

Molecular Detection of Rifampicin and Isoniazid Resistance in Multidrug-resistant *Mycobacterium tuberculosis* Complex from Sputum Samples in Patients Attending Teerthanker Mahaveer Medical College and Research Center, Moradabad, Uttar Pradesh, India

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Abstract

Background: The aim of this study is to evaluate the value of line probe assay (LPAs) in the rapid detection of *Mycobacterium tuberculosis* complex antigen 85A and antigen 85B, and their sensitivity pattern by detection of rpoB gene for rifampicin resistance, katG and inhA gene for isoniazid resistance in TB patient and also detect the prevalence of the multidrug-resistance (MDR). Resistance to rifampicin is caused by single point mutation in rpoB gene encoding the RNA polymerase. More than 90% of cases in rpoB gene mutation occur in 81 bp region which is referred to as rifampicin resistant determining region. In katG gene, 50-95% of mutation occur at S315J in codon 315, and in inhA gene, 20-35% mutation occur at C15T in codon -15 s.

Materials and Methods: A total of 70 smear positive sputum samples were processed for the detection of the MDR status and to detect their mutation pattern. Samples were processed by hybridization to detect their susceptibility pattern.

Result: In our study, 70 clinical smear positive sputum samples were taken, out of which, 8 (11.42%) samples were MDR (resistant to rifampicin and isoniazid), 2 (2.85%) samples were rifampicin resistant but isoniazid sensitive, 6 (8.57%) samples were isoniazid resistant but rifampicin sensitive, and rest 54 (77.14%) samples were sensitive to both rifampicin and isoniazid.

Conclusion: The GenoType MTBDRplus (LPAs) assay is a rapid and reliable diagnostic test on positive sputum samples, and LPAs are highly sensitive ($\geq 97\%$) and specific ($\geq 99\%$) for the detection of rifampicin and isoniazid resistance. Earlier diagnosis of MDR tuberculosis by hybridization and give the proper treatment to infected patients.

Key words: Line probe assay, inhA, katG, Polymerase chain reaction, rpoB

INTRODUCTION

Tuberculosis (TB) disease is caused by infectious organism *Mycobacterium tuberculosis* which mainly affects the lungs and respiratory system. Drug-resistant

M. tuberculosis is a global health problem. The World Health Organization (WHO) estimates that one-third of the world's population is infected by *M. tuberculosis*, 9 million new cases of active TB, and 2-3 million deaths occur annually.¹

The term multidrug-resistance (MDR) refers to resistance to rifampicin and isoniazid with or without resistance one or more other drugs, which are the most effective drugs recommended by the WHO.²

Resistant to rifampicin can be used as a marker for MDR-TB. Rifampicin resistant is caused by single point

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mutations in *rpoB* gene encoding the RNA polymerase. In 98% cases, mutations occur in 81-bp region which is referred to as rifampicin resistance determining region (RRDR) in *rpoB* gene.³

The mutation causing isoniazid resistance are located in 2 genes and regions and reported that 50-95% of isoniazid resistant contain mutation at S315J in codon 315 of the *katG* gene encoding the catalase-peroxidase and 20-35% mutation in the *inhA* region.^{4,5}

On smear positive sputum samples, detection of the drug resistance mutation pattern by molecular methods can be performed within 1-2 days, traditionally drug susceptibility testing takes 6-8 weeks.

MATERIALS AND METHODS

Specimen Collection

During the period of July 2015 to April 2016, 70 smear positive sputum samples were collected from the DOTS center of Teerthanker Mahaveer Hospital, and these samples were processed for the drug susceptibility testing for rifampicin and isoniazid in the Molecular Laboratory of the Microbiology Department, Teerthanker Mahaveer Medical College and Research, Moradabad, Uttar Pradesh, India.

