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Original Article

Microbiology Section

# Role of Line Probe Assay for Rapid Detection of *Mycobacterium tuberculosis* Complex and Drug Resistance Directly from Clinical Samples

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## ABSTRACT

**Introduction:** The global burden of Multi Drug Resistant Tuberculosis (MDR TB) is increasing worldwide. Smear microscopy has low sensitivity, culture based tests for identification of tubercle bacilli and drug susceptibility testing take weeks to give results. Molecular tests allow rapid detection of *Mycobacterium tuberculosis* Complex (MTBC) and drug resistance in 4-8 hours. Real-time-PCR based GeneXpert provides only RIF susceptibility. In this study newer GenoType MTBDRplus VER 2.0 assay is utilized which gives sensitivity of both the first line anti-tubercular drugs.

**Aim:** To evaluate the performance of Line Probe Assay (LPA) for rapid identification and detection of drug resistance in MTBC in respiratory and non-respiratory samples.

**Materials and Methods:** All the samples (respiratory and non-respiratory) received from clinically suspected cases of Tuberculosis (TB), admitted in various wards, ICUs and outdoor patients of Dayanand Medical College and Hospital during the study, were processed in the Department of Microbiology. Samples other than Cerebro Spinal Fluid (CSF) were digested and decontaminated by NALC-NaOH method. Microscopy by ZN (Ziehl-Neelsen) staining, liquid culture by MGIT (Mycobacterial Growth Indicator Tube, BD BBLTM), and LPA by the GenoType MTBDRplus assay, VER 2.0, HAIN Life Science) were performed on all the samples.

**Results:** A total of 70 samples were considered which includes 34 respiratory samples and 36 non-respiratory samples. Out of the 70 samples, 13 (18.6%) were smear positive, 23 (32.8%) grown in culture and 29 (41.4%) were detected positive for MTBC by LPA. Considering culture as gold standard the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the LPA for diagnosis of TB in respiratory and non-respiratory samples were found as (83.33%, 72.73%), (72.73%, 80%), (62.5%, 61.54%) and (88.89%, 86.96%) respectively while sensitivity of LPA in smear positive and smear negative samples were found as 90% and 69.23% respectively. All the samples except two showed sensitivity to Isoniazid (INH) and Rifampicin (RIF). Two samples were found resistant to INH only.

**Conclusion:** LPA performed directly on samples is a wonderful tool for fast detection of MTBC in the respiratory samples along with INH and RIF resistance. In comparison, the sensitivity of LPA is less in non-respiratory samples. Still, many patients can be diagnosed and can start appropriate treatment till the culture report is received.


**Keywords:** Extra pulmonary tuberculosis, Isoniazid, Mycobacterial growth indicator tube, Rifampicin

## INTRODUCTION

TB is global health problem, with almost 9.6 million new cases and around 1.5 million deaths occur every year [1]. Approximately, 15-20% of cases are due to Extra Pulmonary Tuberculosis (EPTB) and among HIV positive patients; it constitutes up to 50% of TB cases [2]. Clinical presentation of EPTB is diverse and it may lead to delayed diagnosis due to paucibacillary nature of the samples. For prevention of TB transmission, rapid and accurate diagnosis of the disease is very crucial to allow prompt initiation of anti-tubercular therapy. Although, the conventional procedures are gold standard and cannot be replaced by the newer diagnostic tools. Sensitivity of acid-fast bacillus detection by microscopy is poor (0-40%) and smear positive samples requires $10^4$ bacilli/ml of the sample [3,4]. One fifth of TB transmission occurs due to smear negative pulmonary TB [5]. Culture is more sensitive method and 10-100 bacilli/mL are required for growth [6]. Long incubation period is required to grow the *M.tuberculosis*, limit the usefulness of culture methods for diagnosis of TB. Use of liquid culture media (MGIT, Bactec) has increased the yield by 10% as compared to solid conventional LJ (Lowenstein-Jensen) media [2]. Recently, nucleic acid amplification tests,

which can be used for direct detection of TB from samples, have emerged as potentially useful tools for rapid diagnosis of TB [7].

