International Journal of Medical and Pharmaceutical Research

Online ISSN-2958-3683 | Print ISSN-2958-3675
Frequency: Bi-Monthly
Available online on: https://ijmpr.in/

Original Article

Drug Resistance Patterns in Tuberculosis: Molecular Analysis Using Line Probe Assay in CBNAAT-Positive Samples from North-West India

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Received: 01-04-2026
Accepted: 05-05-2026
Available online: 19-05-2026

ABSTRACT

Background: Drug-resistant tuberculosis (DR-TB) poses a significant threat to global tuberculosis control efforts. Rapid molecular diagnostic methods, particularly Line Probe Assay (LPA), are essential for early detection and management of drug resistance patterns.
Aim: To determine first and second-line drug resistance patterns using LPA in CBNAAT-positive sputum samples from a tertiary care centre in north-west Rajasthan, India.
Method: A cross-sectional study was conducted from August 2022 to January 2024 at Sardar Patel Medical College, Bikaner. A total of 300 CBNAAT-positive sputum samples were included in the study. All CBNAAT-positive samples were first subjected to fluorescent staining for smear microscopy, after which Line Probe Assay (LPA) was performed. Drug-resistance profiling was carried out using first-line and second-line LPA (GenoType MTBDRplus and GenoType MTBDRsl assays) according to standardized laboratory protocols.
Results: The study population had a mean age of 42.77±18.34 years, with 61.67% males and 62.3% from rural areas. First-line drug resistance was detected in 16.67% of samples: rifampicin mono-resistance (3%), isoniazid mono-resistance (7.33%), and multidrug resistance (6.33%). Second-line drug resistance was observed in 29% of samples, with fluoroquinolone resistance being most common (24.67%).
Conclusion: LPA demonstrates excellent diagnostic performance for rapid detection of drug resistance patterns in tuberculosis. The high prevalence of fluoroquinolone resistance (24.67%) in this region is concerning and necessitates enhanced surveillance and infection control measures.

Keywords: *Tuberculosis, Drug resistance, Line probe assay, CBNAAT, Molecular diagnostics, MDR-TB.*

INTRODUCTION

Tuberculosis (TB) remains one of the leading infectious disease killers globally, with approximately 10 million new cases annually[1]. The emergence of drug-resistant tuberculosis, particularly multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), poses a significant challenge to global tuberculosis elimination goals[2]. India accounts for 27% of the global tuberculosis burden and harbours the highest number of MDR-TB cases worldwide[3].

Drug resistance in *Mycobacterium tuberculosis* arises primarily through chromosomal mutations in genes encoding drug targets or enzymes involved in drug activation[4]. Rifampicin resistance is predominantly associated with mutations in the *rpoB* gene, specifically within the 81-base pair rifampicin resistance-determining region (RRDR)[5]. Isoniazid resistance occurs through mutations in the *katG* gene, which encodes catalase-peroxidase, or in the *inhA* promoter region[6].

Second-line drug resistance involves fluoroquinolones and second-line injectable drugs (aminoglycosides). Fluoroquinolone resistance is mediated by mutations in the *gyrA* and *gyrB* genes encoding DNA gyrase subunits[7]. Resistance to second-line injectable drugs (amikacin, kanamycin, capreomycin) is associated with mutations in the *rrs* gene encoding 16S rRNA, the *eis* promoter region, or the *tlyA* gene[8].

The World Health Organization (WHO) recommended Line Probe Assay (LPA) in 2008 as a rapid molecular diagnostic tool for detecting TB and drug resistance[9]. LPA combines polymerase chain reaction (PCR) amplification with reverse hybridization technology, providing results within 24–48 hours compared to 6–8 weeks for conventional culture-based drug susceptibility testing[10].

Aim: To determine first and second-line drug resistance patterns using LPA in CBNAAT-positive sputum samples from a tertiary care centre in north-west Rajasthan, India.

METHODS

Study Design and Setting

This cross-sectional study was conducted in the Department of Clinical Microbiology and Immunology at Sardar Patel Medical College and Associated Group of Hospitals, Bikaner, Rajasthan, India, between August 2022 and January 2024. The laboratory functions as an Intermediate Reference Laboratory (IRL) under the National Tuberculosis Elimination Programme (NTEP) and is accredited for both molecular and culture-based tuberculosis diagnostics.

Processing of Sputum Specimens

Fresh sputum specimens were processed using the N-acetyl-L-cysteine–sodium hydroxide (NALC–NaOH) method for decontamination and homogenization. NALC acts as a mucolytic agent to liquefy the sputum, while NaOH serves as a decontaminating agent to eliminate non-mycobacterial organisms. Sodium citrate was included to chelate heavy metal ions that may inactivate NALC.

