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Research Article

Mutations in *rpo*B gene and their association with Rifampicin-resistance levels in clinical isolates of *Mycobacterium tuberculosis*

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Abstract: Present study was aimed to identify most frequent mutations in *rpo*B gene region and to evaluate the association between mutations in *rpo*B gene and resistance levels to Rifampicin in clinical isolates of *Mycobacterium tuberculosis* of different geographical regions of India. A total of 100 clinical isolates of *Mycobacterium tuberculosis* were included in this study. Drug susceptibility testing against first line anti-tuberculosis drugs was performed on LJ medium by conventional minimal inhibitory concentration (MIC) method and the mutation(s) in *rpo*B gene of *M. tuberculosis* isolates were analyzed by sequencing method. Of the 100 *M. tuberculosis* isolates, 31 (31.0%) and 18 (18.0%) were found resistant and susceptible for all four first-line anti-tuberculosis drugs. The genetic mutations were observed in 96% (72/75) rifampicin-resistant *M. tuberculosis* isolates, while 4% (3/75) of rifampicin-resistant isolates did not have any mutation in *rpoB* gene. The mutation TCG531TTG (Ser531Leu) was found as most common and frequent mutation in 69.3% (52/75) of rifampicin-resistant isolates of *M. tuberculosis* with MIC level (≥ 512mg/l). The mutation at codon 511 was associated with low degree (128mg/l) of rifampicin-resistance, deletions at codons 514-516 or substitution at codon 516 were found to be associated with moderate degree (256mg/l) of rifampicin-resistance and mutations at codon 526, 531 were associated with the high degree (512mg/l) of rifampicin-resistance in *M. tuberculosis* isolates of Indian origin. The findings of this study will be useful for the development of raid and more specific indigenous molecular tools for the early diagnosis of multidrug-resistant tuberculosis in the country.

Keywords: *Mycobacterium tuberculosis*; Rifampicin; *rpo*B gene; Multidrug-resistance; DNA sequencing.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is the leading cause of death in the world from a preventable and curable infectious disease. It has been estimated that there were 9.6 million new TB cases (5.4 million men, 3.2 million women, and 1.0 million children) and 1.5 million TB deaths (890 000 men, 480 000 women, and 140 000 children) in 2014, worldwide [1]. India has the highest burden of TB cases and accounted for 27% of global TB notifications in 2014 [1]. In recent years, the rise of multidrug-resistant tuberculosis (MDR-TB; resistance to both isoniazid and rifampicin with or without for other drugs) is the major hurdle for the control and effective management of TB worldwide. The case of MDR-TB is difficult to cure and requires prolonged treatment with expensive and often toxic multidrug regimens.

Rifampicin (RIF) is an important first-line anti-TB drug which shows bactericidal action against *Mycobacterium tuberculosis*. Resistance to RIF is very vital and is considered as a surrogate marker of MDR-TB, as about 90% of rifampicin-resistant isolates were also found resistant to isoniazid (INH) drug [2,3]. RIF target β-subunit of bacterial RNA polymerase, encoded by *rpo*B gene and inhibits the early steps of translation of *M. tuberculosis*. Mutations in 81bp rifampicin-resistance-determining (RRD) region of the *rpo*B gene have been reported in 94-96% rifampicin-resistant *M. tuberculosis* isolates [4-6].

In the current scenario, diagnosis of active TB and MDR strains are essential to interrupt the transmission and initiation of effective treatment and therefore better TB control in the community. Detection of

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M. tuberculosis using conventional solid culture and drug susceptibility testing (DST) is considered as the 'gold standard' method of MDR-TB diagnosis. However, conventional methods for diagnosing MDR-TB are expensive, labour intensive and time-consuming [4]. Hence, efforts have been made to develop rapid molecular assays for early detection of rifampicin-resistance. These assays rely on the information available for the *rpo*B gene mutations from different settings. Previous studies have reported that the prevalence of individual mutations in *M. tuberculosis* differs geographically and may affect the sensitivity/specificity of molecular test [7-9]. Therefore, deeper understanding on the most frequent or novel mutations and their correlations with phenotypic drug susceptibility profile of clinical isolates is necessary for the development and/or implementation of molecular assay as routine diagnostics tests, in particular, geographical region. The present study reported the frequency and distribution of mutations in RRD region of *rpo*B gene and highlights the association of *rpo*B mutations with the level of resistance to RIF in clinical *M. tuberculosis* isolates of Indian origin.

