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Detection of Mutations in rpob Gene of Clinically Isolated *M. tuberculosis* by DNA Sequencing

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Abstract

Aim: A population-based study was performed to detect the mutations in 81-bp rifampicin resistance determining region (RRDR) of rpoB gene in clinically isolated *M. tuberculosis* strains.

Methods: Polymerase chain reaction (PCR) mediated direct DNA sequencing was used for rapid detection of rifampicin resistance of *M. tuberculosis*.

Results: Among the 150 isolates, 115 were rifampicin sensitive and exhibited a wild-type pattern on PCR mediated direct sequencing, and remaining 35 isolates were found to be resistant. The codons most frequently involved in mutation were codon 531 (40%), 526 (23%), and 516(15%).Total twenty four kinds of mutation, which 18 point mutation, 4 insertion & 2 deletion were observed in 81-bp RRDR region of rpoB gene.

Conclusion: The sequencing analysis for genotypic evaluation of rifampicin resistance is a highly sensitive assay and provides a practical alternative to in vitro testing in *M. tuberculosis*. Information on the profile of rpoB mutations in *M. tuberculosis* provides an improved diagnosis of rifampicin resistance by increasing the efficacy of gene sequencing based test.

Keywords: *M. tuberculosis*; rpoB mutations; DNA Sequencing

Introduction

The diagnosis and control of tuberculosis (TB) is very significant problem in global health. A recent report by the world health organization (WHO) estimated that India is the highest TB-burden country in the world in terms of absolute numbers of incident cases that emerge each year and it contributed one fourth of the estimated global incident TB cases in 2010. In July 2011, national consultations to estimate the TB burden took place. Revised estimated prevalence and incidence rates of all forms of tuberculosis were respectively 256 and 185 per 100,000 populations in 2010 [1]. The emergence and spread of MDR-TB, is an increasing public problem in India with an estimated number of 110,000 cases spread across the country [2].

Multidrug-resistant tuberculosis (MDR-TB), defined as TB that is resistant to at least isoniazid (INH) and rifampicin (RIF). Treatment of MDR isolates requires the use of more costly and more toxic second-line drugs [3]. Inappropriate treatment can result in the development of resistance to additional antibiotics [4,5] and increase mortality [6]. The detection of rifampicin resistance serves as surrogate marker for detecting MDR-TB [7]. Yang *et al.* [8] showed the contribution of different mutations (insertion, deletion and missense) within a hypervariable region of rpoB gene, the gene encoding the β-subunit of RNA polymerase of *M. tuberculosis* [8]. It has been observed that 95% of rifampcin–resistant strains of *M. tuberculosis* have a mutation within an 81 bp region of rpoB gene, this region is called rifampcin-resistance-determining region (RRDR) corresponding to codons 507-533. [9-11].

Early diagnosis of MDR-TB is most effective means of TB control. Culture-based identification remains the 'gold standard' method for TB diagnosis [12]. However, culture-based identification and conventional drug susceptibility testing of *M. tuberculosis* complex, can take up to 6 weeks due to the low growth rate of the organism. A delay in diagnosis of MDR-TB associated with the conventional methods is likely to contribute to the transmission of resistant isolates. Molecular bases of drug resistance have been identified for all the main antitubercular drugs, and drug resistance can be detected by changes in the several target genes, some of which are still undefined. The mutations can be detected indirectly or directly, by different molecular techniques, such as polymerase chain reaction (PCR)-DNA sequencing, PCR-single strand conformation polymorphism (SSCP) [13,14] PCR-reversed line blot hybridization (PLH) [15], PCR-heteroduplex [16], and line prob assay (LIPA) [17,18].

In this study, mutations in the rpoB genes of *M. tuberculosis* were analyzed by automated DNA sequencing. *M. tuberculosis* isolates were obtained from Sputum samples collected from the patients reporting with pulmonary tuberculosis over a 2 year period from 2010-2012.

Material and methods

Sample collection

Sputum samples were collected from pulmonary tuberculosis patients at the TB-Chest department of Subharti Medical College, Meerut (U.P. INDIA). The samples were processed by standard method; Firstly the sputum was subjected to routine Ziehl-Neelsen staining and the rest of the sputum was digested and decontaminated by N-Acetyl-L-Cysteine-2% NaOH method (NALC-2% NaOH) and

concentrated by the centrifugation at 3000 g for 20 min, from the pellet two Lowenstein-Jensen medium slants were inoculated and incubated at 37˚C for 6-8 weeks. The inoculated LJ media were examined every second day during the first week and then weekly for up to 8 weeks for the presence of growth.

