# The diagnostic utility of line probe assays for multidrug-resistant tuberculosis

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Owing to the burden of multidrug-resistant tuberculosis, molecular techniques have been approved by the WHO for the rapid diagnosis of the same. The objectives of this prospective, diagnostic study, conducted at Christian Medical College, a tertiary care center in South India, were to compare the performance of line probe assay (GenoTypeMTBDRplus) with culture, as well as the Xpert MTB/Rif assay on sputum samples. Ninety-one consecutive suspects of multidrug-resistant pulmonary tuberculosis patients from January 2013 to June 2013 were enrolled in this study and the results of line probe assay compared to culture and Xpert MTB/Rif. Compared to culture, the assay demonstrated a sensitivity and specificity of 81.5% (95%CI 67.4–91.1%) and 87.5% (95%CI 71–96.5%) for the detection of tuberculosis, with sensitivity and specificity of 100% (95%CI 85.2–100%) and 93.8% (95%CI 69.8–99.8%), respectively, for rifampicin resistance. For isoniazid resistance, sensitivity and specificity were 89.3% (95%CI 71.8–97.7%) and 100% (95%CI 71.5–100%), respectively. Compared to Xpert MTB/Rif assay, the assay showed a sensitivity of 80% (95%CI 68.2–88.9%) and specificity of 100% (95%CI 85.8–100%) for the detection of tuberculosis a sensitivity of 94.3% (95%CI 80.8–99.3%) and specificity of 94.1% (95%CI 71.3–99.9%) for rifampicin resistance was attained. This assay performed well on smear positive samples, but poorly on smear negative and scanty samples, and can serve as a rapid diagnostic tool, particularly in isoniazid monoresistant cases of tuberculosis, which are not diagnosed by Xpert MTB/Rif.

**Keywords:** Tuberculosis, Drug resistance, Diagnostics

## Introduction

Tuberculosis continues to be a global crisis. The greatest burden of the disease is borne by low and middle income countries. India and China together account for 35% of cases worldwide. It ranks eighth among the leading causes of death, despite being a curable disease.[1] Multidrug-resistant tuberculosis(MDRTB), defined as the resistance of *Mycobacterium tuberculosis* to the two main first-line drugs in therapy, rifampicin and isoniazid, has plagued the treatment of tuberculosis for over two decades.[2] In addition, resistance to two other classes of drugs, the fluoroquinolones and one of the second-line injectable antibiotic drugs (amikacin, capreomycin and kanamycin), is termed extensively drug-resistant tuberculosis (XDRTB). Several countries are now reporting XDRTB.[3]

Significant setbacks in the management of resistant tuberculosis are the lack of a rapid and accurate diagnostic modality, cost of treatment, and adherence to treatment. Though drug susceptible tuberculosis can be cured with appropriate and regular therapy within six months, drug resistant tuberculosis poses a challenge to the treating clinician with cure rates between 50–70%.[4]

Rapid testing of both isoniazid and rifampicin at the time of diagnosis is the most cost-effective testing strategy for any patient group or setting, and likely benefits of rapid drug susceptibility testing (DST) include increased cure rates, decreased mortality, reduced development of additional drug resistance, and a reduced likelihood of failure and relapse.[5]

Molecular techniques have revolutionized the diagnosis of tuberculosis – both susceptible and resistant. Rising rates of drug-resistant tuberculosis in India necessitate a rapid, low-cost, robust test to detect the same.

