

Study of Rifampicin and Isoniazid resistance mutation genes of *M. tuberculosis* isolates in Nepal

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ABSTRACT

We studied the rifampicin (RIF) and isoniazid (INH) resistant genes in *Mycobacterium tuberculosis* isolated from sputum samples of re-treated TB cases using phenotypic drug susceptibility test (DST) and rapid molecular method (Genotype; MTBDR plus, Hains Life Science, Nehren, Germany). Of the total 207 isolates included in this study, 90 (42.0%) were RIF resistant, 107 (50.7%) were INH resistant whereas 86 (41.5%) were multi-drug resistant (MDR) by DST. According to genotypic analysis, 37.0% (77/207) were RIF resistant, 50.2% (105/207) INH resistant and 70 (33.8%) were MDR. Among the total 90 RIF resistant isolates, 85.5% (77/90) showed mutation in 81 bp of rpoB gene and the mutation locus were distributed as follows; MUT1 Asp516Val (16.7%; 15/90), MUT2A His526Tyr (6.6%; 6/90), MUT2B His526Asp (5.5%; 5/90) and MUT3 Ser531Leu (56.7%; 51/90). Among the total 107 INH resistant isolates, the mutation located in katG gene was 98.1% (105/107) and in inhA MUT1 gene was (16.8%; 18/107). The mutation locus were distributed as follows; MUT1 Ser315Thr (77.6%; 83/107), MUT2 Ser315Ile (3.7%; 4/107) in katG and in inhA MUT1Cys15Thr (16.8%; 18/107). The highest frequency of mutations in rpoB gene was found at the region of codon Ser531Leu (56.7%) while the frequency of mutation in katG and inhA gene were found at the region of codon Ser315Thr (77.6%) and Cys15Thr (17.1%), respectively. The rapid molecular test kit used in this study was found to be sensitive as well as specific for detection of RIF and INH resistant gene and also helpful in early detection of MDR-TB.

Keywords: *M. tuberculosis*, RIF, INH, gene mutation, drug resistance, MDR-TB, Nepal.

INTRODUCTION

Tuberculosis (TB), the world's most serious public health problem, is caused by bacteria called *Mycobacterium tuberculosis*. This is the most common cause of death due to single organism among the persons over 5 years of age in low-income countries and 80% of deaths due to TB occur in young to middle-age man and woman.^{1,2} Worldwide about 16 million people are living with active TB with appearance of 8 million new cases every year and 2 million dies just due to TB.³⁻⁵ TB, therefore, has greatest impact on youth and adults (aged 15-59 years) and constitute the most common cause of death among adults.⁶⁻⁸

Nepal, a poor Himalayan country, has high burden of TB and associated complications. Nearly half (45%) of the total population is infected with *M. tuberculosis* out of which nearly two-third (60%) are of productive age group.⁹ Every year, 44,000 people develop active TB; among them approximately 20,000 are infectious (smear positive) cases.¹⁰ The TB situation has become even worse due to the emergence of multi-drug resistant *M. tuberculosis* strains (MDR-TB), that is, resistant to at

least rifampicin (RIF) and isoniazid (INH) the two most active first line drugs against TB.^{11,12} The estimated MDR-TB rates in new cases and retreated cases are 2.9% and 15.3% respectively.¹³⁻¹⁵

Drug resistance in *M. tuberculosis* occurs due to random genetic mutations in particular genes responsible for resistance.¹⁶ The mechanism of RIF resistance was first characterized in the rpoB (â sub-unit of the RNA polymerase) gene.^{17,18} RIF acts on RNA polymerase inhibiting the transcription that results into bacterial cell death.¹⁹⁻²² Mutations in rpoB (within 81-bp core region of the rpoB gene corresponding to 507-533 amino acid codons coding for the â-subunit of bacterial RNA polymerase)^{23,24} results into drug resistance diminishing the RIF binding to RNA polymerase.²⁵ INH, a pro-drug (inactive when it enters inside the bacterial cells), in presence of catalase-peroxidase enzyme is converted into oxidizing organic toxic radicals that inhibits synthesis of mycolic acid on the bacterial cell wall.²⁶ Hence, the molecular mechanism of INH resistance is more complex than that RIF (mutation is more common katG, inhA, and less common kasA, ahpC, and oxyR genes).²⁷

Table-1: Comparison of phenotypic and genotypic DST results of RIF and INH (n=207)

Results	RIF resistant	INH resistant	MDR
Phenotypic results	90 (42.0%)	107 (50.7%)	86 (41.5%)
Genotypic results	77 (37.6%)	105 (50.2%)	70 (33.8%)
Discordant result	13 (14.4)	2 (1.9%)	16 (18.6)

