ISSN 0973-2063 (online) 0973-8894 (print)

Bioinformation 22(10): 6390-6395 (2026)

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BIOINFORMATION
Discovery at the interface of physical and biological sciences

BIOMEDICAL INFORMATICS

www.bioinformation.net

Volume 22(10)

Research Article

Received October 1, 2026; Revised October 31, 2026; Accepted October 31, 2026, Published October 31, 2026

DOI: 10.6026/973206300226390

SJIF 2026 (Scientific Journal Impact Factor for 2026) = 8.478
2022 Impact Factor (2023 Clarivate Inc. release) is 1.9

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Citation: Kumari *et al.* Bioinformation 22(10): 6390-6395 (2026)

Line probe assay for isoniazid mono-resistant pulmonary tuberculosis at IGIMS, Patna, India

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ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 22(10): 6390-6395 (2026)
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Abstract:
Isoniazid mono-resistance is a common form of drug-resistant pulmonary tuberculosis and rapid detection is essential to improve treatment outcomes and limit disease transmission. Therefore, it is of interest to evaluate the performance of line probe assay for detecting isoniazid mono-resistance and associated genetic mutations in 81 rifampicin-sensitive pulmonary tuberculosis patients over 18 months, using culture-based drug susceptibility testing as the reference standard. Clinical specimens were analyzed by Ziehl–Neelsen staining, GeneXpert, Mycobacteria Growth Indicator Tube 960 culture, drug susceptibility testing and Line Probe Assay, with diagnostic performance assessed using sensitivity, specificity and chi-square analysis. Line Probe Assay showed rapid and reliable detection of isoniazid mono-resistance and its associated genetic mutations, facilitating earlier identification than conventional culture-based methods. Thus, data support the incorporation of line probe assay into routine diagnostic algorithms for timely initiation of appropriate therapy and improved management of drug-resistant pulmonary tuberculosis.

Keywords: Tuberculosis, isoniazid mono-resistance, line probe assay, MGIT 960, GENEXPERT, katG, inhA

Background:
Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), which remains one of the major causes of morbidity and mortality in the world. The World Health Organization (WHO) estimates that in 2022, there were an estimated 10.6 million new TB cases worldwide, of which nearly 27% were in India **[1].** Although the diagnosis and treatment of TB has improved, the development of drug-resistant TB (DR-TB) has complicated the control of the disease and is a significant global health threat **[2]**. Isoniazid (INH) is one of the most powerful first line anti-tubercular drugs and is an important drug in intensive and continuation phase of TB treatment **[3]**. Isoniazid resistance, especially mono-resistance (resistance to INH without resistance to rifampicin) is now known to be a major clinical issue. Research has shown that the prevalence of INH mono-resistance is 5-15% in different regions, including India **[4]**. Patients who are INH mono-resistant are at greater risk of treatment failure, relapse and progression to multidrug-resistant TB (MDR-TB**) [5]**. Mutations in the katG gene and the promoter region of the inhA gene are the major molecular mechanisms of isoniazid resistance **[6]**. High-level resistance is associated with the katG gene mutation, particularly S315T, while low-level resistance is associated with mutations in the inhA promoter and is also cross-resistant with ethionamide **[7]**. The knowledge of these mutations is essential to help direct the right treatment plan. The gold standards are conventional diagnostic methods like culture on Lowenstein-Jensen medium or liquid culture systems like MGIT 960 and phenotypic drug susceptibility testing (DST). These approaches, however, are labor intensive and take weeks to produce results, thereby postponing treatment decisions **[8]**. Molecular diagnostic methods provide alternative rapid and reliable methods, however. The GeneXpert MTB/RIF assay has transformed TB diagnosis with the ability to detect *M. tuberculosis* and rifampicin resistance in less than two hours. It is not, however, able to detect isoniazid resistance and thus fails to identify a substantial number of drug-resistant cases **[9]**. This restriction highlights the need for other diagnostic tools. Line Probe Assay (LPA) is a fast molecular method that can detect resistance to rifampicin and isoniazid by detecting specific mutations in the genes, endorsed by WHO. It is rapid (24–48 hours) and has been demonstrated to be highly sensitive and specific in several studies **[10]**. LPA detects mutations in the rpoB, katG and inhA genes, which allow the distinction between high-level and low-level INH resistance **[11]**. The early detection of isoniazid mono-resistance by LPA enables timely adjustment of treatment regimens, avoiding treatment failure and transmission of resistant strains. Moreover, understanding of mutation patterns can help in epidemiological surveillance and regional resistance trends **[12]**. Therefore, it is of interest to assess the usefulness of LPA for detection of IMO, to compare LPA with conventional DST and to study the mutation pattern in the pulmonary TB patients.