The line probe assays detect drug-resistant tuberculosis in clinical samples. With the ability to detect low-level isoniazid resistance (*inh A*), the WHO-endorsed GenoType MTBDR*plus (*Hain Lifescience, Germany) version 1 is an improvement over the MTBDR (Hain Lifescience, Germany) assay which had only rpoB probes to determine

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rifampicin resistance along with katG probes to determine high-level isoniazid resistance. Hain introduced a second version of GenoType MTBDR*plus (*Hain Lifescience, Germany), in 2011, optimized to detect mutations This assay proved to be non-inferior to the first version in a head-to-head multicenter evaluation and more robust in detecting lower MTB concentrations.[6]

We evaluated this molecular assay on sputum samples of drug-resistant TB suspects, irrespective of smear status. Conventional culture is the accepted gold standard; however, the Xpert MTB/Rif is part of the standard of care that is now used under the programmatic management of drug-resistant tuberculosis (PMDT) under the Revised National Tuberculosis Control Programme (RNTCP).[7] Therefore, we compared the GenoType MTBDR*plus,* henceforth LPA, both against culture and Xpert MTB/Rif to assess its accuracy in a high-burden tertiary care center.

## Materials and methods

### *Study setting*

Christian Medical College and Hospital (CMCH) is a 2600-bed tertiary referral center in Vellore, a city in Tamil Nadu, Southern India. CMCH has an outpatient turnover of 7000 cases per day. Patients suspected of TB are seen at the DOTS center and the outpatient service of the departments of General Medicine, its Infectious Diseases Unit and the department of Pulmonary Medicine. The Mycobacteriology laboratory is an accredited center for culture and drug susceptibility testing since 2009, and performs Xpert MTB/Rif as part of the programmatic management of drug-resistant tuberculosis (PMDT) program under the Revised National Control Programme for Tuberculosis (RNTCP).

### *Participants*

Following approval from the institutional review board, 91 sputum samples from adult suspects of drug-resistant tuberculosis, who fit the inclusion and exclusion criteria, (Refer Table 1) between January 2013 and June 2013, were collected after an informed consent. Patients were naïve to second-line antituberculous treatment. All samples were split equally between the three tests. Ninety-one samples underwent LPA and Xpert MTB/Rif. Eighty were processed by culture, as 11 samples, once confirmed by Xpert MTB/Rif as *M. tuberculosis,* did not have a culture and DST as a follow-on request. Two samples were indeterminate by Xpert MTB/Rif. Therefore, 89 samples were available for comparison with the Xpert MTB/Rif (Refer Table 3) and 80 samples were available for comparison against culture (Refer Table 2). Statistical analysis was by Stata 13. Sensitivity, specificity, positive and negative predictive values, ROC, and likelihood ratios were calculated for all variables -LPA, Xpert MTB/ Rif, culture, and smear microscopy.

**Table 1 Inclusion and exclusion criteria**

| | Inclusion criteria |
|---|---|
| 1. | Age >18 years |
| 2. | Sample volume of at least 3 ml |
| 3. | Signs and symptoms >2 weeks of pulmonary tuberculosis |
| | *And any one of the following* |
| 4. | Patients previously treated with first-line ATT |
| 5. | New cases of tuberculosis not responding to first-line ATT |
| 6. | Failures of new tuberculosis cases |
| 7. | Smear positive patients who remain smear positive at the 4th month of treatment |
| 8. | Contact of an MDRTB case with symptoms of pulmonary TB |
| | *Exclusion criteria* |
| 1. | Patients with extrapulmonary TB |

### *Sample processing*

Smear microscopy by Auramine staining was done on all unconcentrated sputum samples. As per our laboratory standard operating protocol, an LJ culture is included for every sample requested for MGIT culture since contamination rates are marginally higher for MGIT than LJ. Concentration and decontamination were carried out using the Petroff's method preceding solid culture on Lowenstein Jensen (LJ) medium and the NALC/NaOH method preceding liquid culture on Mycobacteria Growth Indicator Tube 960, Becton Dickinson Biosciences, Sparks, MD, USA, (MGIT) as per manufacturer's instructions. Growth on MGIT tubes and/or LJ slants was examined microscopically for acid-fast bacilli (AFB). All contaminated cultures were repeated, and once resolved, included for analysis. Confirmation of *M. tuberculosis* was performed with an immunochromatographic test (SD, Bioline). The WHO-recommended critical concentrations for rifampicin (1 μg/mL) and isoniazid (0.1 μg/mL), were used for DST using the MGIT. Samples that underwent Xpert MTB/ Rif assay were processed according to manufacturer's instructions. All samples underwent LPA to detect *M. tuberculosis* complex strains, and mutations conferring resistance to isoniazid and rifampicin, using DNA extracted from clinical specimens, as per manufacturer's instructions. The person performing the tests was blinded to the reference standard results. A valid LPA result was defined by a *Mycobacterium tuberculosis* complex-specific control (TUB), conjugate controls (CC) and amplification control (AC) bands in conjunction with the target gene locus control. All discrepancies in results were included for analysis.

