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# Band Pattern Analysis of the rpoB, inhA, and katG Gene in *Mycobacterium tuberculosis* through Line Probe Assay in Patients from Western Rajasthan, India

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## A B S T R A C T

Summary: Tuberculosis is globally a leading cause of death with increasing rates of drug resistance which has become a serious topic of concern. Early diagnosis and properly administered treatment regimen are the key elements to combat Tuberculosis and its transmission. Rapid molecular methods have been recommended for early detection of drug resistant Tuberculosis. Objectives: This study aimed to analyze the mutation band pattern in rpoB, inhA, katG gene of drug resistant pulmonary tuberculosis through Hybridization technique using Genotype MTBDR plus V.2 assay in Western Rajasthan. Methodology: Positive sputum samples were collected from MDR/ Mono DR Patients. Sputum decontamination was performed after the quality assurance then DNA extraction, amplification, hybridization and band pattern analysis of line probe assay strips was performed as per manufacturer's instructions by GenoType MTBDRplus kit from Hain Lifescience. Result: Out of 215 study participants, 174 (80.9%) were male and 41 (19%) were female, with a mean age group of 41 years, where (146/215; 67.9%) were rural dwellers. 56.2% of the population was sensitive to both the drug (Rifampicin and Isoniazid). Of the 215 isolates, 12 (5.58%) were hetero-resistant to both RIF and INH i.e. the overall proportion of MDR-TB was 12 (5.58%) There were 6 isolates (2.79%) mono-resistant to RIF and 16 isolates (7.44%) mono-resistant to INH. In rpoB gene, WT 7 (2%) and WT 8 (7.2%) band were the most common band to be absent. In katG gene, MUT 1 band showed maximum mutation (14.9%). In inhA gene, WT 1 band was most common band to be absent (4.6%) and MUT 1 band showed 2.5% of mutation. In the present study analysis of MUT 2 band of rpoB, katG and inhA genes did not show any presence. Conclusion: High frequencies of mutations in rpoB, inhA, and katG gene by LPA were observed. The most common resistance-conferring mutations to RIF occurred at rpoB gene with absence of band WT8 and presence of MUT3 band. In katG gene absence of WT1 and presence of MUT1 band was most common pattern of mutation and in inhA gene, WT1 band was prominently absent.

## Introduction

Tuberculosis (TB) is an airborne infectious disease caused predominantly by *Mycobacterium tuberculosis* species of pathogenic bacteria which was first discovered by Robert Koch in 1882. (Central TB Division, 2020)

More than one fourth of the global TB burden is accounted by India i.e. 27 Lakh out of 1 crore new cases annually, among other countries like Indonesia, South Africa and Philippines. (Data, 2021)

Active, drug-susceptible TB disease is treated with a standard 6-month course of 4 antimicrobial drugs (Rifampicin, Isoniazid, Pyrazinamide and Ethambutol). Drug resistant TB is defined as TB disease where the bacilli is resistance to one or more anti TB drugs.

Multi Drug Resistance (MDR-TB) is defined as tuberculosis disease where the bacilli are resistant at-least to isoniazid (H) and rifampicin (R), with or without resistance to other first line drugs (Ethambutanol, Pyrazinamide). The emergence of multidrug-resistant (MDR) strains has proved to be a major challenge in the management of TB. (Central TB Division, 2020)

## Materials and Methods

A cross sectional hospital-based study was carried out in TB C & DST Lab, Department of Microbiology, Dr. S. N. Medical College, Jodhpur, Rajasthan. For tuberculosis diagnosis standard protocol designed NTEP was followed. Identification of tuberculosis was done using manual and automated methods which involved microscopy, CBNAAT, Liquid culture MGIT 960. For drug resistance pattern analysis Hybridization technique was used. (MDR plus V.20 Hain life science Germany).

## Sputum sample collection and transportation

5–10 mL sputum sample was collected from study participants using a labelled and sterile 50-mL Falcon tube. ZN staining was used as primary microscopy.