An equal volume of freshly prepared NALC–NaOH–sodium citrate solution was added to the sputum specimen and mixed thoroughly by vortexing. The mixture was allowed to stand at room temperature for approximately 10 minutes for digestion and decontamination. Subsequently, phosphate buffer (pH 6.8) was added up to the 50 mL mark to neutralize the reaction and reduce the specific gravity of the mixture. The samples were then centrifuged at 3000 × g for 15–20 minutes.

After centrifugation, the supernatant was carefully decanted, and the sediment was resuspended in approximately 1.5 mL of phosphate buffer (pH 6.8). The resuspended pellet was used for smear preparation for fluorescent microscopy and for DNA extraction for Line Probe Assay (LPA)[11].

Fluorescent Microscopy

Smears were prepared from the processed specimen sediment on clean, grease-free glass slides. The smear was spread evenly in an oval area at the center of the slide and allowed to air dry at room temperature. The slides were then heat fixed by passing them briefly over a flame.

Smears were stained using the Auramine-O fluorescent staining method. Slides were flooded with 0.1% Auramine-O stain and allowed to stain for approximately 20 minutes. After rinsing with water, the slides were decolorized using 0.5% acid-alcohol for 3 minutes, followed by washing with water. The smears were then counterstained with 0.5% potassium permanganate for 1 minute, rinsed, and allowed to air dry.

The stained smears were examined under a fluorescence microscope, initially using the 20× objective for focusing and subsequently the 40× objective for examination. Acid-fast bacilli (AFB) appeared as bright yellow fluorescent bacilli against a dark background (Fig.1). Smear results were recorded according to the standard grading criteria for fluorescent microscopy[12].

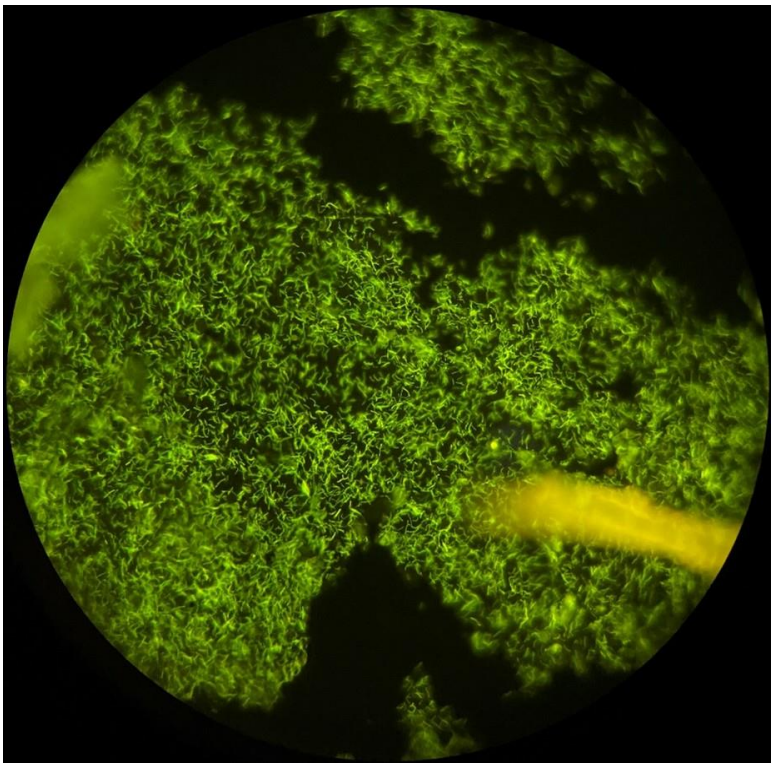


Fig 1 Fluorescence microscopy of sputum smear stained with Auramine-O showing numerous bright yellow-green, slender acid-fast bacilli against a dark background, suggestive of *Mycobacterium tuberculosis*. Image captured at 40× magnification at the Intermediate Reference Laboratory (IRL) for Tuberculosis.