The duration of MDR-TB treatment with second line drugs is long (up to 2 years) and have side effect as well and constitutes the great problem in poor countries.²⁸ It is, therefore, better to know the drug-resistant pattern in *M. tuberculosis* isolates isolated from the cases of TB. In this context, availability of the rapid molecular technique to study the RIF and INH resistance mutation genes has become a very useful for early detection of MDR-TB over the conventional culture and drug-sensitivity test (DST) that takes 6-8 weeks.²⁸ In Nepal, there is very limited information on the study of *rpoB*, *katG* and *inhA* gene mutations associated with RIF and INH resistance in *M. tuberculosis* isolates using rapid molecular techniques. In paper, we report the mutation pattern in the *rpoB*, *katG* and *inhA* gene associated with RIF and INH resistant *M. tuberculosis* isolates.

MATERIALS AND METHODS

Sampling and DST: A total of 207 *M. tuberculosis* isolates isolated from sputum samples of re-treated tuberculosis patients (collected in 2010) were used in this study. DST of these isolates was performed following a standard method as described by Canetti *et al.*²⁹ The drug concentration used were as follows: RIF (40µg/ml), INH (0.2 µg/ml), ethambutol (2.0 µg/ml) and streptomycin (4.0 µg/ml). The quality of all in-house

prepared drug media was checked by using H₃₇RV *M. tuberculosis* control strain. The tubes were incubated at 37°C for 4 weeks. The "resistant results" were reported on 4th week while "susceptible results" were reported on 6th week. Resistant result was expressed as the percentage of colonies on drug containing media comparing with the growth on drug free medium at the critical concentration of drugs. More than one percent of bacillary population resistant to the both the critical concentration of a drug was considered as resistant.²⁹

Molecular detection of drug resistance: The molecular assay for detection of RIF and INH resistant mutations and identification was carried out by using of Genotype MTBDR plus kit (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer’s instructions.³⁰

DNA extraction: A few colonies of pure culture were suspended in 300µl of DNA free water in an extraction tube. The suspension was heated at 95°C for 20 minutes and then incubated for 15 minutes in an ultrasonic bath. The content was centrifuged at 12,000 rpm for 5 minutes. A clear supernatant containing DNA genome was transfer to new tube and used for PCR.

Amplification process in multiplex PCR: For amplification, the mixture contained 35µl of the primer nucleotide mix, 5µl of 10x polymerase incubation buffer, 2µl of 25 mM MgCl₂, 0.2µl of AmpliTaq Gold polymerase (5 U/l; Applied Biosystems), 3µl of DNA free water and 5µl of the supernatant of the cell lysate, for a final volume of 50µl. The amplification protocol consisted of 5 minutes of denaturation at 95°C, followed by 10 cycles comprising 30 seconds at 95°C and 2 minutes at 58°C, an additional 20 cycles comprising 25

Table-2: Summary of mutations associated with RIF and INH resistant by Genotype MTBDRplus.

RIF resistance patterns(<i>rpoB</i> gene)		no= 90					
Falling wild type probe	Type of mutation	Codon analyzed	Location of codons	Nucleotide changes	Amino acid changes	No. of isolates resistant to relevant drugs	%
rpoBWT3/WT4	MUT1	513-519	D516V D516Y	GAC→GTC	Asp→Val	15	16.7
rpoBWT7	MUT2A	526-529	H526Y	CAC→TAC	His→Tyr	6	6.6
	MUT2B	526-529	H526D	CAC→GAC	His→Asp	5	5.5
rpoB WT8	MUT3	530-533	S531L	TCG→TTG	Ser→Lue	51	56.7
Total mutation						77	85.5
INH resistance pattern(<i>katG</i> and <i>inhA</i> genes)		no=107					
katG WT	MUT1	315	S315T1	AGC-ACC	Ser→Thr	83	77.6
	MUT2	315	S315T2	AGC-AAC	Ser→Ile	4	3.7
inhA WT	MUT1	-15	C15T	UGC- ACC	Cys→Thr	18	16.8
Total mutation						105	98.1

Table-3: Sensitivity and specificity of Genotype MTBDRplus DST comparison with conventional (Phenotypic) DST as a gold standard.