Decontamination

Sputum specimens were processed for decontamination by mixing N-acetyl-L-cysteine and NaOH in the specimen and incubate for 15 min. After that, phosphate buffer was added to the specimen and centrifugation was done for 15 min at 3000 g. Then, discard the supernatant and re-suspend the pellet in 1 ml phosphate buffer.^{6,7}

DNA Extraction

About 500 µl of the decontaminated specimen was processed in the microcentrifuge (13,000 rpm for 15 min at room temp). The supernatant was discarded, and the pellet was re-suspended in 100 µl of distilled water and then inactivates the bacteria by incubating in a heating block for 20 min at 95°C. After that, cells were sonicated in an ultrasonic bath for 15 min and centrifuge for 5 min at 13,000 rpm.^{6,7}

DNA Amplification

Amplification was performed by combining 35 µl of primer nucleotide mix (PNM A) and 10 µl of primer nucleotide mix (PNM B). 5 µl extracted DNA was mixed in the master mixture (A and B). After that, this mixture was kept in the thermocycler for the amplification of the bacterial DNA.^{6,7}

15 min	95°C	1 cycle
30 s	95°C	
2 min	65°C	20 cycles
25 s	95°C	
40 s	50°C	
40 s	70°C	30 cycles
8 min	70°C	1 cycle

Hybridization

Hybridization was performed manually using twin cubator/shaking water bath at 45°C.

Procedure for Hybridization⁸

1. About 20 µl of denaturation solution (DEN blue) is dispensed in an each corner of the wells used
2. About 20 µl of amplified samples are added to the denaturation solution by the help of pipette up and down and incubate for 5 min at room temperature
3. About 1 ml of pre-warmed hybridization buffer (HYB, green) is added to each well and shakes the try gently until the solution has a homogenous color
4. A strip is placed in an each well
5. Place the try in shaking water bath/twin cubator and incubate at 45°C for 20 min
6. Completely aspirate hybridization buffer by pipette
7. Add 1 ml of stringent wash solution (SAT, red) to each

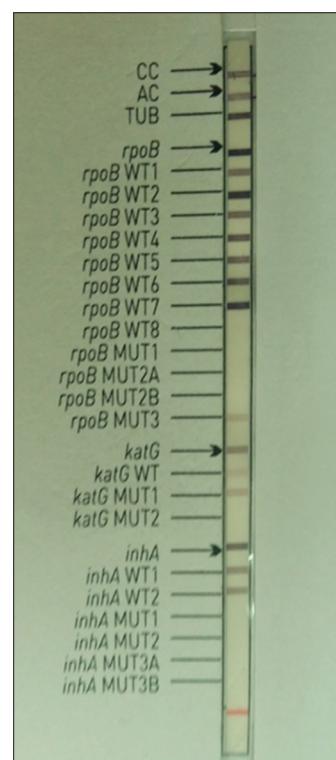


Figure 1: *Mycobacterium tuberculosis* complex is encoded by antigen 85A and antigen 85B. CC: Conjugate control, AC: Amplification control. *rpoB*, *katG*, and *inhA* are the genes of *M. tuberculosis* that can be detected by hybridization

- strip and incubate at 45°C for 15 min in shaking water bath/twin cubator
8. Work at room temperature from this step forwards and removes completely stringent wash solution
 9. Wash each strip with 1 ml of rinse solution (RIN) once for 1 min on shaking platform/twin cubator (pour out RIN after incubation)
 10. Add 1 ml diluted conjugate to each strip and incubate on shaking platform/twin cubator for 30 min
 11. Remove the solution and wash each strip twice with 1 ml of rinse solution (RIN) for 1 min and once with approximately 1 ml of distilled water for 1 min, on shaking platform
 12. About 1 ml of diluted substrate is added to each strip and incubate protected from light without shaking
 13. As soon as bands are clearly visible, stop the reaction by briefly rinsing twice with distilled water
 14. Using tweezers remove strips from the try and dry them between two layers of absorbent paper.

