International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2019): 7.583

Mutation Profile Assessment of *rpoB*, *KatG* and *inhA* Genes in Rifampicin and Isoniazid Resistant *Mycobacterium tuberculosis* in India

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Abstract: ***Line probe assay (GenoTypeMTBDRplus) is the world health organization (WHO) endorsed molecular technique for rapid screening of rifampicin, isoniazid and multi-drug resistant (MDR) tuberculosis (TB).The aim of this study was to assess the mutation profile of rpoB, KatG, and inhA genes in rifampicin and isoniazid resistant Mycobacterium tuberculosis (M.tb) using the line probe assay (LPA). In this study, acid-fast bacilli (AFB) positive sputum samples were subjected to phenotypic culture, and direct line probe assay (GenoType MTBDRplus ver1/ver2) as per manufacturer's instructions. Between 2013 to 2015, 878 AFB smear-positive sputum samples of previously treated TB patients were received at Department of Medicine, All India Institute of Medical Sciences, New Delhi. Of these, 168 MDR-TB, 29 RIF resistant/INH sensitive and 52 resistant/RIF sensitive samples were available for mutation analysis. It was observed that mutation of rpoB gene, S315L (MUT3) were more frequent (statistically significant) in MDR-TB (121/168=72%) patients than RIF resistant/INH sensitive (14/29=48%). Mutation involving codon 315 (S315T1) were also more frequent in MDR-TB. These results indicate possibility of association of MDR-TB with KatG S315 T1 and rpoB S531L in Indian population. However, additional studies with large number of sample are needed to confirm these observations.***

Keywords: *Mycobacterium tuberculosis*, mutation profile, drug resistance

1. Introduction

Besides accounting for highest number of TB cases in the world, India is also witnessing rapid spread of drug resistant forms of *M.tb*, the causative agent of TB [1]. Transmission of MDR-TB, defined as resistance to at least two first line anti-TB drugs rifampicin (RIF) and isoniazid (INH), is potential challenge to eliminate TB worldwide [2]. Drug resistance is mainly acquired by spontaneous mutation in the chromosomal genes and making the selection of resistant *M.tb* strains during interruption of drug therapy [3],[4]. Resistance to RIF is mainly caused by mutation in 81 bp (codon 507-533) hot spot region of *rpoB* gene that encode the β-sub unit of RNA polymerase [5]. Mechanism of isoniazid resistance resulting from mutation in many genes including *KatG*, *inhA.kasA*, *ndh* etc is a complicated process. *KatG* mutations causes loss of catalase-peroxidase activity and causes high level of INH resistance [6], whereas *inhA* mutation results in low level of INH resistance. MDR-TB is not caused by single mutation, rather it is a result of acquisition of series of mutations. Assessment of frequency of various types of mutations is important because it may vary with geographical area.

Detection of drug resistant TB by conventional culture-based method has limitation because of long turnaround time [7]. Therefore, significant emphasis has been given to molecular methods, offering rapid and accurate identification of drug resistant strains of *M.tb*. The detection of mutations associated with drug resistance is crucial for more specific and accurate diagnosis of drug resistance [8]. LPA(MTBDR*plus*), detecting mutation in hot spot region of *rpoB* gene, *katG* and promoter region of *inhA* gene, are recommended molecular method for rapid screening of RIF and INH resistant TB. In the present study, the mutation profile of drug resistant *M.tb* was investigated by using LPA in sputum samples collected from previously treated TB patients.

2. Materials & Method

The cross-sectional study was conducted at Intermediate Reference Laboratory (IRL), Department of Medicine, AIIMS, New Delhi as per institute ethics committee of AIIMS New Delhi. The previously treated TB patients, who visited the department between 2013 to 2015 and provided written informed consent were recruited in the study.

Sample collection and processing

Two samples from each patient was collected as per national TB elimination programme (NTEP), previously called revised national tuberculosis control programme (RNTCP). Each sample was subjected to Zheil-Neelsen (ZN) AFB microscopy and phenotypic culture using Lowenstein-Jensen (L-J) solid media as per NTEP/RNTCP protocol [9]. The microscopy results were recorded in the form of grading as per standard operating procedure of NTEP/RNTCP. LPA was performed with single samples with high bacillary load, for each patient. All three steps of LPA like DNA extraction, amplification and hybridization were performed in three separate laboratories with unidirectional movement.

