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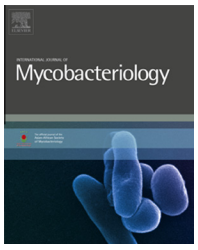

Mutation pattern in rifampicin resistance determining region of *rpoB* gene in multidrug-resistant *Mycobacterium tuberculosis* isolates from Pakistan

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ABSTRACT

The current study was undertaken to characterize the RRDR *rpoB* gene mutations among the rifampicin-resistant *Mycobacterium tuberculosis* (MTB) isolates from Pakistan. Rifampicin mutation patterns were analyzed by using PCR followed by *rpoB* gene sequencing. Among the 1080 referred TB cases, 63 (6%) were resistant against at least one first-line TB drug. Out of these 63 resistant isolates, 24 isolates (38%) were found to be resistant to isoniazid and rifampicin. Sequence analysis of multidrug-resistant tuberculosis (MDR-TB) isolates detected a single mutation in the RRDR region of the *rpoB* gene at codon 531, 516, 512, 528 and 533; however, 5 MDR-TB isolates lack any mutation in the RRDR region. A double mutation was observed in 1 MDR-TB isolate at codon 512 and 516 which are reported for the first time from Pakistan. Moreover, in 1 isolate a novel silent mutation was observed at codon 528. Further studies about these mutations may be helpful in the development of diagnostic tools for the detection of MTB in a high TB endemic area like Pakistan.

Introduction

Tuberculosis (TB) is one of the leading fatal diseases among humans mostly caused by *Mycobacterium tuberculosis* (MTB). It is estimated that one third of the world's population is latently infected with TB. Pakistan ranks fifth among the high TB burden countries in the world [1,2]. Antimicrobial therapy is one of the effective strategies used to control TB. First-line drugs, including rifampicin (RIF), isoniazid (INH), ethambutol, pyrazinamide and streptomycin are the preferred choice for TB control [3]. Recently, the emergence of multidrug-resistant TB (MDR-TB) strains has become a serious public health

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problem in developing countries, including Pakistan, and has consistently been contributing to increased annual TB incidence rates [4–6]. MDR-MTB strains are resistant to RIF and INH and thus limit the use of these available drugs [3].

RIF is an effective drug against MTB [7] which interferes with transcription by the DNA-dependent RNA polymerase [4]. In most cases, resistance to RIF is linked with mutations within 81 bp *hypervariable* or hot spot regions of RIF resistance determining region (RRDR) of the *rpoB* gene [4]. Detection of *rpoB* gene mutations is considered a surrogative marker for MDR-TB detection and can be used as a tool in MDR-TB diagnostics [8–11].

The patterns and frequency of mutations in the *RRDR* region of the *rpoB* gene in the MTB clinical isolates vary significantly according to the geographical location [12]. Limited data is available regarding the pattern of *rpoB* mutation in the MDR-TB patients in Pakistan [13,14].

In the present study, PCR-based molecular detection of *rpoB* gene mutations followed by DNA sequencing was performed. Findings from the present study might be helpful in the development of diagnostic tools for the rapid detection of MDR-TB.

Materials and methods

Sample collection

A total of 1080 suspected TB referred cases from TB centers and hospitals of Punjab were included in the study. The study was conducted at the Bacteriology Laboratory, Institute of Public Health, Lahore. The study was approved by the institutional research ethical committee. Sputum samples from these patients were collected in designated sterile bottles and processed immediately.

Sputum smear microscopy, and In vitro drug susceptibility testing (DST)

Sputum smears were Ziehl–Neelsen (ZN) stained and acid fast bacilli (AFB) were examined under *a* bright field microscope. Parallel to microscopy, all the collected sputum samples were also processed for *in vitro* drug susceptibility testing. Sputum samples were decontaminated using N-acetyl L-cysteine and neutralized with sodium hydroxide [15]. MTB isolates were cultured on a Lowenstein–Jensen (LJ) slant followed by incubation at 37 °C for at least 6–8 weeks. MTB colonies were identified by the para-nitrobenzoic acid (PNB) test and the nitrate reductase test (NRT). Confirmed MTB isolates were tested for *in vitro* drug sensitivity testing for RIF, INH and ethambutol using the standard LJ proportion method as described earlier [15,16]. Isolates were tested for resistance to RIF, INH and ethambutol using concentrations of 40 μg/ml for RIF, 0.2 μg/ml for INH and 2.0 μg/ml for ethambutol. Briefly, the diluted suspension of isolates were inoculated onto LJ medium with and without drugs and incubated at 37 °C. Results were read up to 42 days of incubation. An isolate was considered resistant to a given drug when growth of 1% or more as compared with the control was observed in drug-containing medium. MTB H37Rv wild type strain (ATCC 27294) was used as a control for drug susceptibility testing [16,17].