RESULT AND OBSERVATION

During this study period, total 70 smear positive samples were taken, out of this, 48 were male and 22 were female. 8 sputum samples were MDR (resistant to rifampicin

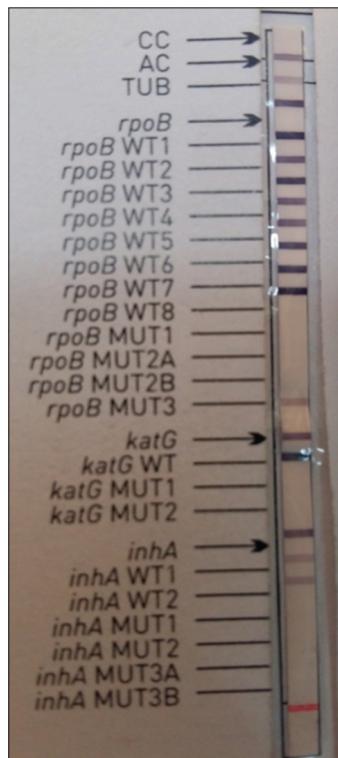


Figure 2: In *rpoB* gene, wild type 8 band is absent and mutation band 3 is developed that's mean the test strain is resistance to rifampicin. In *katG* and *inhA*, all wild type bands are developed with locus control and no mutation band is developed that's mean the test strain is sensitive to isoniazid

and isoniazid) (Figure 1) among with 6 were male and 2 were female. Rifampicin resistant was found in 2 samples (Figure 2) among with 1 was male and 1 was female. Resistant to isoniazid was found in 6 (Figure 3) samples among with 5 were male and 1 was female out of 70 samples and rest 48 samples were sensitive to both rifampicin and isoniazid (Figure 4) among with 36 were male and 18 were female.

Total sample	MDR	RIF resistant	INH resistant	Sensitive to RIF and INH
70	8	2	6	54
Male - 48	Male - 6	Male - 1	Male - 5	Male - 36
Female - 22	Female - 2	Female - 1	Female - 1	Female - 18

In *rpoB* gene, wild type 8 band is absent and mutation band 3 is developed that's mean the test strain is resistance to rifampicin, and in *inhA* gene, mutation band 1 is developed that's mean the test strain is resistance to isoniazid. In this test strain, both drugs are resistance that's called MDR.

Mutation Pattern in *rpoB* Gene for Rifampicin⁸

Out of 70 sputum samples, 10 were resistant to rifampicin. Mutation found in *rpoB* at S531L, codon analyzed 530-533 in 8 samples (Figure 1 and 2). In 1 sample mutation found at D516Y, codon analyzed 513-519 and 1 sample mutation found at L511P, codon analyzed 510-513.

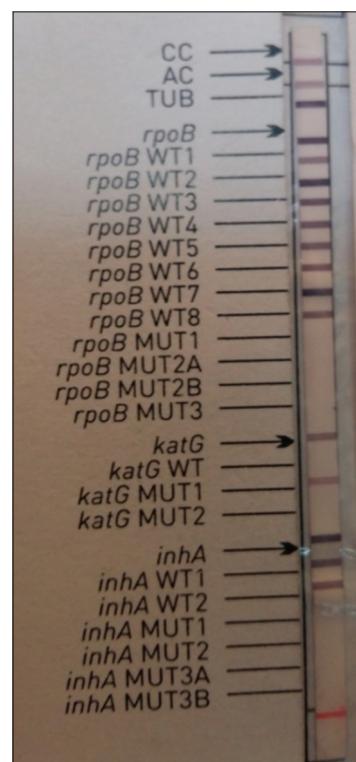


Figure 3: In *rpoB* gene, all wild type bands are developed and no mutation band is developed that's mean the test strain is sensitive to rifampicin. In *katG*, wild type band is absent and mutation 1 is developed that's mean the test strain is resistance to isoniazid