## Sample decontamination procedure

Sputum samples were digested and decontaminated using freshly prepared N-acetyl-L-cysteine (NALC-NaOH) method which liquefies and decontaminated the sputum and helps in homogenization were used to perform liquid culture in MGIT 960, samples were decontaminated using NALC-NaOH method.

Following centrifugation at 3000 rpm for 15 min, the sediment was re-suspended in 2 mL of sterile phosphate-buffered saline (PBS) (pH 6.8).

An aliquot of 0.5 mL of sediment was inoculated into a Mycobacterium Growth Indicator Tube (MGIT) 960 and was loaded onto a BACTEC MGIT 960 instrument. (7890638455 Flourescence_Microscopy Manual, 2021)

## Drug susceptibility testing

For DST, a strip-based technology Line Probe Assay was performed. Drug resistance pattern was studied in first line anti tuberculosis drugs (Rifampicin, Isoniazid). It targeted by targeting *rpoB, inhA and katG* genes for Rifampicin, Isoniazid and High level Isoniazid respectively.

500µl aliquot of the processed sputum sample was processed.

## Line probe assay technology involves the following steps

1. **Extraction:** DNA was extracted from *M. tuberculosis* isolates or directly from clinical specimens.
2. **Amplification:** Amplification of the resistance-determining region of the gene under question is performed using biotinylated primers by the polymerase chain reaction (PCR).
3. **Hybridization:** Binding of labeled amplicons to probes present on the nitrocellulose strip which is termed as hybridization. If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations.

## MTBDR plus strip

The MTBDR strip contains 27 reaction zones including (6) controls for verification of the test procedure (conjugate, amplification, MTB complex, rpoB, KatG and inhA controls).
(7890638455 Flourescence_Microscopy Manual, 2021).

## Results and Discussion

A total of 215 MTB culture-positive participants were included in the study, of which 174 (80.9%) were male and 41 (19%) were female, with a mean age group of 41 years.

The majority of the study participants (146/215; 67.9%) were rural dwellers.

Maximum number of MDR-TB patients had Unknown status of HIV infection which was 156/215 (72.5%) and around 59/215 (27.4%) were Non-reactive to HIV infection.

## Frequency of drug resistance

Maximum study participants were sensitive to both the drug (Rifampicin and Isoniazid) which was 56.2%. Of the 215 isolates in this study, 12 (5.58%) were hetero-resistant to both RIF and INH i.e. the overall proportion of MDR-TB was 12 (5.58%). There were 6 isolates (2.79%) mono-resistant to RIF and 16 isolates (7.44%) mono-resistant to INH. Pattern of rpoB, katG and inhA gene detected by LPA Genotype MTBDR plus V.2 assay.

In rpoB gene, WT 1, WT 2, WT 3 and WT 6 band were most common band to be present (100%) whereas WT 7 (98%) and WT 8 (92.8%) band were the most common band to be absent and were the case of mutation. In katG gene, MUT 1 band showed maximum mutation (14.9%). In inhA gene, WT 1 band was most common band to be absent (95.4%) and MUT 1 band showed 2.5% of mutation. In the present study analysis of MUT 2 band of rpoB, katG and inhA genes did not show any presence.

To the best of our knowledge this study thoroughly focuses on band pattern analysis of the rpoB, inhA, and katG gene in Mycobacterium tuberculosis through hybridization technique. In the present study, out of 215 participants, male population accounted for 80.9% and 19% participants were female population.

Similar observations were seen where male outnumbered the female population by a high percentage in study performed by Raganath *et al.,* (2014), 82.5% of their study population were males and about 17.4% were the female patients, which was highly concordant with our study. In majority of studies in males outnumbered females, Ashok Singh Charan *et al.,* (2020); Obaidullah Qazi *et al.,* (2014); Jain *et al.,* (2013); Kumar *et al.,* (2015) and Sharma *et al.,* (2020) also reported similar gender distribution in their studies. In contrast, Archiaa *et al.,* (2019) showed female preponderance constituting 64% of the study group.

