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Original Research Article

Determining *RPO* Gene mutation responsible for rifampicin resistance in pulmonary tuberculosis assessed by line probe assay

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Abstract

Introduction: *Mycobacterium tuberculosis* causes TB illness, which is increasingly widespread. The *Geno Type MTBD Rplus* assay or line probe assay (LPA) detects the most prevalent genetic changes generating rifampicin (RIF) resistance at *(rpoB* gene mutations) codons 516, 526, and 531, as well as *Mycobacterium tuberculosis*.

Materials and Methods: The study was conducted between February 2023 and October 2023. The sample was obtained from RMCH and processed at the Intermediate Reference Laboratory (IRL) at KGMU Lucknow, Uttar Pradesh, India. A total of 98 acid-fast bacilli (AFB) smear-positive sputum samples were processed by LPA.

Result: This *Geno Type MTBDR plus assay* was employed for analyzing 98 acid-fast bacilli smear-positive sputum samples, and of these, 25 samples had no *Mycobacterium tuberculosis* detected, 8 samples were RIF resistant, 2 indeterminate samples were found, and 63 samples were found to be susceptible to RIF. The assay indicated corresponding resistance rates of 10.95 % (8/73). The most frequently identified genetic mutation responsible for RIF's resistance was the 531 codon of the *rpoB* gene, and that was found in 10.95 % (08/73) of rifampicin-resistant strains.

Conclusion: In conclusion, the Bareilly region had a higher prevalence rate of rifampicin resistance, reaching 10.95%. This study underscores the use of LPA with its short turnaround time and excellent specificity and sensitivity for detecting MDR TB.

Keywords: Rifampicin resistance, Line probe assay, *Mycobacterium tuberculosis*, Pulmonary tuberculosis, *rpo* gene mutation.

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1. Introduction

Mycobacterium tuberculosis causes an illness that is increasingly widespread in every nation because it spreads by aerosol when speaking, coughing, or singing.[1] Every country is operating a campaign for controlling and vigilance of tuberculosis (TB) called the National Tuberculosis Eradication Plan (NTEP) [2] to combat this illness because of the frequent genetic mutation, which has resulted in many drug resistance instances, making treatment harder and causing more deaths.[2] The recent rise of drug-resistant strains as a result of ineffective treatment and enhanced resistance diffusion presents a significant challenge to international efforts to reduce tuberculosis.[1] According to the World Health Organization, around half a million cases of multidrug- resistant tuberculosis (MDR-TB) occur each year.[2] Drug-resistant TB strains must thus be diagnosed as soon as possible.[3] Rifampicin (RIF) is the primary drug used to treat tuberculosis.[2] It acts as a surrogate marker for MDR-TB detection.[2] But results from traditional culture-based drug susceptibility testing techniques are obtained in 4-6 weeks.[4] Patients run the danger of immediately spreading the resistant illness to others while using totally useless chemotherapy at this period. As well as strong laboratory testing and knowledgeable, committed staff are necessary for the diagnosis of tuberculosis (TB), as well as for treatment accuracy and oversight.[5]

Thus, the *GenoTypeMTBDRplus test (Hain Life Science, Nehren, Germany)*, a publicly accessible LPA, has been

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approved by the World Health Organization (WHO).[2] The *GenoType MTBDRplus assay* detects the most prevalent genetic changes generating rifampicin resistance at (*rpoB* gene mutations) codons 516, 526, and 531, as well as *Mycobacterium tuberculosis*.[6]

Ser531Leu of the *rpoB* gene was observed in 63.3% (62/98) of the patients. The next most common *rpoB* mutation reported was Asp516Val, which was found in 21.4% (21/98) of the studied specimens. His526Tyr was found in 12.2% (12/98), and His526Tyr and Ser531Leu together in 3.1% (3/98).[5]

Rifampicin binds to the RNA polymerase subunit within the DNA channel but away from the active site and, by a steric-occlusion mechanism, prevents the production of the second and third phosphodiester bonds between the nucleotides in the nucleotide backbone.[8,14]

This prevents the 5′ end of the RNA transcript from becoming elongated, which inhibits host bacterial protein production and kills mycobacteria.[2] A component of the bacterial RNA polymerase is encoded by the *rpoB* gene. Although this gene is present in a small number of bacterial strains, different bacterial species, including mycobacteria, have different nucleotide sequences and lengths.[9]

Drug (rifampicin) resistance in bacteria, including mycobacteria, is caused by a mutation in the *rpoB* gene that changes the structure of proteins, particularly in the 81 bp hotspot core region called the "rifampicin resistance determining region," or RRDR. [10] The bulk of rifampicin resistance mutations in *Mycobacterium tuberculosis* occur in cluster I, in the 81 bp hotspot core region known as the RRDR.[8]

2. Materials and Methods

2.1. Study duration and region for sample processing

The investigation was conducted between February 2023 and October 2023. The sample was obtained from Rohilkhand Medical College and Hospital Bareilly Uttar Pradesh, India and processed at the Intermediate Reference Laboratory (IRL) at KGMU Lucknow, Uttar Pradesh, India

2.2. Ethical consideration

The study was approved by the university Ethics Committee before commencing the study vide memo no. BIU/REG/PhD/390 dated 26 05, 2022. Individual patient consent was taken as it was a retrospective record-based study.