Materials and Methods:
Study design and setting:
It was a cross sectional study done in the Department of Pulmonary Medicine and Department of Microbiology of Indira Gandhi Institute of Medical Sciences (IGIMS), Patna, which was hospital based.

Study duration:
The study was conducted for 18 months, which included 12 months of sample collection and laboratory investigations**.** The data analysis took 6 months.

Study population and sample size:
Based on the predefined inclusion and exclusion criteria, 81 patients were included in the study that was diagnosed with pulmonary tuberculosis.

Inclusion criteria:
[1] Patients aged >18 years
[2] Confirmed cases of pulmonary TB
[3] The GeneXpert was unable to detect MTB, but it did detect rifampicin resistance.

Exclusion criteria:
[1] Extra-pulmonary tuberculosis cases
[2] Failure to respond to first-line treatment (except for isoniazid)

Sample collection:
[1] **Sputum Samples:** Two samples (spot and early morning) were collected in sterile Falcon tubes (2–5 mL).
[2] **BAL Fluid:** Collected by bronchoscopy (20–50 mL) in selected cases.

Laboratory procedures:
[1] **Smear microscopy (Ziehl-Neelsen Staining):** Performed with standard hot ZN staining technique for detection of acid-fast bacilli (AFB). Slides were viewed under oil immersion (1000×) and results were scored according to the NTEP guidelines.
[2] **GeneXpert MTB/RIF assay:** All samples were tested for the presence of M. tuberculosis and rifampicin sensitivity by real-time PCR of the rpoB gene on GeneXpert.
[3] **Liquid culture (BACTEC MGIT 960):** Processed samples were added to MGIT tubes with OADC and PANTA. Tubes were grown at 37°C and automatically checked for growth by measuring the fluorescence.

[4] Drug susceptibility testing (DST):
DST was carried out for the following first-line drugs in MGIT 960 system:
[1] Isoniazid (0.1 µg/mL)
[2] Rifampicin (1.0 µg/mL)
[3] Ethambutol (5.0 µg/mL)
[4] Pyrazinamide (100 µg/mL)
[5] **Line probe assay (LPA) procedure added:**

[1] Extraction of DNA from processed samples.
[2] PCR amplification with biotin labeled primers
[3] Nitrocellulose strips are used for hybridization.
[4] The presence of mutation bands was detected. Target Genes:
  a) katG → High-level INH resistance
  b) Low level INH resistance (inhA)
  c) rpoB → Rifampicin resistance

The results of the LPA are interpreted as follows:
The resistance pattern was determined by the presence or absence of the wild-type (WT) and mutant (MUT) bands. High-level resistance is due to katG S315T1 mutation InhA promoter mutations = low level resistance

Quality control:
[1] Positive and negative controls were used in each batch
[2] Test performance was validated with internal controls (Amplification and Conjugate control)
[3] Standard biosafety precautions were followed

Statistical analysis:
The data were analyzed using SPSS version 26.0.

Continuous variables:
Mean ± SD**.** Categorical variables → Percentage (%)**.** The chi-square test was used for association.

Ethical considerations:
Institutional ethical clearance was obtained. All participants gave written informed consent. Confidentiality of patient information was respected.