	RIF	INH	MDR
Sensitivity	85.5%	98.1%	81.3%
Specificity	100.0%	100.0%	100.0%
Positive predictive value	100.0%	100.0%	100.0%
Negative predictive value	100.0%	100.0%	100.0%

seconds at 95°C, 40 seconds at 53°C, and 40 seconds at 70°C, and then a final extension at 70°C for 8 minutes. Hybridization and detection were performed with a Twincubator (Hain Lifescience GmbH, Nehren, Germany). The hybridization procedure included following steps: chemical denaturation of amplification products at room temperature for 5 minutes, hybridization of single-stranded biotin-labeled amplicons to membrane-bound probes at 45°C for 30 minutes, stringent washes, addition of a streptavidin-alkaline phosphatase (AP) conjugate at room temperature for 30 minutes, and an AP staining reaction to detect colorimetric bands. To detect RIF resistance, eight wild-type (WT) *rpoB* probes encoding amino acids 505 to 533 and four probes for common mutations were utilized. Probes used for INH resistance detection were designed to recognize a WT S315Thr, with two mutant probes for the highly resistant *katG* gene and two probes specific for WT regions, as well as four mutant probes for the *inhA* gene, which demonstrates low-level resistance. When all WT probes showed positive staining for an isolate and mutant probes demonstrated no staining, the isolate was considered susceptible. In contrast, the isolate was considered resistant when either any one of the WT probes was absent or any one of the mutant probes was present. The results were segregated against different parameters and were presented in percentage as shown in Tables.

RESULTS

Out of 207 *M. tuberculosis* isolates analyzed, 42.0% (90/207) showed phenotypic resistant to RIF while 50.7% (107/207) showed INH resistance. Of these (197 resistant strains either to RIF or INH) 41.5% (86/197), were resistant

to both the drugs (multi-drug resistant) by phenotypic DST. According to genotypic results, 37.6% (77/207) were found to be resistant to RIF whereas 50.2% (105/207) were resistant to INH. Of the single drug resistant (197 resistant strains either to RIF or INH) 33.8% (70/197) were resistant to both the drugs (multi-drug resistant). Genotypic findings were lower than the results obtained by phenotypic DST method [with the difference of 13 (14.4%) in RIF resistant, 2 (1.9%) in INH resistant and 16 (18.6%) in MDR] (Table-1).

RIF resistance associated with gene mutation was 85.5% (77/90). This was distributed in the gene locus as follows; 16.7% (15/90) in *rpoB* MUT1, Ser516Val 6.6% (6/90) in MUT2A, His526Tyr 5.5% (5/90) in MUT2B His526Asp and 56.7% (52/90) in MUT3 Ser531Lue. Similarly, INH resistance associated with gene mutation was 98.1% (105/107) and was distributed in two genes i.e. *katG* and *inhA*. Mutation distribution in *katG* gene was 77.6% (83/107) in MUT1 Ser315Thr, 3.7% (4/107) in MUT2 Ser315Ile; and in case of *inhA* gene it was 16.8% (18/107) in MUT1 Cys15Thr (Table-2). Of the various regions of RIF resistance gene mutation, the