2.3. Statistical analysis

The data was analyzed with SPSS 23.0 version. To find the Significance in categorical data, Chi square was used.

2.4. Specimen collection and processing

At Rohilkhand Medical College and Hospital, 98 sputum samples were taken from the Direct Microscopy Centre unit. Diabetic, HIV and smoker patient also considered in the study.

2.5. Specimen collection

The 2 sputum samples was collected as per the NTEP.[6,7]

In the Pulmonary Tuberculosis two sputum samples were recommended spot sample (collected on the same day under supervision) and early morning sample collected on the next day) alternatively 2 spot samples at least one hours apart can be collected. Sputum was at least 2- 5 ml in quantity and preferably mucopurulent.[12]

2.6. Specimen processing

These sputum samples that tested positive for Ziehl-Neelson staining and fluorescence microscopy were sent to an intermediate reference laboratory for further processing in order to detect multidrug-resistant TB using a line probe assay.[6]

2.7. Transportation of specimen for line probe assay

Using a cold chain, the specimens were delivered in a 50 ml centrifuge tube to the IRL at KGMU Lucknow in 7 days without the use of any preservative.[6]

2.8. Line probe assay

1. The decontamination and digestion of sputum sample by equal volume of combine solution of Trisodium citrate buffer and NAOH solution added with N- Acetyl-L-cysteine (NALC 0.25mg) after 15 minute add phosphate buffer solution (PBS) after that centrifuge then supernated part discarded and base pallet added with PBS mix than transfer into cryo vial 1 ml. this ready for DNA extraction.[12]
2. DNA extraction: DNA extraction was conducted with the Genotype *MTBDRplus* (Hain Lifescience GmbH) Genolyse version 1.0 kit from the processed specimen.[21]
3. Making master mix: Making master mix for the first LPA master mix in accordance with the manufacturer's instructions for the Genotype *MTBDRplus* (Hain Lifescience GmbH).[21]
4. PCR amplification: The mixture was then placed in a thermocycler to amplify the bacterial DNA.[21]
5. Hybridization using GT- BLOT: A quick reference to hybridization of amplified DNA to specific DNA probes bound on membrane strips. Conjugate binding and substrate reaction to detect colored precipitate on membrane strip. This method allows for the visualization of specific DNA sequences, enabling researchers to identify the presence of targeted genes quickly and efficiently. The resulting colored precipitate provides a clear indication of successful hybridization, facilitating easier analysis and interpretation of the results.[6]

Amplification cycle

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

3. Result

This *GenoType MTBDRplus assay* was employed for analyzing 98 acid-fast bacilli smear-positive sputum samples. The assay indicated corresponding resistance rates of 10.95 % (8/73) as shown in figure no 2. The most frequently identified genetic mutation responsible for RIF's resistance was the 531 codon of the *rpoB* gene. All finding are illustrated in figures and tables.

This finding highlights the importance of genetic testing in identifying resistant strains of *Mycobacterium tuberculosis*, which can significantly impact treatment strategies. Further research is needed to explore additional mutations and their association with resistance to other first-line antitubercular drugs.(**Table 1**)

In our study, MTB is present 73 i.e. 74.48% (resistance 8, {5 female and 3 male} sensitive 63 and 2 sample indeterminate) among 46 males and 27 females. There is significant statistical association of gender with MTB (p-value is 0.001).(**Table 2**)

In our study, 8 MTB cases are rifampicin resistant. There is highly significant statistical association of rifampicin resistance with MTB (p-value is 0.001).(**Table 3**)

In our study, smoking is present in 19 MTB cases. There is highly significant statistical association of smoking with MTB (p-value is 0.001).(**Table 4**)

In our study, diabetes is present in 13 MTB cases. There is highly significant statistical association of diabetes with MTB (p-value is 0.001).(**Table 5**)

Table 1: Master chart of whole result

Age Group	Total	Gender based	Rifampicin sensitive	Rifampicin resistance associated gene interpretation by line probe assay	Smoke Status (Yes/No)	Diabetes Status (D/ND)	HIV status (R/NR)
1-10	02	M – 1	Sensitive	-	-	-	-
		F – 1	Sensitive	-	-	-	-
11-20	14	M – 8	Sensitive	-	-	-	-
		F – 6	Sensitive	WT8, MUT3	No	D	NR
21-30	14	M – 7	-	WT8, MUT3	Yes	D	NR
		F – 7	-	WT 3,4 WT3,4	No	ND	NR
31-40	14	M – 10	Sensitive	-	-	-	-
		F – 4	-	WT 3,4, MUT 1 WT8	No No	ND D	NR NR
41-50	15	M – 11	-	WT3,4 MUT1	No No	ND ND	NR NR
		F – 4	Sensitive	-			
51-60	16	M – 13	Sensitive	-			
		F – 3	Sensitive	-			
61-70	18	M – 16	Sensitive	WT8, MUT3 WT3,4	Yes	ND	NR
		F – 2	-				
71-80	04	M – 4	Sensitive	-			
		F – 0	Sensitive	-			
81-90	01	M – 1	Sensitive	-			
		F – 0	Sensitive	-			