Results:
A total of 81 microbiologically confirmed cases of Tuberculosis were included in the present study for evaluation of isoniazid mono-resistance using the MGIT 960 system and Line Probe Assay (LPA). Demographic variables, clinical risk factors, microbiological findings, molecular mutations and diagnostic performance of LPA were analyzed in detail. The majority of participants belonged to the younger age group of 19–38 years, accounting for 49.38% of the study population. A statistically significant association was observed between younger age and isoniazid mono-resistance ($p<0.001$). Male patients constituted 62.96% of cases, indicating male predominance among resistant TB patients. Rural residents represented 65.43% of the cohort, suggesting a greater burden of disease in rural settings, although the association was not statistically significant ($p=0.191$). Lower-middle socioeconomic class formed the largest subgroup (55.56%). Underweight patients accounted for 23.46% of participants and demonstrated relatively higher resistance rates compared with overweight individuals **(Table 1).** MGIT 960 detected isoniazid mono-resistance in 6 patients (7.41%), whereas LPA identified resistance in 7 patients (8.64%). The slightly higher detection rate by LPA suggests its superior ability to identify molecular mutations associated with resistance before phenotypic expression becomes evident. Both methods demonstrated high concordance in detecting susceptible isolates **(Table 2).** Among resistant isolates detected by LPA, katG mutation was the most common mutation, observed in 6.17% of patients, followed by inhA mutation in 3.70%. Combined mutations involving both inhA and katG genes were observed in one patient (1.23%). The predominance of katG mutations suggests the presence of high-level isoniazid resistance in the majority of resistant isolates **(Table 3).** Smoking and alcohol use were identified in 19.75% and 11.11% of patients respectively, but no

ISSN 0973-2063 (online) 0973-8894 (print)
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statistically significant association with resistance was observed. Diabetes mellitus was present in 32.10% of patients and demonstrated higher frequencies of resistance compared with non-diabetic individuals. Previous anti-tubercular treatment history showed an important clinical trend, with all previously treated cases demonstrating resistance on MGIT. Most samples were sputum specimens (96.30%). ZN smear grading revealed that 29.63% of patients had 1+ smear positivity, whereas 9.88% demonstrated heavy bacillary load (3+) **(Table 4).** CBNAAT showed low bacterial load in 41.98% of patients and bacterial load distribution showed statistical significance (p=0.037). No rifampicin resistance-associated rpoB mutations were identified, confirming the presence of isolated isoniazid mono-resistance. LPA demonstrated excellent diagnostic performance compared with MGIT 960, with sensitivity of 100%, specificity of 98.67% and overall accuracy of 98.77%. The negative predictive value was 100%, indicating strong reliability of LPA in excluding resistance. Positive likelihood ratio of 75 further highlighted the high diagnostic strength of the assay **(Table 5).**

Table 1: Distribution of isoniazid mono-resistance by demographic and clinical variables (n = 81)

Variable	Category	Total n (%)	MGIT Resistant n (%)	LPA inhA n (%)	LPA katG n (%)	p-value
Age Group	19–38 yrs	40 (49.38%)	37 (45.68%)	~15	~22	<0.001*
	39–58 yrs	24 (29.63%)	2 (2.47%)	0	2	
	59–78 yrs	17 (20.99%)	1 (1.23%)	0	1	
Sex	Male	51 (62.96%)	5 (6.17%)	3 (3.70%)	4 (4.94%)	–
	Female	30 (37.04%)	1 (1.23%)	0	1 (1.23%)	
Residence	Rural	53 (65.43%)	4 (4.94%)	2 (2.47%)	4 (4.94%)	0.191
	Urban	28 (34.57%)	2 (2.47%)	1 (1.23%)	1 (1.23%)	
Socioeconomic Status	Lower	11 (13.58%)	2 (2.47%)	0	2 (2.47%)	0.092
	Lower- middle	45 (55.56%)	1 (1.23%)	2 (2.47%)	0	
	Upper- middle	12 (14.81%)	1 (1.23%)	0	2 (2.47%)	
	Upper	13 (16.05%)	2 (2.47%)	1 (1.23%)	1 (1.23%)	
BMI	Underweight	19 (23.46%)	3 (3.70%)	1 (1.23%)	2 (2.47%)	0.235
	Normal	58 (71.60%)	3 (3.70%)	2 (2.47%)	3 (3.70%)	
	Overweight	4 (4.94%)	0	0	0	

Table 2: Overall diagnostic comparison between MGIT 960 and LPA

Method	Isoniazid Sensitive Detected	I soniazid Mono-resistance Detected	Total
MGIT 960	75 (92.59%)	6 (7.41%)	81
LPA (inhA & katG)	74 (91.36%)	7 (8.64%)	81