DNA extraction and molecular detection of MTB

Chromosomal DNA from MDR-MTB colonies was extracted using QIAamp DNA Mini Kit (QIAGEN, Toronto, Canada). The polymerase chain reaction (PCR) based amplification of *rpoB* gene of MTB was achieved as described earlier by using 5′-GAGGCGATCACACCGCAGACGT-3′ and 5′-GATGTTGGGCCCCT CAGGGGTT-3′ as forward primer (RP4T) and reverse primer (RP8T), respectively [18]. The amplified product was separated on 1.5% agarose gel and ethidium bromide stained followed by visualization under ultraviolet light in a gel documentation system.

rpoB gene sequencing

The PCR product was processed for the DNA sequencing from both ends using BigDye terminator cycle sequencing kit on ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). Bioinformatics analysis was done using ClustalW (http://embnet.vital-it.ch/software/ClustalW.html) and NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequences were translated to amino acid sequences using ExPASy translate tool (http://web.expasy.org/translate/). The obtained sequence data was compared with the reference sequence of *rpoB* gene of MTB H37Rv.

Results

Susceptibility patterns of first-line TB drugs

Among the 1080 samples, 63 (6%) were resistant to at least one TB drug. Out of these 63 resistant isolates, 24 isolates (38%) were found to be resistant against INH and RIF. Among 24 MDR-TB cases, 53% were male while 47% were female patients.

Profile of rpoB gene mutations in MDR-TB isolates

Amplification of the 157 bp RRDR region of *rpoB* gene was achieved and processed for DNA sequence analysis (Fig. 1). *rpoB* gene mutations were detected in 19 out of 24 (79%) isolates when compared with *rpoB* gene sequence of the reference strain. Out of 19, 11 (58%) isolates showed a mutation at codon 531(TCG to TTG), 4 (21%) isolates exhibited a mutation at codon 516 (GAC to GTC, GAC to TAC), 2 (11%) MDR-TB isolates showed a mutation in the amino acid codon 512 (AGC to ATC) while 1 (5%) isolate showed a mutation at the codon 533 (CTG to CCG); 1 (5%) isolate exhibited a silent mutation at the codon 528 (CGC to CGT) (Table 1).

Discussion

Drug resistance mostly develops by mutational interplay rather than acquisition of resistant genes transfer from other bacteria. Drug resistance in MTB isolates has widely been investigated and is generally thought to be caused by spontaneous mutations [3,5,19]. Emergence of MDR-TB is a serious challenge for clinicians; it arises mostly due to *rpoB* gene mutations [20]. The frequency of mutations in MTB *rpoB* gene

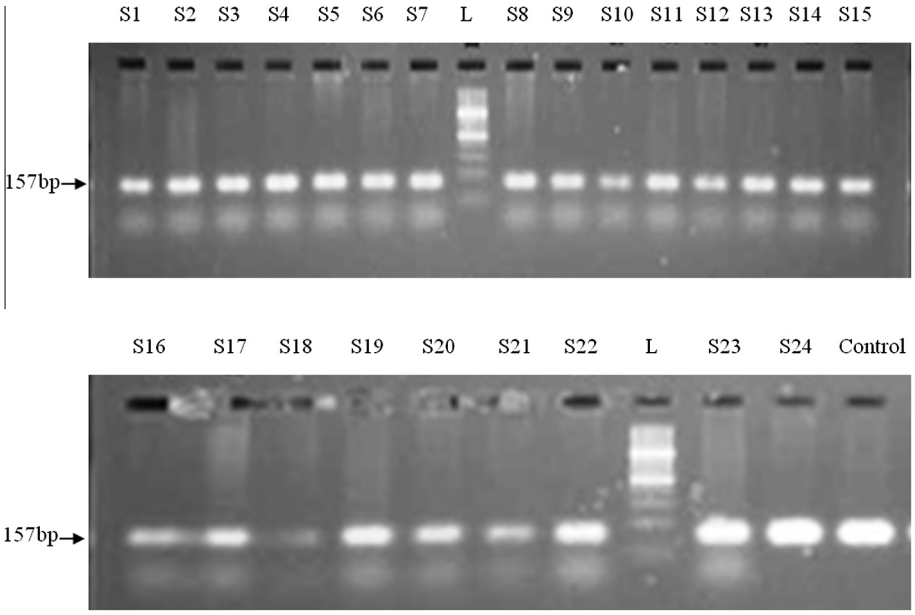

Fig. 1 – PCR amplification of *rpoB* gene of phenotypic MDR-TB isolates. PCR product of *rpoB* was resolved on 1.5% agarose gel. Lane (L) in the figure presents the 100 bp molecular weight DNA ladder. Lane S1 to S24 shows the amplification of 157 bp region of *rpoB* gene from 24 phenotypic MDR-TB isolates.