Sputum decontamination

Sputum samples was decontaminated with N- acetyl, L-cystein – sodium hydroxide (NALC-NaOH) method. Subsequently, sediments were suspended in 1-1.5 ml

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phosphate buffer and two L-J media was inoculated with each sample for culture, as per NTEP/RNTCP protocol. A 500 µl of suspended samples was used for DNA extraction for LPA. Two Lowenstein-Jensen media slopes were inoculated for each sample for phenotypic culture. All Culture isolates were identified by ZN microscopy, P-nitro benzoic acid (PNB) with solid media and biochemical tests (catalase and niacin tes) as per standard protocol [9]

Line probe assay

Both versions (GenoTypeMTBDRplusver, 1 and Ver.2) of LPA were used in the present study. Procedure of both version of LPA was adopted as per manufactures (Hain life science, Germany) instruction. Each strips of LPA test contains 27 reaction probes that hybridize DNA amplicons among which 6 were positive control and 21 were used for detection of resistance to drug rifampicin and isoniazid [10]. For detection of RIF resistance, the strips contain 8 *rpoB* wild type probe (WT1-8) and four *rpoB* mutation probes [MUT1(D516V), MUT2A (H526Y), MUT2B (H526D, MUT3 (S531L). Whereas, for INH resistance, probes of two genes *KatG* and *inhA* are included. For *KatG* it contains one wild type probe (WT1) and two mutation probes [MUT1 (S315T1), MUT2 (S315T2)]. Similarly, for *inhA* it has two wild type and four mutation probes [C15T (MUT1), A16G (MUT2), T8C (MUT3A), T8A (MUT3B). After hybridization and washing, strips were fixed on paper.

Interpretation of LPA results

Absence of wild type (WT) band or presence of mutation (MUT) band was considered as resistant strain. Presence of all WT probe and absence of all MUT probe was identified as sensitive to concern drug. In case of incomplete hybridization results were considered as invalid.

Statistical analysis

All the data obtained in the study were entered in Microsoft excel sheet for analysis. Data were presented in frequency/percentage. Categorical variables were analyzed by Fishers exact/Chi squire test and p value less than 0.05 was considered as statistically significant.

3. Results & Discussion

A total 878 previously treated AFB smear-positive patients (604 male and 274 female) were included in the present study for assessing the different types of mutations in rpoB, KatG and promoter region of inhA. Of 878 samples, 595 were subjected to Geno Type MTBDR plus ver 1 and 283 were with ver2. Valid results were obtained in 781 samples with TUB band (probe specific to *M.tb* complex) and 73 samples in absence of TUB band [of these 73, total 11 were identified as nontuberculous mycobacteria (NTM)] and remaining 24 were invalid results. Of the 781 valid results with TUB band, drug resistance was observed in 249 samples, and remaining 532 were sensitive to both drugs. On further assessment of mutations in 249 samples, it was observed that 168 were MDR, 29 were RIF resistance but sensitive to INH and 52 were INH resistant but sensitive to RIF.

***rpoB* mutation**

The most common mutation of *rpoB* gene was S531L (MUT3), which was more frequently observed in MDR-TB strains (121/168=72%) than RIF resistant/INH sensitive strains (14/29=48%). This difference in the present of mutation in two group was found to be statistically significant (p=0.01). Higher frequency of occurrence of S531L mutation has been also reported previously [11],[12]. The overall *rpoB*, S531L mutation found in RIF resistant strains was 135/197=69%. Other mutations observed in *rpoB* gene were H526Y/MUT2A (18/168=11% in MDR-TB and 2/29=7% in RIF resistant/INH sensitive strains). D516V (MUT1) mutation was found 4/168 (2%) in MDR-TB and 2/29=7% in RIF resistant/INH sensitive strains. Mutation involving H526D (MUT2B) were only observed in MDR-TB (6/168=4%) and not in RIF resistant/INH sensitive strains as detailed in Table 1.