A rapid molecular test known as GenoType MTBDRplus (Hain, Life Science) is a LPA has been approved by WHO in 2008 for the diagnosis of MDR-TB in pulmonary specimens [8]. Initially, it was approved only for smear positive pulmonary samples. The newer version (GenoType MTBDRplus VER 2.0) is recommended for smear positive as well as smear negative pulmonary samples. The test detects amplified DNA by reverse hybridisation on stripes. It identifies the presence of MTBC along with INH and RIF resistance. The RIF resistance is identified by the detection of the most significant mutations of the *rpoB* gene (coding for the β-subunit of the RNA polymerase), The low level INH resistance is identified by the *inhA* gene (coding for the NADH enoyl ACP reductase) and The high level INH resistance detected by the *katG* gene (coding for the catalase peroxidise) [9].

Recently WHO has recommended a cartridge based molecular test, Xpert MTB/RIF (by Cepheid, CA, USA) for extrapumonary samples also but it has limitation of detecting RIF resistance only [10]. As LPA detects resistance to both INH and RIF therefore it needs to be evaluated for EPTB diagnosis also. This study was planned to evaluate the use of LPA for detection of MTBC directly from respiratory as well as non-respiratory samples, to compare with liquid culture and simultaneously, to detect the resistance to INH and RIF in a single day.

## MATERIALS AND METHODS

This prospective study was conducted in the Department of Microbiology, Dayanand Medical College and Hospital, Ludhiana, Punjab, India for the duration of six months (February to August 2017). The Institute's ethics committee approval was taken prior to the study.

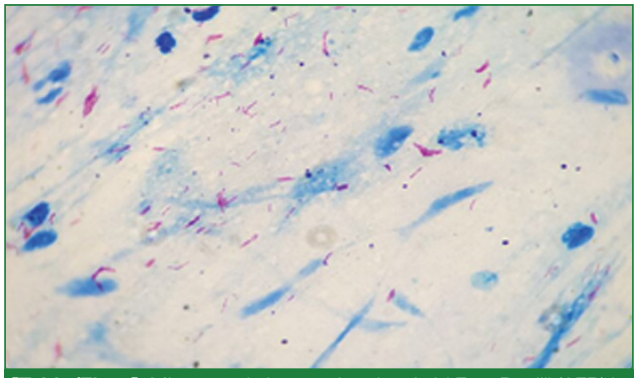
All the respiratory as well as non-respiratory samples except blood and urine, received in sterile containers from clinically suspected cases of TB from all age groups, admitted in various wards, ICUs and outdoor patients during the study period were included. One sample per patient was considered in the study. The samples which showed contamination in culture were also excluded from the study.

### Concentration and Decontamination of Specimen

All the specimens except CSF were concentrated and decontaminated by using the N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) standard method [11]. Tissue biopsy samples were homogenised before decontamination. All the samples were subjected to microscopy, liquid culture and LPA.

**Microscopy:** Single smear was made for each of decontaminated samples, was stained by standard ZN staining method and examined under light microscope for Acid Fast Bacilli (AFB) [Table/Fig-1].

**Culture:** About 0.5 ml of the processed (digested, decontaminated, concentrated) suspension was inoculated into the Mycobacterial Growth Indicator Tube (MGIT, BD Diagnostic Systems)) and mixed well. Before inoculation, each MGIT tube was supplemented with the PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, and Azlocillin) antibiotic mixture and OADC (Oleic Acid Albumin-Dextrose-Catalase) enrichment solution and MGIT cultures were incubated for a total of six weeks and checked daily for increase in fluorescence, before being reported as negative. All positive cultures were confirmed by ZN staining for the presence of acid-fast bacilli.



**[Table/Fig-1]:** Microscopic image showing Acid Fast Bacilli (AFB) in sputum sample (ZN stain, 100X).

**Line Probe Assay:** The test was performed as per manufacturer's guidelines. It is a LPA based on DNA STRIP technology. There are 3 Steps: 1. DNA extraction from decontaminated samples. 2. Amplification by PCR. 3. Reverse hybridisation. All the three steps were carried out in separate rooms to minimize contamination.

### DNA Extraction (Genolyse)

About 500 µL of decontaminated specimen was transferred to the screw cap tube and centrifuged at 1000 rpm for 15 min and the pellet was suspended in 100 µL of lysis buffer (A-LYS). After Vortexing, it was incubated at 95°C for 15 min, 100 µL of neutralisation buffer (A-NB) was added after a brief spin to the lysate and centrifuged at 9600 rpm for 10 min supernatant (5 µL) was used for PCR.