Table 1 – Resistance conferring mutations in MDR-TB isolates.

Codon/nucleotide change	Amino acid change	Sample ID.	No. of Isolates	Rate of mutations (%)	Selected references
TCG → TTG	S531L	1, 2, 3, 4, 9, 12, 14, 16, 17, 20, 22	11	58	[13,23]
GAC → GTC	D516V	5, 13, 15	3	16	[17]
GAC → TAC	D516Y	18	1	5	[17]
AGC → ATC	S512I	18, 21	2	11	[24] and In this study
CGC → CGT	R528R	10	1	5	In this study
CTG → CCG	L533P	24	1	5	[25]

varies geographically [12]. Mutations in *rpoB* gene have been reported earlier in Asian countries, which are generally associated with a high-level of resistance to RIF [21]. Despite Pakistan being a highly TB endemic area, few studies are available regarding molecular characterization of *rpoB* mutations in MDR-TB patients [13,14,17]. The information regarding the prevalence of these mutations might be helpful for better therapy and management of MDR-TB patients.

In the current study, a total of 1080 referred TB cases were processed for first-line drug susceptibility testing; 63 out of 1080 (6%) isolates were resistant against one first-line TB drug. Out of these 63 resistant isolates, 24 isolates (38%) were found to be resistant against isoniazid and rifampicin. Among 24 MDR-TB cases, 53% were male while 47% were female patients, which is in agreement with the previous study [22].

Genotypic analysis was only done for MDR-TB isolates (24 cases) by PCR amplification of 157 bp *rpoB* gene. DNA sequencing of the purified PCR product from all the MDR-TB isolates were accomplished in order to identify mutations in the RRDR region of the *rpoB* gene. DNA sequence data was compared with the sequence of *rpoB* gene of the wild type MTB strain. *Sequencing results showing a mutation in the 157bp region of rpoB in* 19 RIF-resistant isolates out of 24 MDR-TB isolates (79%), reflects the importance of detection of *rpoB* gene mutations as a tool in MDR-TB diagnostics [11]. Out of 19 isolates, 58% of the total MDR-TB isolates showed a mutation at codon 531 (TCG to TTG) that resulted in a change of serine to leucine; these findings are in line with an earlier study which reported codon 531 as a hot spot for *rpoB* gene mutations in MTB [23]. A previous study on MDR-TB isolates from Punjab, Pakistan has reported a 52% prevalence rate of *rpoB* mutation at amino acid codon 531 [13]; 21% of isolates inhabited the mutation in codon 516 (GAC to GTC, GAC to TAC) that resulted in the conversion of two amino acids (aspartate to valine and tyrosine, respectively), which is in line with a previous work which documented a 24% mutation rate at codon 516 [17]. Among MDR-TB isolates, 11% of isolates showed a mutation at codon 512 (AGC to ATC) which replaced serine with isoleucine. This point mutation (AGC to ATC) at codon 512 is observed for the first time only in Pakistani MDR-TB isolates, which may be an addition to a succession of reported mutations; however, a similar point mutation was observed in a study from Poland [24]; 5% of the isolates showed a mutation at codon 533 (CTG to CCG) that shifted leucine with proline as reported in a study from Turkey [25]. In 5% of the isolates a novel silent mutation was observed at codon 528 (CGC to

CGT) that replaces arginine with arginine which is not reported even globally. Silent mutations on other codons were reported by Isfahani et al. [26]. A double mutation was observed in one MDR-TB isolate at codon 512 and 516. Similar mutations were also documented earlier [4,27].

Five (20%) MDR-TB isolates lack any mutation in the RRDR region of the *rpoB* gene, though these isolates were phenotypically resistant to RIF. This difference might persist due to genotype variations prevailing worldwide; however, the presence of mutations in the *rpoB* gene other than the RRDR region could not be excluded as described by Heep et al. [28]. Lack of RRDR mutations in resistant isolates might be due to the presence of other rare *rpoB* mutations or another mechanism of resistance to rifampicin [13,28]. These findings are consistent with previous reports from several countries [13,20,23,28,29].

The frequencies and distribution of mutations at RRDR loci in drug-resistant MTB from Pakistan were investigated. The patterns of mutation in this study coincide with that of the majority of MTB isolates prevalent globally. Further study concerning these mutations might be helpful in the development of diagnostic tools for the rapid detection of MTB.

Conflict of interest

None.

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