Line Probe Assay Methodology

a) First-Line LPA

DNA was extracted from decontaminated sputum sediments using the GenoLyse kit (Hain Lifescience, Germany). The GenoType MTBDRplus assay (version 2.0; Hain Lifescience) was employed to detect resistance to rifampicin (RIF) and isoniazid (INH). The assay targets the *rpoB* gene for RIF resistance, and both *katG* and *inhA* promoter regions for INH resistance[4].

b) Second-Line LPA

Samples with confirmed positivity were further tested using GenoType MTBDRsl (version 2.0; Hain Lifescience) for detection of resistance to fluoroquinolones (FQs) and second-line injectable drugs (SLIDs). This assay identifies mutations in *gyrA* and *gyrB* genes (associated with FQ resistance) and in the *rrs*, *eis* promoter, and *tlyA* genes (linked to SLID resistance)[5]

Laboratory Workflow

The LPA procedure consisted of four steps:

i. **DNA Extraction:** Using GenoLyse reagents according to the manufacturer's instructions.
ii. **PCR Amplification:** Biotinylated primers targeting drug-resistance–associated genes were used in a thermal cycler under recommended cycling conditions.
iii. **Hybridization and Detection:** Amplified products were subjected to reverse hybridization on nitrocellulose strips coated with immobilized oligonucleotide probes. Hybridization and washing steps were performed in automated TwinCubator systems.
iv. **Result Interpretation:** Strips were air-dried and visually interpreted using a template provided by the manufacturer. Absence of a wild-type probe signal and/or presence of a mutation probe indicated resistance.

Quality Control

Positive and negative controls provided by the manufacturer were included with each run. Strict adherence to unidirectional workflow was maintained, with physically separated areas for DNA extraction, PCR setup, amplification, and hybridization to avoid contamination. Internal quality assurance was performed periodically in line with NTEP and WHO recommendations.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation, while categorical variables were presented as frequencies and percentages. The Chi-square test was applied to assess the association between demographic variables and drug resistance patterns. A p-value < 0.05 was considered statistically significant.

RESULTS

A total of 300 CBNAAT-positive sputum samples were included in the study. The mean age of the study population was 42.77 ± 18.34 years. The majority of patients belonged to the 21–40-year age group (40%), followed by 41–60 years (32.67%), while 9% were in the 1–20-year age group (Table 1).

Table 1 Distribution of samples according to Age

Age group	**No.**	**%**
1 – 20	27	9.00
21 – 40	120	40.00
41 – 60	98	32.67
>60	55	18.33
Total	300	100.00
Mean ± SD	**42.77 ± 18.34**	

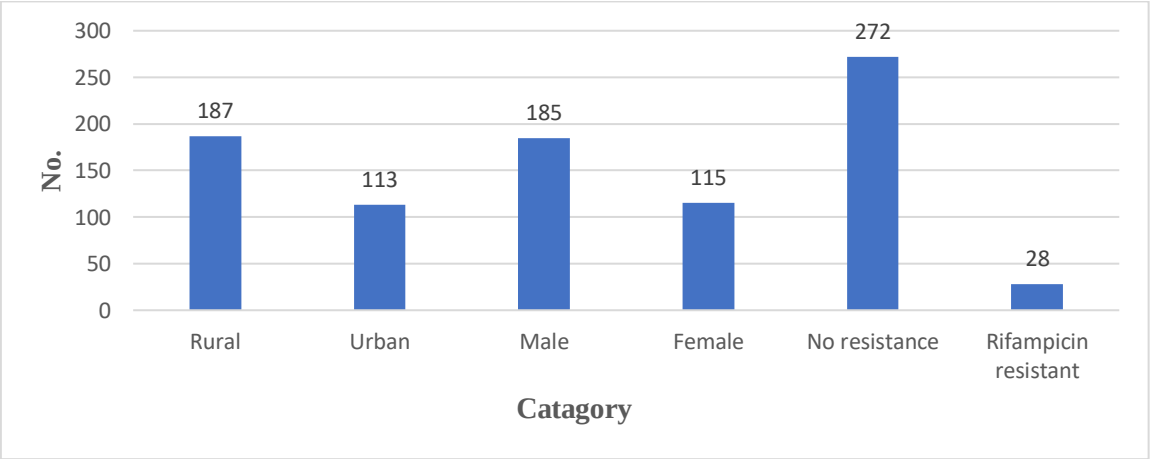
Among the 300 patients, 185 (61.67%) were male and 115 (38.33%) were female. The majority of patients were from rural areas (62.3%), whereas 37.7% belonged to urban areas (Table 2 & Fig 2).

Out of the 300 CBNAAT-positive samples, 272 (90.7%) showed no rifampicin resistance, while 28 (9.3%) were detected as rifampicin-resistant (Table 2 & Fig 2).

Comparison of CBNAAT and first-line Line Probe Assay (LPA) results showed some discrepancies in the detection of rifampicin resistance. While CBNAAT detected rifampicin resistance in 28 (9.3%) samples (Table 2 & Fig 2), first-line LPA identified rifampicin monoresistance in 9 (3%) cases and combined rifampicin and isoniazid resistance in 19 (6.33%) cases, indicating rifampicin resistance in a total of 28 samples (9.33%).

