye terminator method using forward primer RP5T and reverse primer RP13T to know the exact nucleotide sequence of RRDR of *rpoB* gene.

RP5T-(5′-GGTGGTCGCCGCGATCAAGGAG-3′)
RP13T-(5′-CAGCCCGGCACGCTCACGTGAC-3′)

The nucleotide sequence data was compared with the reference sequence of the RRDR (of *rpoB* gene) of *M. tuberculosis* to detect the site of mutation.

Percentage for mutation pattern and frequency of mutation of particular codon was calculated. Sensitivity of method (DNA sequencing) taking culture and drug sensitivity as gold standard for diagnosis of MDR-TB was calculated by SPSS.

Results

Demographic and Clinical Profile

In our study the maximum numbers of patients (70 %) were in the age group of 20–40 years and it was more common in male (56.66 %) than female (43.33 %). Most of the patients presented with complain of cough with sputum, weight loss, fever and haemoptysis. Most of the patients had decreased haemoglobin and total leukocyte count, Lymphocytes and ESR were found to be elevated.

Molecular Study

Results of DNA sequencing was compared with reference DNA sequence of RRDR of *rpoB* gene of *M. tuberculosis*. The reference DNA sequence of RRDR (81 bp or codon 507–533) of *rpoB* gene is shown in Fig. 1 [9].

Table 1 Site of mutation in RRDR region of *rpoB gene* by DNA sequencing ($n = 30$)

Mutation	Site of mutation (codon)	No. ($n = 30$)	Percentage
Single	531	16	53.33
	526	5	16.66
	516	3	10
Multiple	531, 526	1	13.33
	531, deletion 509	1	
	531, 529, 516	1	
	531, 513, deletion 509	1	
No mutation or mutation outside RRDR region		2	6.66

Sensitivity of DNA sequencing of RRDR for detection of rifampicin resistance was 93.33 % taking CDST as gold standard.

Sites and frequency of mutation in RRDR region of *rpoB* gene is detailed in Tables 1 and 2.

In our study, we observed mutation in codon 531, 526, 516, 529, 513 and deletion at 509. No mutation in RRDR region of *rpoB* gene was found in 2 samples though it was rifampicin resistance. 4 samples (13.33 %) showed multiple sites of mutation in its RRDR region.

Discussion

According to Revised National Tuberculosis Control Programme (RNTCP), TB is diagnosed based on sputum microscopy and chest X-ray. For the treatment, patients are divided into two categories base on site and extent of disease, Category-I (new cases of pulmonary and extra pulmonary tuberculosis), Category-II (sputum smear-positive relapse, failure and default). First time diagnosed patients; treatment is given according to category I. Patients who come after treatment failure, relapse or default of category I are given treatment according to category II. Patients who fail to respond even to category II are suspected to have MDR-TB. So MDR-TB is not a

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

Fig. 1 RRDR region of *rpoB* gene

Table 2 Frequency of site of mutation by DNA sequencing

Mutations	Normal sequence	Mutated sequence	Frequency of mutation (out of 100)
531	TCG	T**T**G	66.7
526	CAC	**T**AC	20
516	GAC	G**T**C	10
		AAC	3.3
529	CGA	C**T**A	3.3
513	CAA	C**T**A	3.3
Deletion 509	AGC	–GC	6.7
No Mutation or mutation outside RRDR region			6.7

clinical diagnosis. At present the common protocol for diagnosis of MDR-TB has been subjecting the culture isolates to antibiotic sensitivity test which may take 3–8 weeks time depending on the direct or indirect diagnostic approach. Thus, these techniques have a major limitation with respect to time taken in arriving at a diagnosis which is a huge drawback in MDR-TB patients as it has a very rapid course and may be fatal [18, 19]. Advantage of molecular tests is their rapid turnaround time, which may have implications for patient management and transmission of drug-resistant TB. DNA sequencing of *rpoB* gene in MDR tuberculosis also has added advantage of having knowledge of mutation profile of *rpoB* gene, which may help in development of a screening protocol relevant to geographical area, for early detection of MDR tuberculosis.