2. Materials and Methods

2.1 Culture isolates and drug susceptibility testing

A panel of 100 *M. tuberculosis* isolates, recovered from suspected MDR cases of pulmonary TB and H37Rv (TMC02) strain were obtained from the National Mycobacterial Repository Centre of our Institute for this study. The clinical isolates of *M. tuberculosis* belonged to Agra, Jaipur, New Delhi, Cochin, Port Blair and Haridwar region. All the isolates were screened for the susceptible / resistance pattern to RIF (64mg/l), INH, Ethambutol (EMB) and Streptomycin (SM) drugs using conventional minimal inhibitory concentration (MIC) method as described by Das *et al*., [10]. MIC was determined using standard criteria of counting the colony-forming units (CFUs) and comparing with susceptible reference strain *M. tuberculosis* H37Rv [11, 12]. Isolates found resistant to RIF (64mg/l) were further tested at 128, 256, 512mg/l in order to determine their level to resistance MIC values.

2.2 DNA isolation and PCR sequencing of the *rpo*B gene

DNA was extracted from all isolates of *M. tuberculosis* using lysozyme and proteinase-K treatment as per van Embden *et al*., [13]. A 350bp region of *rpo*B locus was amplified using the PCR primers: forward, 5'- GGGAGCGGATGACCACCC -3' and reverse 5'- GCG GTACGGCGTTTCGATGAAC -3'. Briefly, PCR were set up in volume of 50µl, using 200ng of genomic DNA, 1X reaction buffer (50mM KCl, Tris-HCl, pH-9.0), 0.2mM each of dNTPs, 1.5mM $MgCl_2$, and 1 unit Taq DNA polymerase. The reaction mixture was performed in a 'GeneAmp PCR System 9700' thermal cycler (Applied Biosystems, USA). Thermal cycling was as follows: initial denaturation at 94^0C for 4 min., followed by 35 cycles of denaturation at 94^0C for 1 min., annealing at 60^0C for 1 min., extension at 72^0C for 2 min., and final extension at 72^0C for 10 min. Amplicon sizes of 350bp were considered positive in amplification of PCR, after separation on 2% agarose gel for 1.30 hrs at 50 volts ($3.8V/Cm^2$).

2.3 DNA sequencing and *In-silico* analysis

Amplicon of *rpo*B gene was resolved on 1% agarose gel and band of 350bp was excised. DNA was extracted from the gel slices using QIAEX II Gel Extraction kit (QIAGEN, USA). Each sequencing reaction of 20µl volume consisted of 200ng genomic DNA, 3.5pmol of forwarding primer, 8µl sequencing mix (BigDye Terminator Cycle Sequencing Kit v3.1, Applied Biosystems) and adjusted to a final volume of 20µl by adding distilled water. The cycling parameters were as follows: 30 cycles of 96°C for 30 Sec and 60°C for 4 min. Amplicon was purified by adding 0.1 volume of 3M sodium acetate (pH 4.5) and 2.5 volume of absolute ethanol. The sequencing of the amplicon was carried out using the ABI PRISM 310 DNA sequencer (Applied Biosystems, USA). The generated sequences were compared with wild type *rpo*B gene sequence of *M. tuberculosis* H37Rv using MegAlign program of DNASTAR software (Madison, WI, USA).

3. Results

3.1 Drug-susceptible pattern of *M. tuberculosis* isolates

Of the 100 *M. tuberculosis* isolates, 31 (31.0%) and 18 (18.0%) were found resistant and susceptible for all four drugs (Table 1). Individually, 75 (75.0%), 79 (79.0%), 45 (45%), 47 (47.0%) and 25 (25.0%), 21 (21.0%), 55 (55.0%), 53 (53.0%), 75 (75%) isolates were resistant and susceptible to RIF, INH, EMB and SM, respectively. Of the 75 RIF-resistant isolates, 100, 60 and 55.8% isolates were also resistant to INH, EMB, and SM, respectively. Detail results of drug susceptibility pattern of tuberculosis isolates are summarized in Table 1.