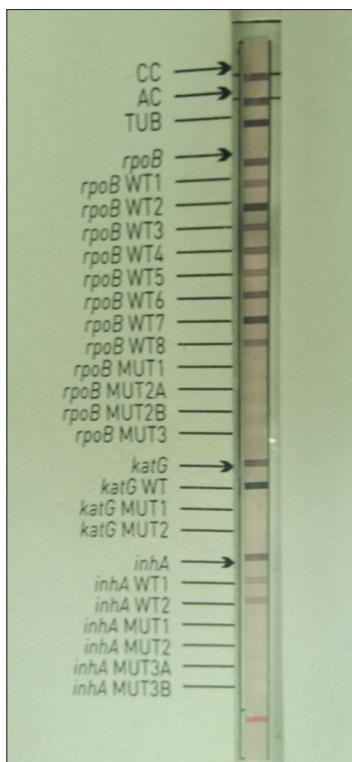


Figure 4: All wild type bands are developed for *rpoB*, *katG*, and *inhA* with locus control and no mutation band is developed that's mean the test strain is sensitive to rifampicin and isoniazid

Mutation Pattern in *katG* and *inhA* Gene for Isoniazid⁸

About 14 sputum samples were resistant to isoniazid out of 70. In 11 samples mutation found in *katG* at S315T1, codon analyzed 315 (Figure 1 and 3) and in 3 samples mutation found in *inhA* at C15T, analyzed nucleic acid position - 15.

DISCUSSION

The WHO reported that 8.8 million new TB cases found every year and 440,000 were forms of the disease that are MDR-TB. MDR-TB is a higher mortality and treatment Failure.⁹ Treatment of the MDR-TB is depending on the quicker detection of the drugs susceptibility pattern, and effective drug therapy is available. Using phenotypic methods, it takes 4-8 weeks to give the drug susceptibility pattern. In the present study, using polymerase chain reaction and line probe assay, it takes 1-2 days to detect the MDR status of the patient. In our study, patients belong to rural areas were highly infected from pulmonary TB and mainly the age group is found 40-70 years. Patients having pulmonary TB are chronic smokers.^{11,12}

Genetic studies has demonstrated that more than 90% of the resistance to rifampicin is associated with single point mutation in the RNA polymerase gene (*rpoB*) comprising a defined region of 81-base pair (bp) which is referred

to as RRDR (Figure 1 and 2) In contrast, resistance to isoniazid is linked to mutation occurring in *inhA* gene and by mutation of the catalase-peroxidase gene (*katG*). However, among them *katG* (catalase enzyme) mutation is more common than *inhA* (isoniazid) resistance which is 50-95% (Figure 1 and 3).^{13,14}

In our study, total 70 samples were collected from DOT's center, and all samples are smear positive. Out of 70 samples, 8 were MDR (resistance to rifampicin and isoniazid), 2 samples were resistance to rifampicin but sensitive to isoniazid, so total 10 samples were resistance to rifampicin and in 8 samples mutation found in *rpoB* at S531L, codon analyzed 530-533. In 1 sample mutation found at D516Y, codon analyzed D516Y, codon analyzed 513-519 and rest 1 sample mutation found at L511P, codon analyzed 510-513. 6 samples were resistance to isoniazid but sensitive to rifampicin. Total 14 samples were resistance to isoniazid, mutation found for *katG* in 11 samples at S515T1, codon analyzed 315 and in 3 samples mutation found for *inhA* at C15T, analyzed nucleic acid position - 15.

CONCLUSION

The conclusion of this study that the GenoType MDR plus assay is rapid diagnostic method to detect the MDR status from clinical sputum samples and also detect the mutation pattern in *rpoB* gene for rifampicin, *katG* gene and *inhA* gene for isoniazid. Mutation in the *rpoB* gene is responsible for the rifampicin resistance, mutation in the *katG* and *inhA* gene is responsible for isoniazid resistance and mutation in all genes at the same time that is resistance to both isoniazid and rifampicin (MDR).

Increasing the rate of the MDR-TB is a big problem, and earlier diagnosis is needed to done by molecular methods and also need, control to spread the infection by giving the proper anti-tubercular therapy.

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