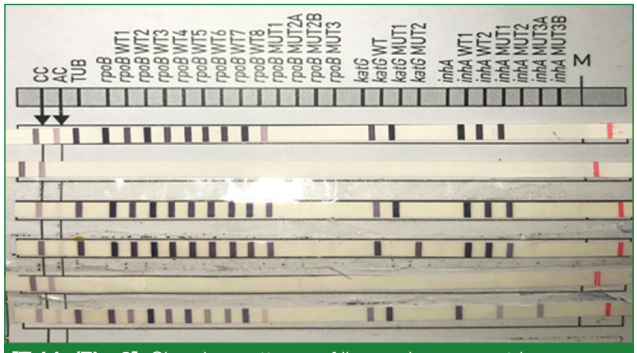
### Amplification by PCR

About 50 µL of PCR Mix for each sample is prepared by adding10 µL AM-A, 35 µL AM-B and 5 µL of DNA solution. Amplification was done in thermo cycler using 52 thermal cycles for clinical samples [1 cycle; (15 min 95°C), 20 cycles; (30 secs 95°C and 2 min 65°C), 30 cycles; (25 sec 95, 40 sec 50°C and 40 sec 70°C) and 1 cycle (8 minute, 70°C)].

### Reverse Hybridisation

After amplification, hybridisation was performed with twin incubator; the biotin-labelled amplicons were hybridised to the single stranded membrane bound probes. After a

hybridisation buffer and stringent buffer washing, freshly prepared conjugate and substrate were added to the strips and an alkaline phosphatase mediated staining reaction was observed in the bands where the amplicon and the probe have been hybridised. The GenoType MTBDRplus assay strip contains 27 reaction zones; 21 of them are wild type and mutations probes and 6 are control probes include a conjugate control, and amplification control, *M.tuberculosis* complex-specific control (TUB), *rpoB* locus control, *katG* locus control, and an *inhA* locus control. The absence of any of the wild-type bands or the presence of any mutation bands in each drug resistance-related gene shows resistance to the respective anti-tubercular antibiotics [Table/Fig-2]. Using culture as a gold standard method, sensitivity, specificity, PPV, NPV of the LPA were calculated.




**[Table/Fig-2]:** Showing patterns of line probe assay strips.
Lane 1, Positive control (sensitive to RIF and INH); Lane 2, Negative control; Lane 3, Sensitive to RIF and INH; Lane 4, high level INH monoresistance (absence of *katG* wild type and presence of *katG S315T1* mutation); Lane 5, absence of TUB band; Lane 6, low level INH monoresistance` (absence of wild type 1 and presence of *inhA* MUT 3A band

## RESULTS

**Specimens:** A total of 70 samples (34 respiratory and 36 non-respiratory) were received during the study. Of the 34 (48.57%) respiratory specimens received were included, 16 (47%) sputum, 8 (23.52%) Endotracheal aspirates (ET aspirates), 6 (17.64%) pleural fluid, 4 (11.76%) Broncho-Alveolar Lavage fluid (BAL). Among the 36 (51.42%) non-respiratory specimens most common was pus 13 (36.11%), followed by CSF 8 (22.22%), lymph node biopsy/aspirates 7 (19.44%), tissue 4 (11.11%), ascitic fluid 3 (8.3%) and peritoneal fluid 1 (2.7%) [Table/Fig-3]. Patient's age ranged between 4 months to 88 years. Samples were received more from males 46 (65.71%) than female patients 24 (34.28%). CSF samples were received mostly from paediatric patients.

Out of the three diagnostic tests highest positivity was observed by LPA in 29 (41.4%) followed by liquid culture 23 (32.8%) and smear microscopy 13 (18.6%) [Table/Fig-3]. A total of 34 (48.5%) samples were found positive for *M.tuberculosis* by any of the three methods whereas, only 9 (12.8%) samples were found positive by all the three methods. Considering culture as gold standard test for diagnosis of TB, sensitivity, specificity, PPV and NPV of the LPA were calculated by using data of [Table/Fig-4,5]. Various test results for the respiratory and non-respiratory samples are shown in [Table/Fig-6]. Sensitivity of LPA in smear positive and smear negative samples were found as 90% and 69.23% respectively.