## Results

The prevalence of TB patients by Xpert MTB/Rif and by culture was 73 and 60%, respectively. The prevalence of MDRTB by Xpert MTB/Rif was 54% and by culture was 48%. Thirty-five patients were naïve to first-line ATT, and 56 had been treated previously. By culture, the prevalence of MDRTB in new patients and in re-treatment patients was 26.6 and 52.1%, respectively. By the Xpert MTB/ Rif assay, prevalence of MDRTB in new and re-treatment patients was 32.1 and 62.9%, respectively. Being a tertiary care center, we see patients with tuberculosis who are referred from different parts of the country as they are refractory to treatment. In addition, we collected samples

**Table 2 Line probe assay results in comparison to culture (n = 80)**

| Detection of MTB by culture | | | | Detection of rifampicin resistance by culture | | | | Detection of isoniazid resistance | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Positive | | Negative | | Positive | | Negative | | Positive | | Negative | |
| 48 | | 32 | | 23 | | 16 | | 28 | | 11 | |
| LPA | | LPA | | LPA | | LPA | | LPA | | LPA | |
| Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| 39 | 9 | 4 | 28 | 23 | 0 | 1 | 15 | 25 | 3 | 0 | 11 |

**Table 3 Line probe assay results in comparison to Xpert MTB/Rif (n = 89)**

| Detection of MTB by Xpert MTB/Rif | | | | Detection of rifampicin resistance by Xpert MTB/Rif | | | |
|---|---|---|---|---|---|---|---|
| Positive | | Negative | | Positive | | Negative | |
| 65 | | 24 | | 35 | | 17 | |
| LPA | | LPA | | LPA | | LPA | |
| Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| 52 | 13 | 0 | 24 | 33 | 2 | 1 | 16 |

from patients who were suspects of MDRTB. This may explain the high prevalence of drug-resistant tuberculosis in this study.

### *Accuracy indices*

Sensitivity for the detection of tuberculosis by LPA compared to conventional culture and Xpert MTB/Rif was 81.5% (95%CI 67.4–91.1%) and 80% (95%CI 68.2–88.9%), respectively. The sensitivity for detection of rifampicin resistance against culture and Xpert MTB/Rif was 100% (95%CI 85.2–100%) and 94.3% (95%CI 80.8–99.3%) respectively and for detection of isoniazid resistance 89.3% (95%CI 71.8–97.7%) against conventional culture.

Specificity for the detection of tuberculosis by LPA compared to culture and Xpert MTB/Rif was 87.5% (95%CI 71–96.5%) and 100% (95%CI 85.8–100%), respectively. Specificity for detection of rifampicin resistance against culture and Xpert MTB/Rif was 93.8% (95%CI 69.8–99.8%) and 94.1% (95%CI 71.3–99.9%), respectively, and for the detection of isoniazid resistance 100% (95%CI 71.5–100%) against culture.

Of 91 samples, 29 samples were AFB smear negative. The sensitivity and specificity in smear positive patients were 80.3% (95%CI 68.2–89.4%) and 100% (95%CI 2.5–100%), respectively, against Xpert MTB/Rif and 82.2% (95%CI 67.9–92%) and 66.7% (95%CI 22.3–95.7%) against culture. In smear negative patients, the sensitivity and specificity against Xpert MTB/Rif were 66.7% (95%CI 22.3–95.7%) and 100% (95%CI 85.2–100%) and against culture were 66.7% (95%CI 9.4–99.2%) and 92.3% (95%CI 74.9–99.1%), respectively (refer Table 4).