Table 2: Gender distribution of study subjects

Gender	MTB Present	MTB Absent	p-value
Male	46 (63.01%)	21 (84%)	0.001
Female	27 (36.98%)	4 (16%)	

Table 3: Rifampicin resistance among study subjects

	Indeterminate	Resistant	Sensitive	p-value
MTB Present	2 (2.73%)	8 (10.95%)	63 (86.30%)	0.001
MTB Absent	0 (0.0%)	0 (0.0%)	25 (100%)	

Table 4: Smoking among study subjects

Smoking	MTB Present	MTB Absent	p-value
Present	19 (26.02%)	10 (40%)	0.001
Absent	54 (73.97%)	15 (60%)	

Table 5: Diabetes among study subjects

Diabetes	MTB Present	MTB Absent	p-value
Present	13 (17.80%)	2 (8%)	0.001
Absent	60 (82.19%)	23 (92%)	

4. Discussion

This study indicates the position of mutation on the *rpoB* gene in rifampicin-resistant patients at a tertiary care centre in Bareilly. A study was done from February 2023 to October 2023 on 98 tuberculosis cases detected via fluorescent microscopy and ZN staining and confirmed by LPA. 8 samples have the rifampicin drug resistance gene common missense mutation at (WT8, MUT3) mutation at 531 531bp {73/3} (2.19%), (WT 3, 3,4,MUT 1), MUT1 {73/2} mutation at 516 bp (1.36%). Interpretation given in the result with or without isoniazid was observed in 8 samples. Singhala R. et al.'s study found that among 137 rifampicin-resistant strains identified, *rpoB* was detected in 100 (73%) strains. The most common known mutation was 531 in 80/137, followed by D516V (8/137) and H526Y (7/137).[18]

The findings highlight the prevalence of specific mutations associated with drug resistance, emphasizing the importance of genetic testing in guiding treatment options. Further research is needed to explore the implications of these mutations on treatment efficacy and patient outcomes.[18]

The study of Nandwani et al. said that out of 141 samples, 59 (41.84%) were found to be RIF resistant, with 54 also showing resistance to LPA. The most prevalent mutation in RIF resistance was missing wild type 8 (codons 530-533) and related MUT3, resulting in S531L amino acid substitution (n=43, 79.6%), followed by codons 526-529-WT7 (n=4, 7.4%), 513-519-WT3/4 (n=3, 5.5%), D517V-MUT1 (n=1, 1.8%), and codons.[19]

According to Yadav et al., the majority of erroneous findings from the genotype MTBDR plus version 2 LPA were found in culture-negative samples or sputum specimens with a reduced bacillary load (1+).[20]

LPA is a rigorous approach for identification of drugresistant TB, which has given the basis for speedy and successful control of drug-resistant TB in India.

The reasons for concomitantly negative LPA and culture findings of smearpositive sputum samples from MDRTB suspects are numerous; nonetheless, the existence of nontubercular mycobacteria in these samples and the likelihood of ineffective treatment in these individuals cannot be ruled out.[17]

5. Conclusion

In conclusion, the Bareilly region had a higher prevalence rate of rifampicin resistance, reaching 10.95%. More research is needed on medication resistance in *Mycobacterium tuberculosis.*[14] In this study, the most prevalent rifampicin resistance mutation was found in the 531 region of the *rpoB* gene. This study underscores the use of the LPA with its short turnaround time and excellent specificity and sensitivity for detecting MDR TB.

Because it spreads rapidly through infected persons from air, it is suspended in air for more time because of its small size. Airborne particles (<5 µm) linger in the air and 'pollute' areas with inadequate ventilation,[16] a more serious issue in the world. In this study, the most common mutation is the S531L amino acid substitution of *the rpoB* gene for rifampicin resistance.[16]

The LPA or *Genotype MTBDRplus* is a ground breaking invention for diagnosing drug-resistant tuberculosis. Which encourage the identification and control of drug-resistant TB. This study underscores the use of LPA with its short turnaround time and excellent specificity and sensitivity for detecting MDR TB. However, LPA cannot totally replace

phenotypic culture techniques, as seen by the rementioned data.[21]

Silent mutations may confound clinical interpretation, and phenotypic DST is recommended in these circumstances. One disadvantage of this work was the inability to conduct phenotypic DST owing to time and manpower restrictions. To summarize, the LPA undoubtedly assists in the earlier identification and effective care of nevertheless, as with other molecular tests, a prudent decision to augment LPA with traditional techniques should be made when necessary.[17]

6. Source of Funding

None.

7. Conflict of Interest

None.

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