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Line Probe Assay as a Rapid Tool for Detection of MDRTB

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Authors' contributions

This work was carried out in collaboration between all authors. Author RR designed the study, performed the data acquisition, data analysis, and statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and manuscript review. Author GSVK managed data analysis, statistical analysis, and manuscript review, analyses of study, manuscript editing and review. Authors VJ and RR managed the analyses of the study, manuscript editing and review. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To evaluate genotypic Line Probe Assay (LPA) for rapid detection of Multidrug Resistant Tuberculosis (MDRTB) directly from sputum samples in comparison with Drug Susceptibility Testing (DST) on phenotypic MBBacT liquid media.
Study Design: Data analysis from 86 *Mycobacterium tuberculosis* (MTB) strains was done using SPSS version 17.
Place and Duration of Study: Department of Microbiology, JSS Medical College, Mysore, Karnataka, between January 2011 to January 2012.
Methodology: MDRTB rate detected by LPA assay from 92 samples by noting the mutations in hot spot region of *rpoB* gene, *katG* and *inhA* regulatory region and compared with DST on MBBacT liquid media.

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Results: Out of 86 MTB isolates, resistant rates for Rifampicin (RIF) and Isoniazid (INH) were 41.8%, 39.53% by LPA and 45.34%, 55.81% by MBBacT. LPA assay showed sensitivity and specificity as 92.35%, 100% for RIF resistance detection and 70.83%, 100% for INH resistance detection, 94.74%, 100% for MDRTB detection compared to conventional DST results.
Conclusion: This study showed that LPA has high detection rate for RIF resistance. However to improve the detection of INH resistance in MTB strains additional probes are to be included in LPA. LPA has good sensitivity and specificity for MDRTB detection with turnaround time of less than 48 hours.

Keywords: LPA; MDRTB; RIF; INH; resistance.

1. INTRODUCTION

MDRTB has emerged as a major public health problem. Annual global MDRTB burden is estimated as 5% of global TB burden. Only 5% of MDRTB patients are currently detected worldwide as a result of serious laboratory capacity constraints [1]. MDR-TB is defined as resistance to at least isoniazid and rifampicin with or without resistance to other first-line drugs. MDR TB requires relatively costly laboratory diagnosis and treatment for at least two-years with drugs that are expensive, toxic and not particularly potent [2]. The main goal of National 12th Five year plan is Universal Access to quality TB diagnosis & treatment for all pulmonary & extra pulmonary TB patients including drug resistant and HIV associated TB [3]. Detection of MDRTB helps in timely and effective disease management to prevent the spread of tuberculosis. Patients infected with MDR strains remain as sources of infection for longer period.

Easy-to-perform, rapid, and cost-effective assays based on molecular techniques that are suitable for application in clinical mycobacteriology laboratories are necessary to evaluate the presence of genomic mutations conferring resistance. Detection of resistance by conventional methods is inadequate due to the slow growth rate of M. tuberculosis; in addition, direct detection of known mutations could be more reliable in predicting the response to therapy [4].

Recently World Health Organization (WHO) recommended the use of molecular LPAs for rapid screening of MDRTB in low and middle income countries [1]. LPA is based on multiplex Polymerase Chain Reaction (PCR) in combination with reverse hybridization to identify either wild type sequences and/or specific mutations [5]. INNO-LiPA RifTB (Innogenetic, Ghent, Belgium) targeting *rpoB* gene and GenoType MTBDRplus (Hain Lifescience GmbH, Nehren, Germany) targeting *rpoB*, *katG* and *inhA* genes in both culture isolates and sputum samples are the two commercially available line probe assays. MTBDRplus assay has an advantage over INNO-LiPA Rif TB in detecting resistance to INH also [6,7,8].

UNITAID (Tous Unis pour Aider) has recently funded a project to introduce new TB diagnostics in selected low income countries and the project partners include WHO Global Laboratory Initiative (GLI), Foundation for Innovative New Diagnostics (FIND), Stop TB partnerships Global Drug Facility (GDF) [9].

A number of studies have evaluated the diagnostic accuracy of LPA for detecting MDRTB in diverse geographic settings. We conducted this study to evaluate LPA assay for rapid

identification of MDR strains by detecting mutations in the *rpoB, katG, inhA* genes directly from sputum samples in comparison with DST on MBBacT media in TB endemic country.