## Discussion

Of the half a million cases of multidrug-resistant TB (MDRTB) estimated to have occurred in 2014, only about a quarter of these were detected and treated.[8] In India, the number of people with tuberculosis amounts to a fifth of cases seen globally. MDRTB in India stands at 2.2% in new cases and 15% in re-treatment cases.[9]

We evaluated the LPA on sputum samples, irrespective of smear status and treatment status, demonstrating its utility in a high-burden country for drug-resistant TB, although it is approved for use only on smear positive samples.[10] To the best of our knowledge, no similar studies on both smear positive and smear negative patients have been performed in India.

### *LPA compared to culture for the diagnosis of tuberculosis*

When compared to the method, LPA showed a sensitivity of 81.3% (95%CI 67.4–91.1%), 100% (95%CI 85.8–100%) and 89.3% (95%CI 71.8–97.7%) for the detection of *M. tuberculosis*, rifampicin resistance, and isoniazid resistance, respectively, and a specificity of 87.5% (95%CI 71–96.5%), 93.8% (95%CI 69.8–99.8%),100% (95%CI 71.5–100%) for the same. A study from South Africa ($n$ = 282) had a sensitivity of 73.1%, and a specificity of 100% for detection of *M. tuberculosis*.[11] Literature from Moldova ($n$ = 348) cites a sensitivity of 88% and a specificity of 98.4% for detection of *M. tuberculosis*, 94.3% and 96% for rifampicin resistance, and 95.8 and 88.9% for isoniazid resistance.[12] A recent study from India[13] which, however, was performed only on smear positive samples, showed that in their testing strategy, the LPA had 100% concordance with MGIT 960.

Among the nine samples that were culture positive but LPA negative (refer Table 2), 5/9 were reported by initial fluorescent microscopy as scanty acid-fast bacilli and 1/9 was reported negative for acid-fast bacilli. The detection limit of the LPA is 10,000 CFU/ml,[14] which is the same as that of smear microscopy, while that of culture is 10–100 CFU/ml.[15] It is likely that LPA has missed samples with a low bacillary load. In addition, six of the nine patients were also on first-line antituberculous treatment at the time the sample was collected, which may affect the quantity of DNA.

**Table 4 Accuracy indices for LPA**

| Reference test | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | +LR | –LR |
|---|---|---|---|---|---|---|
| Culture (*n* = 80) | 81.5 (67.4–91.1) | 87.5 (71–96.5) | 90.7 (77.9–97.4) | 75.7 (58.8–88.2) | 6.5 (2.57–16.4) | 0.214 (0.12–0.4) |
| Xpert (MTB/Rif) (*n* = 89) | 80 (68.2–88.9) | 100 (85.8–100) | 100 (93.2–100) | 64.9 (47.5–79.8) | | 0.2 (0.12–0.33) |
| Culture for rifampicin resistance (*n* = 48) | 100 (85.2–100) | 93.8 (69.8–99.8) | 95.8 (78.9–9.9) | 100 (78.2–100) | 16 (2.4–107) | 0 |
| Xpert (MTB/Rif) for rifampicin resistance (*n* = 65) | 94.3 (80.8–99.3) | 94.1 (71.3–99.9) | 97.1 (84.7–99.9) | 88.9 (65.3–98.6) | 16 (2.39–108) | 0.061 (0–0.23) |
| Culture for isoniazid resistance (*n* = 48) | 89.3 (71.8–97.7) | 100 (71.5–100) | 100 (86.3–100) | 78.6 (49.2–95.3) | | 0.107 (0.37–0.31) |
| Culture (smear positive samples) (*n* = 51) | 82.2 (67.9–92) | 66.7 (22.3–95.7) | 94.9 (82.7–99.4) | 33.3% (9.9–65.1) | 2.47 (.79–7.7) | .267 (.11–.62) |
| Xpert MTB/Rif (smear positive samples) (*n* = 60) | 80.3 (68.2–89.4) | 100 (2.5–100) | 100% (92.7–100) | 7.69% (.2– 36) | | .20 (.12–.33) |
| Culture (smear negative samples) (*n* = 29) | 66.7% (9.4–99.2) | 92.3 (74.9–9.1) | 50 (6.76–93.2) | 96 (79.6–99.9) | 8.67 (1.8–41) | .361 (.073–1.8) |
| Xpert MTB/Rif (smear negative samples) (*n* = 29) | 66.7 (22.3–95.7) | 100 (85.2–100) | 100 (39.8–100) | 92 (74–99) | | .333 (.11–1.0) |