In the present study, majority of the population belonged to the rural group 67.9%, this was similar with studies of Sharma *et al.,* in 2020. Also majority of the patients were in age group of 31-40 years (20.9%) this is concordant with study of Sharma *et al.,* (2020), in which majority of the patience were within same age group of 31-41 years where mean age was 37 and in the present study mean age is 41 years.

Present research did not had evidence of any HIV-Reactive data. On the contrary, other research carried out in this field by Jain *et al.,* in 2015, had evident data of 0.2% HIV-Reactive Rifampicin-resistant TB subjects.

In the present study maximum study participants were sensitive to both the drug (Rifampicin and Isoniazid) which was 56.2%, 2.5% participants were the ones who were Rifampicin mono-resistant and 7.4% of study population was Isoniazid mono-resistant and 5.5% of the population was hetero-resistant to both the drugs Rifampicin and Isoniazid.

This observation was similar to the study done by Vashistha *et al.,* (2017) in 2016, where maximum study participants 67% were sensitive to both the first line drug rifampicin and isoniazid and 3.9%, 12.5% were rifampicin and isoniazid mono-resistant respectively.

Comparing the pattern of gene mutations in INH resistant strains of the present study with the study recently performed at Ajmer by Charan *et al.,* in 2020 revealed that katG gene mutation pattern (which represents high level isoniazid), was high in percentage (65%) as compared to inhA gene mutation pattern (low level isoniazid) (28%) which was highly concordant with the present study where katG gene mutation was 64.2% and inhA gene mutation was 17.8%.

In tuberculosis Drug resistance is acquired resistance is caused by a genetic mutation that makes a drug ineffective against the mutant bacilli. In clinical settings, an inadequate and irrationally administered treatment regimen allows drug-resistant mutants to become the dominant strain in a patient infected with TB. Therefore, it is important to keep vigilante on any change in pattern of mutation in in MTB isolates.

**Table.1** Antibiotic sensitivity patterns

| Total No. of samples | Rifampicin and Isoniazid Sensitive | Rifampicin mono-Resistant | Isoniazid mono-Resistant | Rifampicin Resistant, Isoniazid Resistant |
|---|---|---|---|---|
| 215 | 121 | 6 | 16 | 12 |

Most frequent Mutation pattern of Rifampicin (rpoB), Isoniazid (katG and inhA gene ) detected by Genotype MTBDR plus V.2 assay

**Table.2** Pattern of gene mutations

| Gene | Band | Gene region/Mutation | No. of samples | Frequency% |
|---|---|---|---|---|
| rpoB | WT1 | 506-509 | 154 | 100 % |
| | WT2 | 510-513 | 154 | 100 % |
| | WT3 | 513-517 | 154 | 100 % |
| | WT4 | 516-519 | 153 | 99.3 % |
| | WT5 | 518-522 | 153 | 99.3 % |
| | WT6 | 521-525 | 154 | 100 % |
| | WT7 | 526-529 | 151 | 98 % |
| | WT8 | 530-533 | 143 | 92.8 % |
| | MUT 1 | D516V | 2 | 1.2 % |
| | MUT2A | H526 Y | 0 | 0 % |
| | MUT2B | H526 D | 0 | 0 % |
| | MUT 3 | S531 L | 10 | 6.4 % |
| katG | WT 1 | 315 | 134 | 87 % |
| | MUT 1 | S315 T1 | 23 | 14.9 % |
| | MUT 2 | S315 T2 | 0 | 0 % |
| inhA | WT 1 | 215/216 | 147 | 95.4 % |
| | WT 2 | 28 | 152 | 98.7 % |
| | MUT 1 | C15T | 4 | 2.5 % |
| | MUT 2 | A16G | 0 | 0 % |
| | MUT 3A | T8C | 0 | 0 % |
| | MUT 3B | T8A | 0 | 0 % |