Antibiotic susceptibility testing

DST was carried out by using BACTEC MGIT 960 system (Becton Dickinson Diagnostic Systems). Inocula were prepared following the manufacturer's instructions. All cultures were inoculated/sub-cultured onto Löwenstein-Jensen medium and were used for antimicrobial susceptibility testing no later than 14 days after the first appearance of colonies on the slant. All colonies were transferred into a sterile tube containing 4 ml of Middlebrook 7H9 Broth with 8 to 10 sterile glass beads. The suspension was vortexed for 3 min and left standing undisturbed for 20 min. The supernatant was transferred into a sterile tube and left standing undisturbed for 15 min. Finally, the supernatant was transferred into a third sterile tube, and the turbidity was adjusted to 0.5 McFarland standard with sterile saline. One ml of this adjusted suspension was diluted in 4 ml of sterile saline (1:5 dilution). Lyophilized drugs (BACTEC MGIT 960 SIRE kit) were dissolved in diluent according to the manufacturer's instructions. From the dissolved drug solutions, 100 µl was pipetted into a 7 ml MGIT 960 tube. The subsequent dilutions for all drugs were made in DI water, and aliquots of the stock solution were stored at −20°C for 6 months. The final drug concentrations used were 1.0 µg/ml for STR; 0.1 µg/ml for INH; 5.0 µg/ml for EMB; 1.0 µg/ml for RIF. A 1:5 dilution of the organism suspension in sterile saline was used for inoculating 0.5 ml volumes in to MGIT streptomycin (STR), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB) (SIRE) sets. All inoculated sets were loaded into the BACTEC MGIT 960 instrument within 2 h of inoculation, and readings were automatically interpreted by the BACTEC MGIT 960 instrument and reported as either susceptible or resistant.

PCR

PCR were performed by commercial kit method from Bangalore Genei, Banglore (India). This test is based on the principle of single–tube nested PCR method, which is very sensitive diagnostic tool for the identification of *M. tuberculosis*. This assay is two–step sequential assay. In the first step, the IS region of MTB complex DNA sequence, a 220 bp is amplified by specific external primers. In the second step, the nested primers are added to further amplify a 123 bp amplification product. In sputum sample one aliquot of the sediment obtained after NALC-2% NaOH decontamination was kept frozen at -20°C was used as a source of DNA. DNA extraction is done by commercial kit method from Bangalore Genei, Bangalore (India). The extracted DNA was amplified by two step PCR assay (Bangalore Genei, Bangalore). In the first step, 220 bp DNA segment was amplified by external primer. The amplification was carried out in 12 µl final volume, in which 8.22 µl amplification premix (reaction buffer with MgCl2, dNTPs and Mycobacterium tuberculosis complex specific external primer) 0.33 µl Taq DNA polymerase, 0.5 µl Uracil DNA glycosylase and 3 µl DNA template. The amplification was carried out in a thermocycler (Applied Biosystem). After initial denaturation at 94°C for 5 minute, 35 amplification cycles were performed within thermocycler. Each cycle consisted of denaturation at 94°C for 2 min, annealing of primer at 68°C for 2 min and primer extension at 72°C for 1.30 min. After complete amplification of 220 bp of DNA segment, the amplified product was used as DNA template for amplification of 123 bp segments, 15 µl of master mix (buffer with MgCl2, dNTPs and Mycobacterium tuberculosis specific internal primer) and first PCR product was added in PCR tube, and amplification was carried out in a thermocycler. After initial denaturation at 94°C for 5 minute, 35 amplification cycles were performed within thermocycler. Each cycle consisted of denaturation at 94°C for 2 min, annealing of primer at 68°C for 2 min and primer extension at 72°C for 1.30 min. The second PCR products were analyzed by using 2% agarose gel at 120 volts and the resultant bands were interpreted by UV transillumination. A product of 123 bp was indicative of infection with *M. tuberculosis* and an amplified product of 340 bp was used as an internal control.