Notes: Data in parentheses are 95% CI, unless otherwise indicated; PPV – positive predictive value, NPV – negative predictive value, +LR – positive likelihood ratio, –LR – negative likelihood ratio.

Four samples were positive by LPA, but negative by the culture method. These samples were also positive by the Xpert MTB/Rif assay, which indicates they were probably true positives. The detection of TB DNA from dead bacteria can lead to a higher rate of false positive results when assayed against culture results, and this reduces the specificity.[12] In addition, the culture in itself is an imperfect gold standard, having a sensitivity of only 77%[16] for sputum samples, and thus when culture is taken as a reference standard, a new test under evaluation can appear false positive.

Only one sample showed an inconsistency for rifampicin resistance detection. This was rifampicin-resistant by the LPA, susceptible by conventional DST and susceptible by Xpert(MTB/Rif)assay. In many a case, mutations may be present, but not necessarily lead to the expression of resistance phenotypically.[17] Confirmation by phenotypic methods, thus, is crucial.

Three samples were detected by conventional DST as isoniazid-resistant (one of which was monoresistant) but not by LPA. Literature cites that isoniazid resistance is coded for by several mutations, the majority of which are covered by *katG* S315. Several mutations that code for isoniazid resistance, including *oxyR-ahpC* and *kasA* genes, are not detected by the LPA.[18]

### *LPA compared to Xpert (MTB/Rif) for the diagnosis of tuberculosis*

Of 91 sputum samples, two were indeterminate by Xpert MTB/Rif, leaving 89 samples available for analysis. We report a sensitivity of 80% (95%CI 68.2–88.9%) and a specificity of 100% (95%CI 85.2–100%) for the diagnosis of tuberculosis, and 94.3% (95%CI 80.8–99.3%) and 94.1% (95%CI 71.3–99.9%) for rifampicin resistance detection.

All the samples that were detected as *M. tuberculosis* by LPA were also detected by Xpert MTB/Rif assay. Thirteen samples were not detected by the LPA (refer Table 3), whereas they were detected as *M. tuberculosis*, either susceptible or resistant by the Xpert MTB/Rif assay. Of these 13, nine were smear negative or scanty positive samples by smear microscopy. The LPA has a detection limit of 10,000 CFU/ml, while the Xpert MTB/Rif assay has a detection limit of 131 CFU/ml,[19] based on several studies on analytical detection limit. Samples that have a very low bacillary load may be detected by Xpert MTB/Rif but missed by LPA.

Two samples were rifampicin-resistant by the Xpert MTB/Rif assay but susceptible by the LPA, both of which were isoniazid-monoresistant by LPA and culture. This is possibly because of certain mutations that the LPA, in contrast to the Xpert MTB/Rif assay, does not encompass.[20] DNA sequencing studies demonstrate that more than 95% of rifampicin-resistant *M. tuberculosis* strains have a mutation within the 81-bp hot-spot region (codons 507–533) of the RNA polymerase B subunit (*rpoB*) gene. While the Xpert MTB/Rif detects mutations in all but codon 530, the line probe assay fails to detect mutations from codon 527–530.[21]