Table 1. Drug susceptibility profiles of *M. tuberculosis* isolates.

Drug susceptibility profile				No. of isolates
RIF	INH	EMB	SM	
S	S	S	S	18
S	R	S	S	4
S	S	S	R	3
R	R	S	S	17
R	R	R	S	14
R	R	S	R	13
R	R	R	R	31
Total				**100**

S: Sensitive; R: Resistant

3.2 Mutations in *rpo*B gene of *M. tuberculosis* and their association with RIF resistance levels

Out of 75 rifampicin-resistant isolates, 72 (96%) isolates were found to have mutations at different codons of *rpo*B gene region. Whereas remaining 3 (4%) RIF-resistant isolates and all RIF sensitive isolates (n=25) did not have any mutation in targeted region. The following mutations in *rpo*B gene region were observed in RIF-resistant isolates: CTC511CCG (Leu511Pro) in 2.6% (2/75) isolates with MIC level ≤ 128 mg/l; deletions 514 –TC, 515-ATG (Met) and 516-G. in one RIF-resistant isolate (1/75, 1.3%) with MIC level ≤ 256mg/l of RIF-resistant. The mutation CAC516GTC (Asp516Val) was found in 6 (8%) RIF-resistant isolates with MIC level ≤ 256mg/l. Mutations were found as CAC526CGC (His526Arg) in 1.3% (1/75) isolate, CAC526TAC (His526Tyr) in 8% (6/75) isolates, and CAC526AAC (His526Asn) in 2.6% (2/75) isolates, all of them exhibiting MIC level ≤ 512mg/l. The mutation TCG531TGG (Ser531Trp) was exhibited in 2.6% (2/75) of RIF-resistant isolates and the most common and frequent mutation of TCG531TTG (Ser531Leu) was observed in 69.3% (52/75) RIF-resistant isolates with MIC level (≥ 512mg/l) (Table 2).

Table 2. Results of correlation between MICs of Rifampicin and mutations in the *rpo*B gene of *M. tuberculosis* isolates.

Sequence/ amino acid alteration	RIF MIC	RIF + INH	RIF + INH + EMB	RIF + INH + SM	RIF + INH + EMB	No. Isolates (%)
CTC511CCG (Leu511Pro)	≤ r64	1	0	1	0	2(2.6)
Deletions 514 –TC; 515-ATG (Met); 516-G	≤ r64	1	0	0	0	1(1.3)
CAC516GTC (Asp516Val)	≤ r128	3	1	1	1	6(8)
CAC526CGC (His526Arg)	≤ r256	0	0	1	0	1(1.3)
CAC526TAC (His526Tyr)	≤ r512	4	1	0	1	6(8)
CAC526AAC (His526Asn)	≤ r512	0	1	0	1	2(2.6)
TCG531TTG (Ser531Leu)	≥ r512	6	10	9	27	52(69.3)
TCG531TGG (Ser531Trp)	≤ r512	0	1	0	1	2(2.6)
Wild type	≤ r64	2	0	1	0	3(4)
Total		**17**	**14**	**13**	**31**	**75**

R-rifampicin; H-isoniazid; E-ethambutol; SM-streptomycin; r-resistant

4. Discussion

Multidrug-resistant tuberculosis has been emerged as a global problem, with high incidence in low-income countries including India. The diagnosis of drug-resistant isolates depends on conventional methods, which required long (4-5 weeks) incubation period. Molecular methods have shown the promises for rapid detection of MDR. However, the sensitivity and specificity of these methods depend on the genetic diversity of isolates [14]. These problems can be overcome by DNA sequencing of the core region of the *rpo*B gene. Present study employed DNA sequencing as 'gold standard' test for the detection of genetic mutations in RDR region of *rpo*B gene of clinical isolates of *M. tuberculosis*. Previous studies have also used DNA sequencing method for the study of genetic mutation and genetic diversity of *M. tuberculosis* isolates in different settings [15-17].