PCR amplification of rpoB gene

The isolated DNA was amplified by using rpoB (Gene accession no. L27989) primer, rpoB forward (5´-GGG AGC GGA TGA CCA CCC-3´) and reverse (5´-GCG GTA CGG CGT TTC GAT GAA C-3´) Siddiqi *et al.* [19], in a thermocycler (Applied Biosystem). PCR reactions were carried out in 50 µl total volumes, containing 5 µl of 10X PCR reaction buffer, 5 µl of 10 mM dNTP mix, 2.5U of Taq polymerase, 1 mM of $Mgcl_2$, 2 µl of each primer (10 pmol/mol) and 5 µl of genomic DNA. The reaction mixture were then subjected to initial denaturation, 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and final extension of 72°C for 10 min. PCR products were analysed by electrophoresis through 1.5% agarose gel and ethidium bromide staning, 350 bp band of amplified DNA on agarose gel were excised. DNA was extracted from gel slices with a QIA quick gel extraction kit (Qiagen) according to the manufacturer's instruction. The purified DNA was resuspended in sterile double-distilled water and was used for the sequencing studies.

DNA sequencing

Sequencing of the purified product was carried out by automated DNA sequencer (ABI 3130xl, Applied Biosystem) with Big Dye terminator v 3.1 sequencing kit, using the same primers as used for amplification. The sequence data were analysed by using the sequencing analysis software, version-5.2. The nucleotide sequence obtained was analysed using Basic Local Alignment Search Tool (BLASTn) Bioinformatics tool available at National Center for Biotechnology Information (NCBI) [20] 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 change in comparison with wild type *M. tuberculosis* (H37Rv).

Results

A total of 150 clinical isolates of *M. tuberculosis* were obtained from culture positive pulmonary TB patients. All the 150 isolates were tested with the BACTEC MGIT 960 SIRE susceptibility test, 115 isolates were sensitive to MGIT streptomycin (STR), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB) (SIRE) sets and remaining 35 isolates were found as MDR. Drug susceptibility profiles of these MDR isolates are shown in Table 1.

No. of Strains	Susceptibility to indicated drug			
	RIF	INH	STR	EMB
28	R[b]	R	R	R
4	R	R	S[c]	S

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2	R	R	R	S
1	R	R	S	R

Table 1: Drug susceptibility patterns of MDR *M. tuberculosis* strains; aRIF-rifampcin; INH-isoniazid; EMB-ethambutol; STR-streptomycin, bR-resistant, cS-sensitive

As a result of DNA sequencing of 35 rifampicin resistant strains, rpoB positive PCR products showed that all 35 isolates displayed mutation in the rpoB gene. Our analysis detected a total 24 mutated positions distributed among 10 codons within RRDR of the rpoB gene (Table 2). The codons most frequently involved in mutation were codon 531 (40%), 526 (23%) and 516 (15%). Mutation at codon 531 of TCG (Ser-Leu) occurred in 11 isolates and changes of TCG (Ser-Trp) occurred only in 8 isolates (figure 1). Other resistance associated mutation in rpoB gene occurred at codon 512 (one isolate), 514 (one isolate), 517 (Three isolates), 518 (Three isolates), 519 (one isolate), 520 (one isolate), 524 (one isolate). Twenty four kinds of mutations were observed in 81-bp RRDR region of rpoB gene, of which 18 were point mutation, 4 insertion and 2 deletion.

Codon	Nucleotide change	Amino acid change	Mutation type
512	AGC-TGC	Ser-Cys	Substitution
514	TTC-TTT	Phe-Phe	Silent
516	GAC-GTC	Asp-Val	Substitution
	GAC-GGAC	Asp-	Insertion
	GAC-TAC	Asp-Tyr	Substitution
	GAC-GAG	Asp-Glu	Substitution
517	CAG-DEL	Gln-	Deletion
	CAG-CAA	Gln-Gln	Silent
	CAG-CCAG	Gln-	Insertion
518	AAC-AA_	Asn-	Deletion
	AAC-AAAC	Asn-	Insertion
	AAC-GAC	Asn-Asp	substitution
519	AAC-ACC	Asn-Thr	Substitution
520	CCG-CCCG	Pro-	Insertion
524	TTG-ATG	Leu-met	Substitution
526	CAC-TGC	His-Cys	substitution
	CAC-TAC	His-Tyr	substitution
	CAC-CTT	His-Leu	substitution
	CAC-CTC	His-Leu	substitution
	CAC-GAC	His-Asp	substitution
	CAC-CGC	His-Arg	substitution
	CAC-ACC	His-Thr	substitution
531	TCG-TTG	Ser-Leu	substitution
	TCG-TGG	Ser-Trp	substitution