The absence of a wild-type 8 band without a corresponding mutation band was seen in 16 samples, and in 15 of these, the Xpert MTB/Rif assay also demonstrated rifampicin resistance, which gave us a high concordance for rifampicin-resistant tuberculosis. This is in striking contrast

to a study from Delhi[13] that had a much lower concordance, though the latter was in a subgroup of rifampicin-monoresistant patients, and thus a skewed population. The interpretation for the absent wild-type 8 band is dependent on correlation with culture and clinical criteria, as per manufacturer's instructions. The absence of a wild type band is suggestive of one of two scenarios – either it is a silent mutation that does not lead to an amino acid change, or a mutation not detected by LPA/an unknown gene.[22]

We report rifampicin monoresistance of 5.7% (five samples), which was corroborated by Xpert MTB/Rif assay. Of these, three were non-viable and did not grow on culture. The prevalence of rifampicin monoresistance varies from country to country, with high rates from South Africa, and in association with HIV patients.[23] In India, the prevalence of rifampicin monoresistance varies from 0.5–2%.[24]

The LPA is complementary to the rapid detection of drug susceptible tuberculosis by the Xpert MTB/Rif assay, detecting isoniazid resistance in just a matter of 6 h. One of the mutations that is detected by the line probe assay (i*nh A* locus) encodes low-level isoniazid resistance. Low-level resistance suggests the minimum inhibitory concentration is higher than in a susceptible population of organisms, leading to variable treatment results. Thus, it acts as a marker for multidrug resistance.[25]

### *The performance of LPA on smear negative and smear positive samples*

The sensitivity and specificity in smear positive patients were 80.3% (95%CI 68.2–89.4%) and 100% (95%CI 2.5–100%), respectively, against Xpert MTB/Rif and 82.2% (95%CI 67.9–92%) and 66.7% (95%CI 22.3–95.7%) against culture. In smear negative patients, the sensitivity and specificity against Xpert MTB/Rif were 66.7% (95%CI 22.3–95.7%) and 100% (95%CI 85.2–100%) and against culture were 66.7% (95%CI 9.4–99.2%) and 92.3% (95%CI 74.9–99.1%), respectively.

All four samples that were LPA positive but smear negative were also detected by Xpert(MTB/Rif) assay. However, 13 smear positive samples were not detected by LPA. Nine of these patients had a scanty AFB in their smear. Eight of these grew *M. tuberculosis* on culture and 10 were detected by the Xpert MTB/Rif assay.

Culture, though a reference standard, has a low sensitivity in itself, which can lead to apparent false positive results by LPA. In smear negative patients, the sensitivity and specificity against Xpert MTB/Rif were 66.7 and 100% and against culture were 66.7 and 92.3%, respectively. This is in tune with literature that describes the sensitivity of LPA as poor in smear negative patients.

## Limitations

The main limitation was a small sample size. Furthermore, it would have been useful to sequence isolates to resolve discrepant results. Future work should focus on the MTBDRsl which can be used to identify XDRTB from the same DNA extract, and its utility in a high- burden, low-resource setting.

## Conclusion

The assay showed a moderate sensitivity of 81.5 and 80% for detection of tuberculosis against culture and Xpert MTB/Rif, respectively, missing predominantly scanty and smear negative samples. Though sensitivity for detection of rifampicin resistance against culture and Xpert MTB/Rif were 100 and 94.5%, respectively, detection of Isonizid resistance was poorer and stood only at 89.3%. The turnaround time is short for LPA (6 h), as compared to culture and DST, though it is longer than for the Xpert MTB/Rif assay. However, it has the added advantage of also detecting isoniazid monoresistance. In a clinical setting, this test can be applied to smear positive samples with excellent sensitivity and specificity, and incorporated into existing diagnostic practice. A bonus is that the same DNA extract can be directly used for the MTBDRsl (Hain Lifesciences, Germany), detecting XDRTB where called for, which may prove game-changing in the management of drug-resistant tuberculosis.

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