**Figure.1**

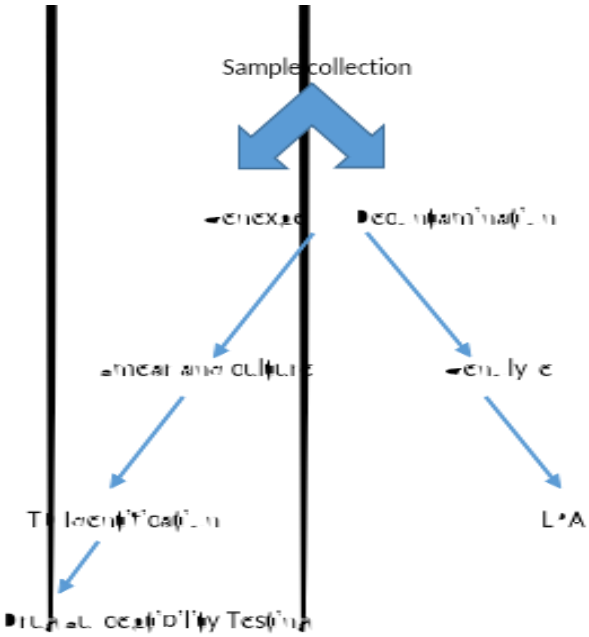
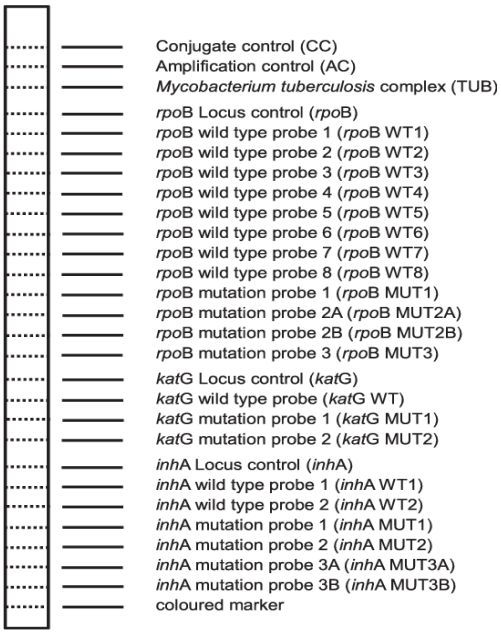



**Figure.2**




The most common resistance-conferring mutations to RIF occurred at rpoB gene with absence of band WT8 and presence of MUT3 band. In katG gene absence of WT1 and presence of MUT1 band was most common pattern of mutation and in inhA gene, WT1 band was prominently absent. The high percentage of mono-resistance in rifampicin and isoniazid is alarming especially when we have target to End TB by 2025. It is also important to follow mono-isoniazid resistance patients at they are very much likely to convert in MDR case as reported by other authors.

MDR-TB patients should be kept under a close watch as its transmission to close contacts will lead to prominent

diagnosis of MDR-TB strain. Also there should be continue survey by which adverse effect of individual drug can be noted and according to the findings, required action could be taken by the doctor.

We recommend more studies with large number of samples are required to validate these preliminary findings, and define the exact pattern of mutation hot spot in drug resistant strains so any shift in strains can be traced before it becomes epidemic.

Greater efforts to reinforce TB control programmes are done and should be continued to ensure universal access to MDR-TB diagnosis and reduce the development and transmission of drug-resistant TB.

## Author Contribution

Mahima Chouhan: Investigation, formal analysis, writing—original draft. R. S. Parihar: Validation, methodology, writing—reviewing.

## Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Research Funding:** Not applicable

**Ethical Approval:** Not applicable.

**Consent to Participate:** Not applicable.

**Consent to Publish:** Not applicable.

**Conflict of Interest:** The authors declare no competing interests.

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Ranganath and co-authors in Department of Microbiology, JSS Medical College, Mysore, Karnataka, from January 2011 to January 2012 reported a total of 92 smear positive sputum samples from which 86 samples were identified as MTB in 2014. Susceptibility pattern on LPA showed that 36 (41.80%) were resistant, 50 (58.13%) were sensitive to RIF and 34 (39.53%) were resistant, 52 (60.46%) were sensitive to INH. - Google Search [Internet]. [cited 2021 Dec 15]. Available from: https://www.google.com/search

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