Table 3: Mutational patterns in isoniazid mono-resistance by LPA

Gene Mutation	Resistant Cases	Percentage (%)
inhA	3	3.70
katG	5	6.17
Both inhA and katG	1	1.23
Total	7	8.64

Table 4: Isoniazid resistance distribution by clinical and risk factors (n = 81)

Variable	Category	Total n (%)	MGIT Resistant n (%)	LPA inhA n (%)	LPA katG n (%)	p-value
Smoking	Yes	16 (19.75%)	2 (2.47%)	1 (1.23%)	1 (1.23%)	0.323
	No	65 (80.25%)	4 (4.94%)	2 (2.47%)	4 (4.94%)	
Alcohol Use	Yes	9 (11.11%)	2 (2.47%)	1 (1.23%)	1 (1.23%)	0.401
	No	72 (88.89%)	4 (4.94%)	2 (2.47%)	4 (4.94%)	
Comorbidities	Diabetes	26 (32.10%)	3 (3.70%)	1 (1.23%)	2 (2.47%)	0.219
	Hypertension	20 (24.69%)	3 (3.70%)	1 (1.23%)	2 (2.47%)	0.298
	HIV Positive	81 (100%)	–	–	–	–
Previous ATT History	Yes	4 (4.94%)	4 (4.94%)	2 (2.47%)	2 (2.47%)	0.467
	No	77 (95.06%)	1 (1.23%)	0	1 (1.23%)	
TB Contact History	Yes	1 (1.23%)	1 (1.23%)	0	1 (1.23%)	0.492
	No	80 (98.77%)	5 (6.17%)	3 (3.70%)	4 (4.94%)	0.476
Sample Type	Sputum	78 (96.30%)	–	–	–	
	BAL	3 (3.70%)	–	–	–	
ZN Staining	Negative (Mucus)	35 (43.21%)	–	–	–	0.050
	Negative (Saliva)	11 (13.58%)	–	–	–	
	1+	24 (29.63%)	–	–	–	
	2+	3 (3.70%)	–	–	–	
	3+	8 (9.88%)	–	–	–	

Table 5: Molecular findings and diagnostic performance of LPA (n = 81)

Parameter	Category	Frequency n (%)	p-value
CBNAAT Bacterial Load	Very Low	12 (14.81%)	0.037*
	Low	34 (41.98%)	
	Medium	25 (30.86%)	
	High	10 (12.35%)	

LPA – rpoB Mutation	Detected	0 (0%)	–
	Not Detected	81 (100%)	
LPA – inhA Mutation	Present	3 (3.70%)	–
	Absent	78 (96.30%)	
LPA – katG Mutation	Present	5 (6.17%)	–
	Absent	76 (93.83%)	
Second-Line Drug Resistance (LPA)	Levofloxacin Resistance	0 (0%)	–
	Moxifloxacin Resistance	0 (0%)	
	Injectable Drugs Resistance	0 (0%)	
Diagnostic Accuracy (LPA vs MGIT)	True Positive (TP)	6	–
	True Negative (TN)	74	
	False Positive (FP)	1	
	False Negative (FN)	0	

Discussion:

Tuberculosis remains a significant public health issue in the world, especially in developing nations where there is a high burden of disease and socioeconomic inequalities **[2]**. Drug-resistant tuberculosis is associated with higher morbidity, mortality and transmission rates and is a further diagnostic and therapeutic challenge **[10]**. The present study aimed to assess isoniazid mono-resistance by both phenotypic drug susceptibility testing (DS) using MGIT 960 and molecular Line Probe Assay (LPA) with a focus on mutation patterns and diagnostic accuracy. Most of the patients in the present study were in the younger age group (19-38 years), similar to the previous epidemiological studies which showed that TB mainly affects the economically productive age group **[3]**. Younger people might be more likely to be exposed to the community, to be mobile at work, or to have risk factors for transmission. The male predominance in the present study is consistent with the world statistics on tuberculosis and could be explained by occupational exposure, smoking, health seeking behavior and biological susceptibility **[3]**. The rural dominance in the current study is due to the differences in access to health care, overcrowding, malnutrition and delayed diagnosis in the underserved population **[5].** The observed nutritional deficiencies and low BMI of resistant patients further corroborate the link between malnutrition and poor host immunity in TB **[7]**. The prevalence of resistance in underweight people highlights the importance of nutrition support as part of TB care. Resistant cases tended to smoke and drink more, but this was not statistically significant. Impaired mucociliary clearance, altered immune response and increased susceptibility to tuberculosis infection and treatment failure have been associated with tobacco smoking **[8]**. Likewise, alcohol consumption is associated with poor adherence to treatment and immunosuppression. Almost a third of the participants were diagnosed with diabetes mellitus, which is consistent with the fact that diabetes is associated with increased risk of active tuberculosis and negatively impacts treatment outcomes **[9]**. An interesting result of the present study was the high percentage of resistance in previously treated patients. It is well established that prior exposure to anti-tubercular therapy is a risk factor for development of drug resistance, either because of incomplete treatment, poor adherence or inadequate regimens **[10]**. This emphasizes the need for early detection of resistance and close monitoring of treatment within the national tuberculosis control programmes. Isoniazid mono-resistance was detected by the MGIT 960 system in 7.41% of patients, while LPA detected resistance in 8.64% of patients. The prevalence observed in the present study was comparable to that reported by Chandrasingh and Nair, who identified INH mono-resistance in 6.7% of extrapulmonary TB isolates using LPA, with no rifampicin resistance detected **[13]**. The higher detection rate by LPA could be because the assay is able to detect genetic mutations prior to the full expression of phenotypic resistance. Raizada *et al.,* in a multisite validation study in India, showed high accuracy of the GenoType MTBDRplus assay compared with conventional culture-based DST and substantially shorter turnaround time, supporting the utility of LPA for rapid molecular detection of drug resistance **[14]**. Günther *et al.* also found high agreement between LPA and traditional culture-based methods for rapid resistance detection **[15]**. The molecular analysis showed that katG mutations were predominant over inhA mutations. KatG mutations are associated with high-level isoniazid resistance, while inhA mutations usually confer low-level resistance and cross-resistance to ethionamide **[12]**. The predominance of katG mutations observed in the present study is consistent with previous LPA-based investigations of isoniazid resistance, which have showed the importance of mutations in katG and the inhA promoter in defining molecular resistance patterns **[16]**. High-level resistance is still the primary mechanism of isoniazid mono-resistance in the study population, as evidenced by the high prevalence of katG mutation in the current study. Mutations in both inhA and katG were relatively rare and might suggest more complex resistance patterns that would necessitate individual therapeutic approaches. No rifampicin resistance associated rpoB mutations were found, indicating that the resistant isolates were not multidrug-resistant TB, but rather isoniazid mono-resistant tuberculosis. Second-line LPA were also found to be susceptible to all the anti-tubercular drugs that are considered as reserve drugs, as they lacked resistance to fluoroquinolones and injectable drugs. The most important finding in this study was the high diagnostic accuracy of LPA compared to MGIT 960. The assay showed 100% sensitivity, 98.67% specificity and 98.77% accuracy. The negative predictive value of 100% means that LPA is very reliable to rule out isoniazid resistance. The speed of turnaround and the accuracy of LPA make it an invaluable diagnostic tool for early initiation of effective therapy and prevention of transmission. This is consistent with earlier studies which have shown the value of molecular assays for the rapid detection of drug resistance **[10, 15 and 17].** The present study highlights the increasing role of molecular diagnostics in TB management. Prompt detection of isoniazid mono-resistance enables physicians to tailor therapy and prevent the development of MDR TB. The use of LPA in routine

diagnostic algorithms could have a major impact on the effectiveness of TB control in high-burden countries.

Conclusion:

Line Probe Assay showed rapid, sensitive and specific detection of isoniazid mono-resistant tuberculosis, enabling earlier diagnosis than conventional culture-based methods. Younger age, male sex, rural residence, undernutrition, diabetes and previous anti-tubercular treatment were common characteristics of resistant cases, with katG mutation indicating predominant high-level isoniazid resistance. Thus, data support the routine use of Line Probe Assay for early detection of isoniazid resistance, facilitating timely individualized therapy and improved tuberculosis control.

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