| Sample (Number) | ZN smear | Culture (MGIT) | LPA |
|---|---|---|---|
| Sputum* (16) | + + + + + + - - - - - - - - - | + + + + + - -+ + - - - - - - - - | + + + + + + + - - + + + - - - - - |
| BAL fluid* (4) | + - - - | + + + - - | + - - - |
| ET aspirates* (8) | + - - - - + + - | + - - - - + + - | + - - - - + + - |
| Pleural fluid* (6) | + - - - - - | + - - - - - | + + - - - - |
| Pus (13) | + - - - - - - - - - - - - | - - + - + + - - - - - - - | + + - + + + - - - - - - - |
| CSF (8) | - - - - - - - - | - - - - - - - - | + - - - - - - - |
| LN aspirates (7) | - - - - - - - | + + + + - - - | + + + + + - - |
| Tissue (4) | + - - - | + + + - - | + - - - |
| Ascitic fluid (3) | - - - | + + - | + - - |
| Peritoneal fluid (1) | - | - | - |
| Total (70) | 13 (+) | 23 (+) | 29 (+) |

**[Table/Fig-3]:** Samplewise positivity of various diagnostic methods for tuberculosis (n=70).
*Respiratory samples, + (positive test), - (negative test)
* BAL= Broncho-Alveolar Lavage fluid; ET= Endotracheal; LN= Lymph Node; CSF= Cerebro Spinal Fluid;

| LPA | Culture | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 10 | 6 | 16 (47%) |
| Negative | 02 | 16 | 18 (53%) |
| Total | 12 (35.3%) | 22 (64.7%) | 34 |

**[Table/Fig-4]:** LIne probe assay vs culture in respiratory samples (n=34).

| LPA | Culture | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 8 | 5 | 13 (36.1%) |
| Negative | 3 | 20 | 23 (63.9%) |
| Total | 11 (30.5%) | 25 (69.4%) | 36 |

**[Table/Fig-5]:** Line probe assay vs culture in extrapulmonary samples (n=36).

| Parameters | Respiratory Samples (n=34) | Non-respiratory Samples (n=36) |
|---|---|---|
| Smear positivity | 11 (32.35%) | 2 (05.55%) |
| Culture positivity | 12 (35.29%) | 11 (30.55%) |
| LPA positivity | 16 (47.05%) | 13 (36.11%) |
| Sensitivity of LPA | 83.33% | 72.73% |
| Specificity of LPA | 72.73% | 80% |
| PPV for LPA | 62.5% | 61.54% |
| NPV for LPA | 88.89% | 86.96% |

**[Table/Fig-6]:** Comparison of respiratory and non-respiratory samples tested by various methods.

Of the 29 LPA positive samples 27 (93%) samples showed sensitivity to RIF and INH both and two (6.9%) were found resistant to INH alone. One showed high level INH resistance and other had low level of INH resistance [Table/Fig-2]. All the samples were found sensitive to RIF.

## DISCUSSION

In this study we evaluated the LPA for simultaneous identification of MTBC and detection of drug resistance to INH and RIF in pulmonary as well as EPTB cases. In our study, 18.6% (5.5% non-respiratory and 32.4% respiratory) samples were found smear positive. All the smear positive samples were further found positive either by LPA or culture or both. One meta-analysis study reported 0.5% smear positivity in 0-4 year age 14% among 5-15 year and 52% among adults and concluded children are at more risk of missing the diagnosis by microscopy [12]. Sanker P et al., reported, 46.5% smear positivity by ZN smears in extrapulmonary samples [13]. While, 9.1% sensitivity of ZN staining for non-respiratory samples was reported by Neonakis IK et al., [14].

Total culture positivity in our study was found to be 33% including 35.3% in respiratory samples and 30.5% in non-respiratory samples and corresponds with other studies [15-17]. Lower positivity, 13.8% was reported by some authors [18,19] and higher (51.8%) culture positivity observed in another study on extrapulmonary samples [20]. The smear positive/negative but LPA positive samples which didn't grow in culture can be explained by destruction of bacilli during decontamination procedure or the patients were already on anti-tubercular therapy. Among the respiratory samples maximum isolation was from sputum and ET followed by BAL and pleural fluid. Out of all non-respiratory samples maximum isolation was observed from Lymph Node (LN) aspirates and pus samples, corresponds with other studies [16,18,20]. None of the CSF sample showed growth in culture.