Table 2 Sociodemographic and CBNAAT Profile of Study Participants (n = 300)

Variable	**Category**	**No.**	**%**
Residence	Rural	187	62.3
	Urban	113	37.7
Gender	Male	185	61.67
	Female	115	38.33
CBNAAT	No resistance detected	272	90.7
	Rifampicin resistant	28	9.3
Total	—	**300**	**100**



However, LPA additionally detected mono-isoniazid resistance in 22 (7.33%) samples, which could not be identified by CBNAAT as the assay primarily detects rifampicin resistance only. These findings highlight the added diagnostic value of Line Probe Assay in detecting isoniazid resistance and confirming rifampicin resistance mutations (Table 3 & fig 3).

Table 3 Anti-tubercular drug Resistance pattern according to 1st line LPA

LPA 1st line	**No.**	**%**
Rifampicin only resistant	09	3.00
Mono Isoniazid resistant	22	7.33
Rifampicin isoniazid both	19	6.33
No resistant	250	83.33

Total	**300**	**100.00**

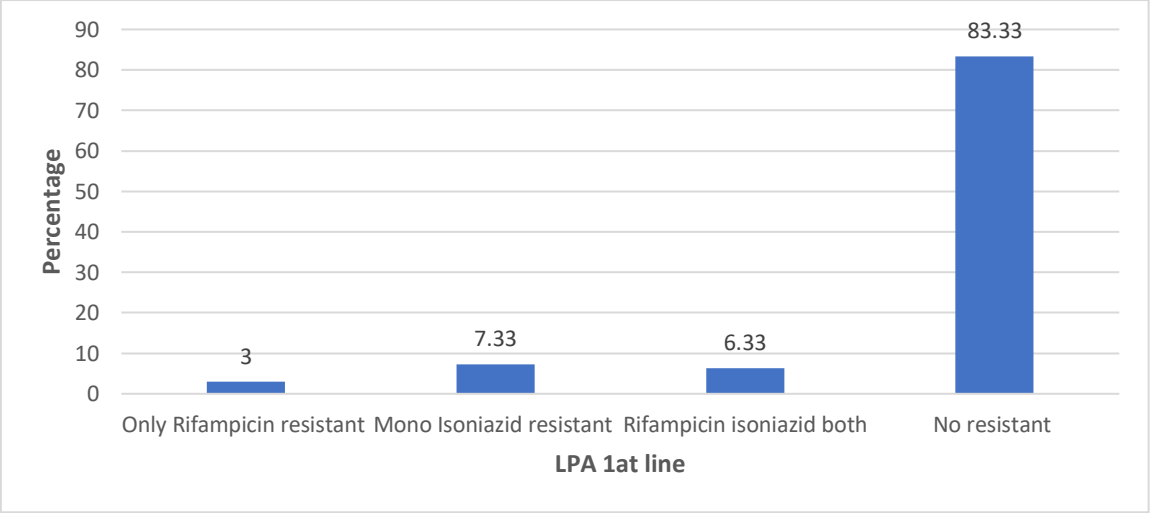


Fig 3- Anti-tubercular drug resistance pattern according to 1st line LPA

Second-line LPA demonstrated that 213 samples (71%) showed no resistance to second-line anti-tubercular drugs. Fluoroquinolone resistance was the most common, detected in 74 cases (24.67%), followed by combined resistance to fluoroquinolones and second-line injectable drugs in 8 cases (2.67%). Resistance to second-line injectable drugs alone was observed in 5 cases (1.67%) (Table 4).

Table 4 Anti-tubercular drug Resistance pattern according to 2nd line LPA

LPA 2nd line	**No.**	**%**
2nd line Injectables resistance	5	1.67
fluroquinolone resistant	74	24.67
fluroquinolone and SLI both resistant	8	2.67
No resistance	213	71.00
Total	**300**	**100%**

DISCUSSION

This study provides crucial epidemiological data on drug resistance patterns in tuberculosis from north-west India using molecular diagnostics. The findings highlight important demographic associations, the burden of first- and second-line drug resistance, and their clinical and public health implications.

In the present study, 300 CBNAAT-positive sputum samples were analyzed. The majority of cases belonged to the 21–40 years age group (40%), followed by 41–60 years (32.67%), with a mean age of 42.77 ± 18.34 years. Similar findings were reported by Mukesh Sharma et al. (2020)[13], who observed the highest prevalence among individuals aged 30–40 years. Comparable age distribution was also reported by R. Singhal et al.[14] and Getu Diriba et al. (2022)[15], where most patients belonged to the economically productive age group. The higher prevalence in this age group may be attributed to increased social and occupational exposure, resulting in greater risk of transmission.