Table 2: Pattern of mutations in RRDR region of rpoB gene in 35 RIFr *M. tuberculosis* strains

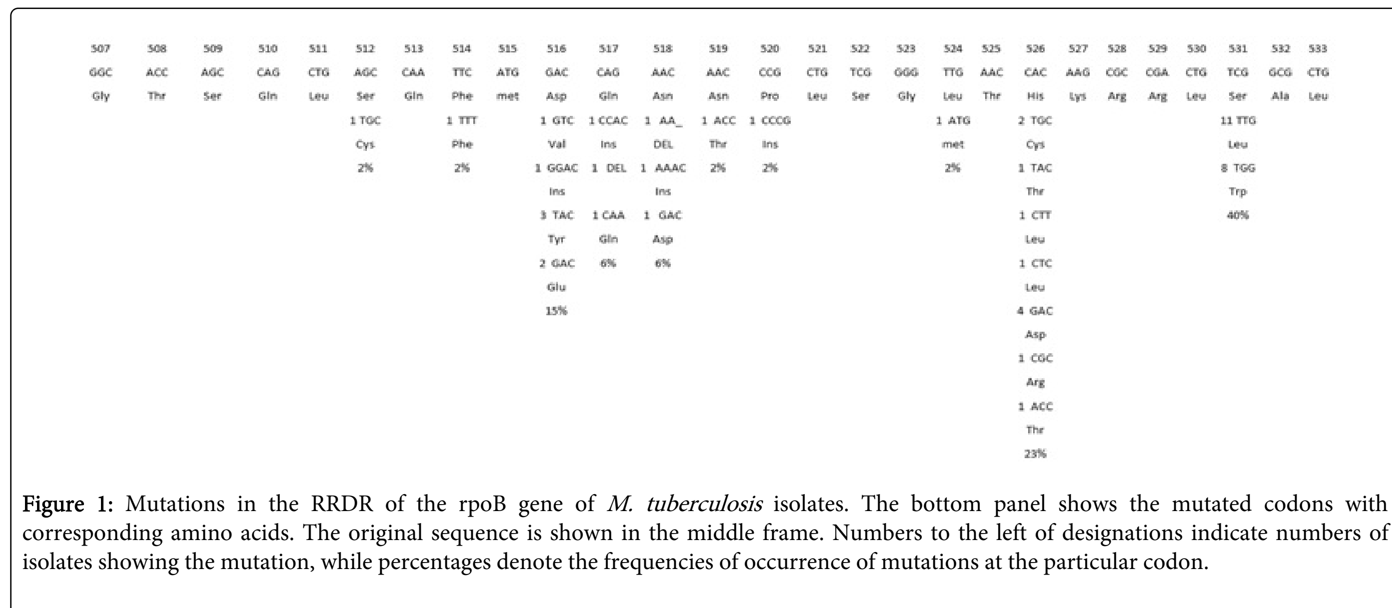

Figure 1: Mutations in the RRDR of the rpoB gene of *M. tuberculosis* isolates. The bottom panel shows the mutated codons with corresponding amino acids. The original sequence is shown in the middle frame. Numbers to the left of designations indicate numbers of isolates showing the mutation, while percentages denote the frequencies of occurrence of mutations at the particular codon.

Our DNA sequencing analysis of the 35 RIFr strains disclosed 24 different positions of mutation and revealed that 23 (66%) of 35 strains had a mutation at only one position, 9 (26%), at two position, 3 (9%) at three positions.