Most of the studies on LPA are done only for respiratory samples or on culture isolates obtained from non-respiratory samples. It has shown that sensitivity of LPA increases with burden of bacilli, a study from South Africa reported the sensitivity of LPA in sputum smear negative, 13.7; smear scanty, 46.2%; smear 1+, 69.1%; smear 2+, 86.3%; smear 3+, 89.8% [21]. We found 90% sensitivity of LPA in smear positive samples and 69.23% in smear negative samples. In another study 98.8% sensitivity of the assay has been reported from respiratory culture positive samples [14]. In our study sensitivity of LPA was observed 83.3% in respiratory samples. Very few studies are available on direct detection of MTBC from non-respiratory samples by LPA. We found 72.73% sensitivity and 80% specificity of LPA in non-respiratory samples which corresponds with other study reported 71.4% sensitivity and 92.8% specificity of LPA for the detection of MTBC in the EPTB [22]. LPA was performed directly on culture positive extrapulmonary samples and reported 100% positivity by Laxmi KR et al., and 90.9% sensitivity of the assay by Neonakis et al., [23,14]. One study from south India done by IRL (Intermediate Referral Laboratory) under RNTCP, evaluated the performance of LPA (GenoType MTBDRplus VER 1.0) directly on the samples, has reported 74.3% positivity of the assay in smear positive and 15.3% positivity among smear negative extrapulmonary samples [13]. The present study showed higher positivity, 35.3% (12/34) by LPA in smear negative non-respiratory samples. This could be because we have used the newer VER 2.0 of MTBDRplus assay which is recommended for smear negative samples also. Overall, 92.3% (12/13) positivity of LPA was seen in smear positive samples and 30% (17/57) positivity was found in smear negative samples and it showed 100% positivity in smear positive respiratory samples.

A total of 10 (14.2%) samples which were grown in culture but found negative by LPA could be NTM (Non Tuberculous Mycobacteria) or presence of some PCR inhibitors in the samples can be responsible for negative LPA results. Another study from India reported 3.4% isolation of NTM from extrapulmonary samples [24].

In the current study 27 (93%) LPA positive samples were found sensitive to both INH and RIF while other studies have reported 60-70% sensitivity to INH and RIF [24,25]. Only 2 (6.9%) samples (CSF, LN aspirate) showed resistance to INH only. High level resistance to INH was seen in case of LN TB. It was smear negative and culture positive whereas, low level INH resistance was seen in tubercular meningitis case which was found negative by smear and culture. In both the cases clinicians started appropriate treatment within 2 days which could have been delayed or missed if the sample were not tested by LPA. Higher resistance was reported by other studies. Singhal R et al., reported 7.1% INH monoresistance, 7.7% RIF monoresistance and 16.9% resistance to both the drugs (MDR) [25]. Similarly, Goyal S et al., reported 19.6% INH monoresistance, 3.6% RIF monoresistance and 14.3% MDR TB [24]. The low level of resistance in our study could be due to the small sample size and the study was done in a private tertiary care hospital whereas, most of the MDR TB cases belongs to low income group usually take treatment from Government Institutes. LPA performed directly on the samples gives results within 6-8 hours. Conventional culture and drug susceptibility test takes 8-12 weeks while MGIT culture and susceptibility takes 25-30 days. Though, sensitivity of LPA in non-respiratory samples is low but it is better than microscopy. Hence, it can be performed directly on non-respiratory samples till the culture report is awaited. Even when this test is performed on the culture isolates obtained from non-respiratory samples saves further 4-6 weeks required for conventional drug susceptibility testing.

GeneXpert gives results within two hours but it provides only RIF sensitivity. LPA set up can be used for second line anti-tuberular drug sensitivity testing also.

## LIMITATION

Limitation of the study was that the sample size was small;

more studies need to be done with bigger samples size. LPA requires dedicated rooms for DNA preparation and amplification and a BSL-2 laboratory for processing samples or a BSL-3 if manipulation of culture is required, limits its use up to referral laboratories only.

## CONCLUSION

Use of LPA directly on samples can diagnose TB and drug resistance in a single day, particularly, from extrapumonary samples, which are missed by microscopy and simultaneously detect drug resistance that allows to start prompt and appropriate treatment till waiting for culture results. We recommend that this test must be performed on all the samples from suspected TB cases along with conventional diagnostic methods.

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