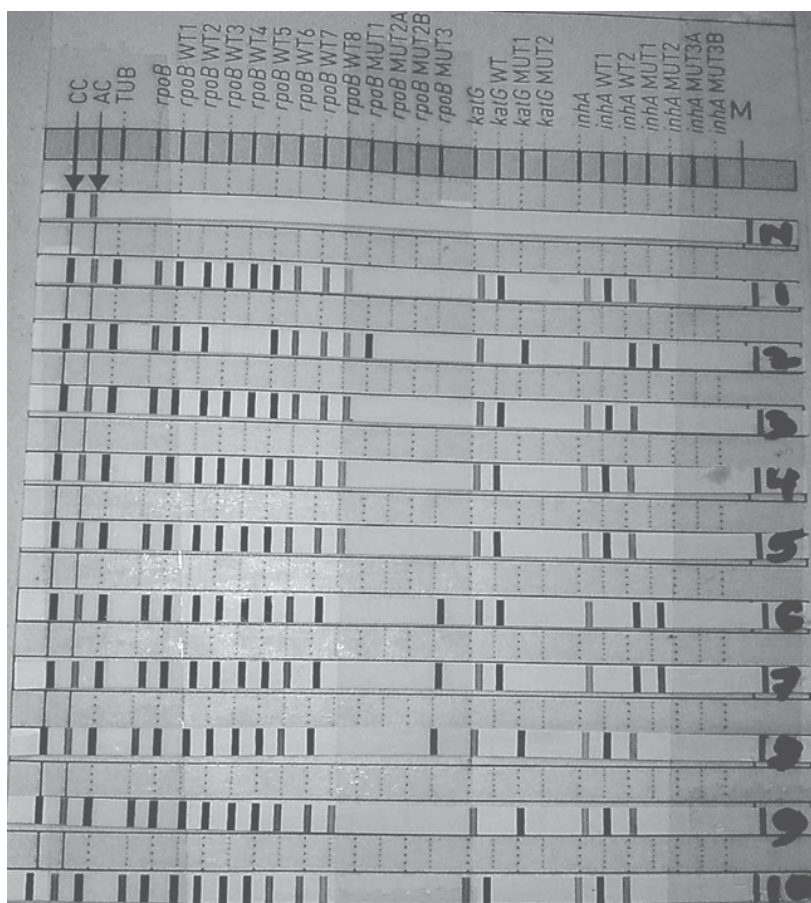


Fig. 1: MTBDR plus test results. Lane-N: Negative control; Lane-1, 3, 5 and 10: *M. tuberculosis* sensitive to RIF and INH; Lane-2: MDR-TB mutation *rpoB* MUT3 (S531L), *katG* MUT1 (S315Thr) and *inhA* MUT1 (C15Thr); Lane-6, 7 and 8: MDR-TB mutation *rpoB* MUT3 (S531L) and *katG* MUT1 (S315Thr) and Lane-9: INH single-drug resistant mutation *katG* MUT1 (Ser315Thr).

highest frequency was observed in the codon Ser531Lue for MUT3 followed by Asp516Val in MUT1, His526Tyr in MUT2A and His526Asp in MUT2B. In case of INH resistance gene mutation, of the two genes (katG and inhA) involved, the mutation in katG gene was mostly located in codon Ser315Thr for MUT1 whereas mutation in inhA gene was in C-15T in MUT1. One isolate had dual mutation associated with RIF resistant in region of Ser531Lue and His526Tyr. The sensitivity, specificity, positive predictive value and negative predictive value of the genotype MTBDRplus test for RIF and INH mutation was 85.5% , 98.1%, 100% and 100% respectively (Table-3). The MTBDR plus test results are shown in Fig. 1.

DISCUSSION

Little information on genomic mutation pattern of *M. tuberculosis* isolated from re-treated cases is available from Nepal. We studied the mutation pattern in rpoB gene, katG gene and inhA gene of *M. tuberculosis* associated with RIF and INH resistance in re-treated cases in Nepal using rapid genotyping method. There was no significant difference in the RIF and INH resistance as detected by phenotypic DST and rapid genotypic methods. The difference in the rates of RIF resistance (42.0% by phenotypic DST and 37.6% by rapid genotypic methods) and INH resistance (50.7% by phenotypic DST and 50.2% by rapid genotypic method) were not significant. No significant differences in RIF and INH resistance rates have also been reported by other investigator.⁸ Genotypic analysis showed high RIF (85.5%) and INH (98.1%) resistant mutation. Such high rate of mutation have also been reported by other workers.^{16,18} The high frequency of mutation associated to RIF resistant seen at region of codon Ser 531Lue in MUT3 was in agreement with the results shown by Huyen *et al*,¹⁹ Lu *et al*,³¹ Lingala *et al*.²⁰ Mutation rate of 16.7% at the region 516 was (as second leading frequency in this study) similar with the findings reported by Bernard *et al*.¹⁷ Some of the previous reports, however, have shown mutation at 526 codon as second commonest region.^{3,20} In the case of INH resistant mutation in katG gene and inhA gene, the most common mutation regions were seen in MUT1 (Ser315Thr) and MUT2 (Ser515Ile) of katG and MUT1 (Cys-15Thr) in inhA genes as have been shown by previous studies.^{17,19,31}

The concordant result of genotypic and phenotypic resistant RIF (85.6%) and INH (98.1%) genes observed in this study were very similar with the findings reported by other investigators.^{20,35} On the other hand, the discordant results (14.4%) of phenotypic and genotypic resistant found in this study was also consistent with previous report.³⁶ The discordant results in RIF (n=13)

and in INH (n=2) could be due to either presence of uncommon mutation outside the RIF and INH resistance regions or additional molecular mechanisms of resistance^{20,24} which could not be detected by this rapid genotypic method used in this study.^{34,37,38} The DNA sequencing method is the only one tool which can detect all range of mutation gene parameters but such a method is not available in poor countries like Nepal. Nevertheless, the rapid molecular test is highly sensitive, specific and reliable and therefore useful in early detection of RIF and INH resistance mutation.³⁹⁻⁴¹ This test kit, however, has limited set of DNA probes that cover most common mutation in rpoB gene, katG, inhA gene regions but not uncommon mutation regions.^{3,33,37}

In this study, attempt was to find out the base line information on the rpoB, katG and inhA mutation genes in *M. tuberculosis* strains isolated from re-treated cases in Nepal. These findings may be helpful for National Tuberculosis Control Program in developing policies for effective treatment and control of MDR-TB in Nepal. Furthermore, these findings may also form the basis for undertaking further studies in future. Though the rapid genotype test kits used in this study reportedly covers only common mutation regions, it is rapid, reliable and useful for quick detection of resistant mutation in rpoB gene, katG gene and inhA gene and is helpful in the early diagnosis of MDR-TB which in turn, help in the management of high burden MDR-TB cases in days to come.

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