Male predominance was observed in the present study, with 61.67% of cases occurring in males compared to 38.33% in females. Similar observations were reported by Getu Diriba et al. (2022)[15] and Mukesh Sharma et al. (2020)[13], who reported male predominance of 54.3% and 77.85%, respectively. The higher prevalence among males may be related to occupational exposure, lifestyle factors, and healthcare-seeking behavior.

The majority of patients in this study were from rural areas (62.3%), whereas 37.7% were from urban areas. This finding is consistent with the observations of Mukesh Sharma et al. (2020)[13], who reported a higher proportion of TB cases in rural populations (62.15%). Factors such as limited healthcare access, lower awareness, overcrowding, and poor treatment compliance may contribute to the higher burden in rural settings.

First-line LPA results demonstrated that 7.33% of isolates were mono-resistant to isoniazid, 3% were mono-resistant to rifampicin, and 6.33% exhibited multidrug resistance (MDR). The majority of isolates (83.33%) were susceptible to both drugs. Comparable findings were reported by Neetha S. Murthy et al. (2021)[16], who documented isoniazid mono-resistance in 6.5%, rifampicin mono-resistance in 1.5%, and combined resistance in 5.8% of isolates. Higher resistance rates have been reported by Mukesh Sharma et al. (2020)[13] and Prabha Desikan et al. (2017)[17], indicating regional variations in drug resistance patterns.

Second-line LPA revealed fluoroquinolone resistance in 24.6% of cases, resistance to second-line injectable drugs (SLID) in 1.67%, and combined resistance in 2.67% of isolates. Similar trends have been reported in studies from India, where fluoroquinolone resistance ranges between 24% and 59.6%. Sunil Sethi et al. (2020)[18] reported overall fluoroquinolone resistance of 38.6%, The high prevalence of fluoroquinolone resistance in the present study may be attributed to widespread and often inappropriate use of fluoroquinolones for other bacterial infections prior to TB diagnosis.

Age-wise analysis revealed that the highest fluoroquinolone resistance occurred in the 41–60 years age group (45.95%), followed by 21–40 years (35.14%). Resistance to second-line injectable drugs was most common in the 21–40 years age group (80%). Similar demographic trends were reported by Radha Gopalaswamy et al. (2020)[19], who observed a predominance of second-line drug-resistant TB among middle-aged adults, particularly males.

Among the 19 MDR cases identified in this study, 21.05% demonstrated additional fluoroquinolone resistance and 10.5% showed resistance to second-line injectable drugs. Interestingly, fluoroquinolone resistance was also observed among isolates without first-line drug resistance (24.8%), suggesting possible prior exposure to fluoroquinolones and highlighting the need for rational antibiotic use.

Overall, the findings of this study demonstrate a considerable burden of drug-resistant tuberculosis, particularly fluoroquinolone resistance, in the study population. The results emphasize the importance of rapid molecular diagnostics such as LPA for early detection of resistance patterns and effective management of DR-TB under the National Tuberculosis Elimination Programme.

CONCLUSIONS

This study provides important epidemiological insights into the pattern of drug-resistant tuberculosis in north-west India. The findings indicate that tuberculosis and associated drug resistance predominantly affect the economically productive age group, highlighting the significant socio-economic impact of the disease. First-line anti-tubercular drug resistance was observed in 16.67% of cases, while a notable proportion of isolates demonstrated second-line drug resistance (29%), with fluoroquinolone resistance being the most frequently detected pattern. These findings underscore the growing challenge posed by emerging resistance to key anti-tubercular drugs, which may adversely affect treatment outcomes and tuberculosis control efforts.

The study also highlights the complementary role of molecular diagnostic techniques in the early detection of drug-resistant tuberculosis. CBNAAT (Xpert MTB/RIF) provides rapid detection of *Mycobacterium tuberculosis* and rifampicin resistance, whereas Line Probe Assay (LPA) enables identification of specific genetic mutations associated with resistance to rifampicin and isoniazid, allowing a more comprehensive characterization of drug resistance.

The combined use of CBNAAT and LPA therefore facilitates rapid, accurate detection of resistance patterns and supports timely initiation of appropriate individualized treatment. Strengthening the integration of these molecular diagnostic tools within tuberculosis control programs is essential for early detection, effective management of drug-resistant tuberculosis, and reduction in disease transmission, thereby contributing to improved patient outcomes and progress toward tuberculosis elimination.

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