Discussion

Rif-resistance is almost exclusively associated with mutation of the rpoB gene, which encodes the β-subunit of RNA polymerase [12,21]. More than 95% of rpoB mutations in Rif-resistant clinical isolates have been found within RRDR. Over 70 distinct rpoB mutation and four

frequent mutation (codon 531, 526, 516, 513) have been reported for RIF–resistant *M. tuberculosis* isolates worldwide [22,23]. Rif-resistance is often regarded as an excellent surrogate marker for MDR tuberculosis [24,25]. In our study all 35 (23%) isolates show resistance to both rifampicin and isoniazid and having a mutation in 81 bp RRDR of rpoB gene. Previously characterized mutations were defined as those associated with drug resistance as listed in the Tuberculosis drug resistance mutation database [26]. Mani *et al.* [27], found 88% (44/50) rifampicin resistant isolates from various parts of India [27]. Khanna *et al.* [28], found 54% (104/194) MDR-TB in Delhi & neighbouring regions in India [28]. Multidrug resistant tuberculosis (MDR-TB) cases have shown a worrying increase in the eight southern districts of Punjab, India in the past one year. The disease has also shown almost 100% rise in eight southern districts–Patiala, Mohali, Fategarh Sahib, Ludhiana, Barnala, Sangrur, Mansa and Roopnagar (India) [Amaninder pal Sharma][29].

In previous studies most of the rpoB mutations found in *M. tuberculosis* strains were single amino acid substitution (single point), at either position Ser-531 (Leu/Gln) or His-526 (Asp/tyr); although for a few strains, double mutation [30,16] and even triple mutation existed [30]. In contrast to this, in our study we found 34% (12/35) of strains had mutation at more than one position in the rpoB gene and mutation frequencies at different position were also different from the earlier studies. In our study we observed that the *M. tuberculosis* isolates with the RIF^r phenotype contain missense mutation that show amino acid substitution at Ser-531(40%), His-526(23%) and Asp-516(15%) residues. This finding is almost similar to result reported by Ramaswamy and Musser [31], who showed frequencies of 41 and 36% for various mutation occurring at codon 531 and 526 respectively, in 478 isolates obtained from various part of the world. Matsiota-Bernard et al. [32] and Pozzi et al. [30] found high frequencies of mutation at codon 531, 53% and 59% respectively. Although Qian et al. [33], reported a low frequency of the mutation at codon 526(4%) in china isolates, we found high frequency of this mutation (23%). Similarly high frequencies of this mutation at codon 526 have found in isolates from japan (33%) [33], Greece (19%) [32], Italy (30%) [30], Korea (38%) [13].

In this study molecular analysis of 81 bp RRDR of rpoB gene of *M. tuberculosis* clinical isolates were carried out and mutations were identified. New mutations reported in this study include mutation from AGC (Ser)-TGC (Cys) at codon 512, TTC (Phe)-TTT (Phe) at codon 514, CAG (Gln)-CAA (Gln) at codon 517, AAC (Asn)-ACC (Thr) at codon 519, TTG (Leu)-ATG (met) at codon 524. Despite the large number of mutations already reported in other studies, the evidence of new mutations in this study indicates that mutations continue to arise, probably due to the ability of *M. tuberculosis* to adapt to drug exposure. For the control of MDR–TB, elimination of uncompleted treatment is critical. Since uncompleted treatment tends to facilitate the rate of evolution of increased levels of drug resistance within MDR-TB clones [34,35] which facilitates increase in prevalence of such clones in *M. tuberculosis* populations, treatment become more difficult, which makes non-compliance more likely, and therefore uncompleted treatments more likely, which completes a vicious self-accelerating cycle. This possibility of uncompleted treatments resulting in escalating rise in prevalence and strength of MDR-TB means that every case of uncompleted treatment counts, and should be avoided if man is to stem the MDR-TB tide. Hence, early detection of the MDR-TB may well be critical to durable control of tuberculosis.

In conclusion, the rifampicin resistance (RIF^r) appears to be an effective marker of MDR-TB, and confirmation with PCR-DNA sequencing can detect the presence of RIF^r *M. tuberculosis* within 2 days, and can clearly differentiate them from RIF^s strains, the technique described in our study may prove to be useful for identifying MDR-TB. Our study demonstrate the feasibility and ease of using automated DNA sequencing to rapidly and unambiguously characterize rpoB mutation associated with rifampicin resistance. Main advantage of this technique is that to allow the identification of all mutations in the target sequence, the analysis of the 81-bp RRDR of rpoB gene of 35 *M. tuberculosis* clinical isolates was performed and mutations were recognized. Five new mutations were recognized. The profile of mutations in the 81-bp RRDR is similar to that the majority of isolates worldwide.

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