In present study, a total of 100 clinical isolates of *M. tuberculosis* were subjected to drug susceptibility testing and 75 (75.0%) were found resistant to RIF using MIC method. All the RIF-resistant isolate were found to be resistant to INH (Table 1). These findings support the previous studies and further confirmed that RIF is a surrogate marker for the multidrug-resistance in TB [18,19]. Similarly, most of the EMB-resistant isolates were INH-resistant, suggesting a high degree of association between EMB and INH-resistance. Present findings supported our previous study which reported 85.18% EMB-resistant isolates were resistant to INH [20]. Madison *et al*., [21] have also reported that EMB-resistance was accompanied by 96.6 percent resistance to INH. This association was not merely a matter of chance as the same trend of EMB-resistance with INH-resistance has also been previously observed in north Indian cities (Agra, Delhi, Jaipur, and Varanasi) by Gupta *et al*., [20].

In the present study, DNA sequencing was employed for the detection of mutations in RRD region of the *rpo*B gene and mutations were observed in *rpo*B locus in 96% (72/75) of RIF-resistant isolates. The frequency of the *rpo*B gene mutations observed in the present study is within the previously reported range of 95-96% [18,22]. In the present study, of 75 RIF-resistant isolates, 3 (4%) isolates did not have any mutation in the targeted region. Previously, other researchers were also not reported any mutations in *rpo*B region of few RIF-resistant isolates [16,23]. These findings indicated that in these isolates alternate mechanisms of drug-resistance (such as efflux pump) might be responsible for RIF-resistant phenotype of the isolates [24].

In the present study, we have observed Leu511Pro, Asp516Val, His526Arg, His526Tyr, His526Asn, Ser531Leu, and Ser531Trp substitution mutations in RIF-resistant isolates. Previous studies have also reported these common mutations in RIF isolates of different geographical regions [18,22,25,26]. In present study, 1 RIF-resistant isolate (1/75, 1.3%) had deletions (514 –TC, 515 –ATG, 516 –G) and correlate with MIC level ≤ 256mg/l. Previously, an isolated study has also reported deletions - 514 TTC (Phe), 515 ATG (Met) and 516 GAC (Asp) in 1 (1.2%) RIF-resistant isolate

[27]. The mutations Asp516Val in 8% (6/75) isolates with MIC ≤ 256mg/l, His526Arg in 1.3% (1/75), His526Asn in 1.3% (1/75), His526Tyr in 8% (6/75) isolates were observed with MIC ≤ 512mg/l in present study. These mutations have also been reported by others researchers- Asp516Val in 1.8-6.2% and His526Arg/ Asn /Tyr in 0.6-11% of RIF-resistant isolates [16,26,28]. In our study, the most common mutation, Ser531Leu, was found in 69.3% (52/75) isolates with MIC ≥ 512mg/l and Ser531Trp in 1.3% (1/75) with MIC ≤ 512mg/l. The frequencies of these mutations reported by other workers were: Ser531Leu in 46.3 - 71% of RIF-resistant isolates and Ser531Trp in 1.2-16.5% of RIF-resistant isolates [14,28].

In present study, strong correlation was found between mutation at specific codon and degree of resistance to RIF; mutation at codon 511 is associated with low degree (128mg/l) of RIF-resistance, deletions at codons 514-516 or substitution at codon 516 are associated with moderate degree (256mg/l) of RIF-resistance and mutations at codon 526, 531 are associated with the high degree (512mg/l) of RIF resistance in *M. tuberculosis* isolates of study settings. Similar to the present study, Bobadilla-del-Valle *et al*., [29] also reported the point mutations in codon 513, 526 and 531 was associated with the high degree of resistance to RIF, whereas mutations in codon 516 were observed in low-level RIF-resistance in isolates of *M. tuberculosis.* Other researchers also reported an association between high and low level of resistance to RIF and of specific mutations in RRDR region of *M. tuberculosis* [30-32]. In conclusion, the findings of this study will be useful for the surveillance of geographical and temporal trends in the prevalence of specific mutations, as well as for research on the implications of geographical differences in mutation frequencies for the development of indigenous diagnostic tools for drug-resistant TB in the country.

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