In our study we found the most common sites of mutation were at codon 531 (TCG→TTG), 526 (CAC→TAC) and 516 (GAC→GTC, AAC) with frequency of mutation at these sites were 66.7, 20 and 13.3 % respectively. Uncommon sites of mutation were in codon 529 (CGA→CTA), 513 (CAA→CTA) and deletion at 509 (AGC→–GC). We found 6.7 % samples with no mutation with in RRDR region of *rpoB* gene. Our finding of mutations at various sites of *rpoB* gene is supported by previous studies reported the most frequent mutations in RRDR of *rpoB* gene were in codon 531 followed by codon 526 and codon 516 [20]. Study in Brazil by Andreia et al. [21], reported that the codons most frequently affected were 531(T**C**G→T**T**G), 526(**C**AC→**T**AC) and 516(G**A**C→G**T**C) with frequencies of 54, 21 and 7 % respectively. Remaining 18 % samples did not show any mutation in RRDR region. Deepa et al. [8], observed two mutation at codons 531 and one at 526. Rest two samples did not show any mutation RRDR region. Study on 50 samples of MDR patients from North India found mutation at codon 531 (60 %), 526 (26.6 %) and 516 (6.6 %) and no mutation (6.6 %) in RRDR region of *rpoB* gene [22].

Finding of no mutations in the RRDR of *rpoB* gene indicating mutations outside the 81-bp segment (RRDR) of *rpoB* or additional molecular mechanisms that may be involved in rifampicin resistance of *M. tuberculosis* like permeability barrier or membrane proteins acting as drug efflux pumps[6, 7, 10].

Interesting finding was out of 4 mutations in codon 516, 3 were observed at 2nd nucleotide (G**A**C→G**T**C) and in 1 sample it was at 1st nucleotide (**G**AC→**A**AC) of that codon. Supportive finding was observed in study done by Mercy et al. [23], showed mutation at codons 511, 513, 516, 518, 519, 526, 528, 529 and 531. In this study the mutated sequence was different for same codon. Codon 531 (TCG) showed mutated sequence TTG, TTC and TGG in 12, 1 and 1 sample respectively. Codon 526 (CAC) showed mutated sequence AAC, TAC and CTC in 2, 2 and 1 sample respectively. Codon 516 (GAC) showed mutated sequence TAC, GTC and GCA in 2, 1 and 1 sample respectively.

It was also observed that 4 samples (13.33 %) had multiple mutations in RRDR region of *rpoB* gene. One sample showed mutation at codons 531 and 526. The other one at codons 531 and deletion at 509. Still another at codons 531, 529 and 516. Last one at codon 531, 513 and deletion at 509. Study in Hyderabad by Mercy et al. [23], showed 2 out of 30 samples with multiple mutation. In one sample, multiple mutations were observed at codons 511, 516 and 528 and in the second at codon 513, 519 and 531. Study in China by Fan et al. [24] reported common site of mutation at codon 531 (53.8 %), 526 (23.1 %) and 516 (15.4 %) and also observed uncommon sites along with multiple mutations in three samples out of 39 samples studied.

Study in same geographical area that is in North India in 2010 by Patra et al. [22] on 50 samples observed no samples with multiple mutation. In contrast, our study carried out at the same place, however showed multiple and uncommon sites of mutations as already described along with the common sites detected by previous study. This is may be due to changing mutation pattern in RRDR of *rpoB* gene of *Mycobacterium tuberculosis*. Henceforth findings of our study highlighted the emerging change in the trend of mutation profile of *rpoB* gene in rifampicin resistant *Mycobacterium tuberculosis*.

Due to low detection rate for MDR-TB its urgent need for development of rapid method for detection of resistance because only way of preventing spread of MDR-TB is to detect and treat as early as possible. Though detection of mutation by DNA sequencing is gold standard, it cannot be used as screening method due to cost and technical complexity of method. WHO recommended Line Probe Assay

(LiPA) and Xpert MTB/RIF as rapid screening method for detection of MDR-TB by detecting rifampicin resistance, both depends on detection of presence of mutation in RRDR region of *rpoB* gene [14, 25]. Designing of probes in both methods depend on knowledge of mutation profile as it is differ in different geographical area, as binding of probe to the mutated sequence may altered as changing pattern of mutation by uncommon sites and multiple mutation.

Conclusion

Knowledge of mutation profile from different geographical area has implication in diagnosis of MDR-TB by developing molecular screening methods. Changing mutation pattern may alter sensitivity and specificity of screening methods which are based on detection of mutation. However large scale studies with adequate sample size from different geographical areas are needed before come to conclusion of changing pattern of mutation profile.

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