Table 1: Mutation patterns of *rpoB*, *KatG* and *inhA* genes in 249 drug resistant *Mycobcterium tuberculosis*

Gene	LPA probe	Mutated gene region/ codon	MDR, n=168 (%)	RIF resistant but INH sensitive, n=29 (%)	INH resistant but RIF sensitive, n=52 (%)
rpoB	$^{\Delta}$WT1	506-509	1 (1)	1 (3)	0 (0)
	$^{\Delta}$WT2	510-513	13 (8)	4 (14)	0 (0)
	$^{\Delta}$WT3	513-517	14(8)	4 (14)	0 (0)
	$^{\Delta}$WT4	516-519	8 (5)	4 (14)	0 (0)
	$^{\Delta}$WT5	518-522	0 (0)	2 (7)	0 (0)
	$^{\Delta}$WT6	521-525	0 (0)	0 (0)	0 (0)
	$^{\Delta}$WT7	526-529	15 (9)	4 (14)	0 (0)
	$^{\Delta}$WT8M	530-533	126 (75)	14 (48)	0 (0)
	MUT1	D516V	4 (2)	2 (7)	0 (0)
	MUT2A	H526Y	18 (11)	2 (7)	0 (0)
	MUT2B	H526D	6 (4)	0 (0)	0 (0)
	MUT3	S531L	121 (72)	14 (48)	0 (0)
KatG					
	$^{\Delta}$WT	315	151 (90)	0 (0)	35 (67)
	MUT1	S315T1	160 (95)	0 (0)	35 (67)
	MUT2	S315T2	2 (1)	0 (0)	0 (0)
inhA					
	$^{\Delta}$WT1	-15/-16	13 (8)	0 (0)	16 (31)
	$^{\Delta}$W2	-8	2 (1)	0 (0)	5 (10)
	MUT1	C15T	16 (9)	0 (0)	10 (19)
	MUT2	A16G	0 (0)	0 (0)	0 (0)
	MUT3A	T8C	2 (1)	0 (0)	1 (2)
	MUT3B	T8A	0 (0)	0 (0)	0 (0)

$^{\Delta}$ Indicate missing RIF= Rifampicin, INH=Isoniazid, MDR= Multidrug resistant

***KatG* mutation**

The leading mutation observed in INH resistant *M.tb* stains was at *KatG* codon 315 (196/220=89%) responsible for high level resistance. High frequency of this mutations has been also documented in previous study [13],[14]. Mutation *KatG* S315T1 was the most commonly observed in INH resistant strains (195/220=89%) which was more frequent in MDR-TB (160/168=95%) than INH resistant/RIF sensitive strain. It is a transitional mutation that results in substitution of serine by threonin (AGC-ACC) [15]. The most common

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patterns in mutated *KatG* gene was absence of WT probe with the presence of MUT1 (S315T1). One MDR-TB strain showed presence of both *KatG* WT and MUT1. Other mutation involving *KatG* codon 315 (S315T2) were less frequent in INH resistant strains. These mutations were observed only in 2 strains (1%) of MDR-TB, and not in INH resistant/RIF sensitive strains. The overall frequency of *KatG* mutations in MDR-TB was 161/168=96% and those in as INH resistant/RIF sensitive was 35/52=67%.

***inhA*mutation**

The low level resistance corresponding to *inhA* -15-promoter region [C15T (MUT1)] was more frequent in INH resistant/RIF sensitive strains (10/52=19%) as compared to MDR-TB strain (16/168=9%). The presence of *inhA* MUT3A (T8C) mutation in study was less frequent (2/168 in MDR-TB and 1/52 in INH resistant/RIF sensitive strains). Moreover, *inhA* MUT2 (A16G) mutation was neither observed in MDR-TB nor in INH resistant/RIF sensitive strains. Overall *inhA* mutation in INH resistant strains were found in 37/220 strains (17%) and was less frequent in MDR-TB strains (18/168=11%) than INH resistant/RIF sensitive strains (19/52=37%). Alone *inhA* gene mutations were found in 7/168 (4%) in MDR-TB strains and 17/52 =28%) of INH resistant/RIF sensitive. Mutations involving both *katG* and *inhA* were found to be 2/52 (4%) in INH resistant/RIF sensitive strains and 13/168 (8%) in MDR-TB strains.

LPA is a rapid promising tool that offers several advantages over conventional TB test, including less turnaround time (2-3 days) [14]. However, LPA cannot replace conventional phenotypic culture completely, as it does not include whole region of concerned gene and can miss some mutations responsible for drug resistance [16]. Next generation sequencing based method have the great \ potential to detect the mutations. These high throughput next generation sequencing technologies are useful to scan large region of gene but the procedures followed are complex requiring many steps and adequate training.

4. Conclusion

The study demonstrated the possibility of association of S315T1 and *rpoB* S315T1 with MDR-TB in Indian populations. However, further studies with large sample size are needed to elucidate these mutations and enhance our understanding of the epidemiology and transmission dynamics of drug resistant-strains in India.

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