2. MATERIALS AND METHODS

2.1 Study Setting

From January 2011 to January 2012, a total of 92 sputum specimens were collected from patients at risk of MDRTB visiting JSS referral Hospital for tuberculosis in Mysore, Karnataka. Each specimen was accompanied by a physician-completed questionnaire that included pertinent patient demographics, clinical history, and MDR-TB risk factors. The three major MDR-TB risk factors reported were prior anti-TB treatment for Pulmonary Tuberculosis, contact with other MDR-TB patients and origin from an area with a known high incidence of MDR-TB. We concentrated our work on pulmonary samples, as these are by far the most important kind of specimen in our laboratory. DST on MBBacT and LPA was carried out at this referral centre.

2.2 Specimen Collection and Processing on MBBacT

Two sputum samples (spot or morning) were collected per patient in 50 ml sterile leak proof wide mouth container. The sputum specimen had a volume of approximately 5ml. All specimens were processed by Ziehl Neelsen (ZN) smear microscopy, cultured on MBBacT liquid medium. Later drug susceptibility testing for the isolates were carried out on MBBacT liquid media by proportion method, which were then analyzed by LPA assay. Entire processing was performed in Biosafety cabinet 2.

N-acetyl L-Cysteine & Sodium Hydroxide (NALC-NaOH) decontamination method was used as a concentration technique [10]. Specimens were then incubated at room temperature for 15 minutes, concentrated, decanted and resusupended. One part of the suspension was inoculated onto MBBacT and residual suspension was kept frozen at -70ºC and used for genotype LPA testing. Those which flagged positive in MBBacT machine were examined for Acid fast bacilli (AFB) using ZN staining, confirmed as MTB using MPT64 antigen detection test [11]. All isolates were subjected to DST on MBBacT for INH and RIF using previously described method [12]. All positive MBBacT vials were stored at -70ºC for the duration of assessment to allow discrepant testing.

2.3 LPA Assay

Assay was performed as per manufacturer's instructions (HAIN Life science GmbH, Germany, Genotype MDRTB assay).

A. DNA isolation from sputum

Quick protocol that yields DNA suitable for amplification is shown below:

1 ml of decontaminated sample was suspended in 300µl Sterile distilled water (SDW)

↓

Bacteria was pelleted by spinning for 15 min at 10000 x g

↓

Supernatant was discarded and bacteria was suspended in 100-300 µl of water

↓

Bacteria were incubated for 30 min at 95℃ in a wat er bath

↓

Spinned for 5 min at full speed and 5 µl of the supernatant was directly used for PCR.

In case DNA solution was to be stored for an extended period, supernatant was transferred to a new tube and stored at -20ºC.

B. DNA amplification

Amplification mix (45 µl) was prepared in a DNA-free room.
Per tube mix:

- 35 µl Primer/Nucleotide mix (PNM)
- 5 µl 10x polymerase incubation buffer
- 2 µl MgCl2solution (1.5 and 2.5 mM)
- 1-2 unit(s) taq polymerase (QIAGEN, Hilden, Germany)
- 3 µl water to obtain a volume of 45 µl
- Add 5 µl DNA solution (20-100 ng DNA) leading to a final volume of 50 µl

Negative control (water instead of DNA solution) was also put up. Master Mix was prepared containing all reagents except for DNA solution and mixed well. 45 µl was aliquot in each of the prepared PCR tubes.

Amplification profile:

Duration & Temp	**Culture samples**	**Direct patient material**
5 min 95ºC	1 cycle	1 cycle
30 sec 95ºC	10 cycles	10 cycles
2 min 58ºC		
25 sec 95ºC	20 cycles	30 cycles
40 sec 53ºC		
40 sec 70ºC		
8 min 70ºC	1 cycle	1 cycle

Amplified products were stored at +4 to –20ºC.

C. Hybridization

20 µl of Denaturation Solution (DEN) was dispensed in a corner of each of the wells used,

↓

20 µl of amplified sample was added to the solution, mixed well

↓

Incubated at room temperature for 5mins

↓

Strips were taken out of the tube using tweezers and marked with a pencil

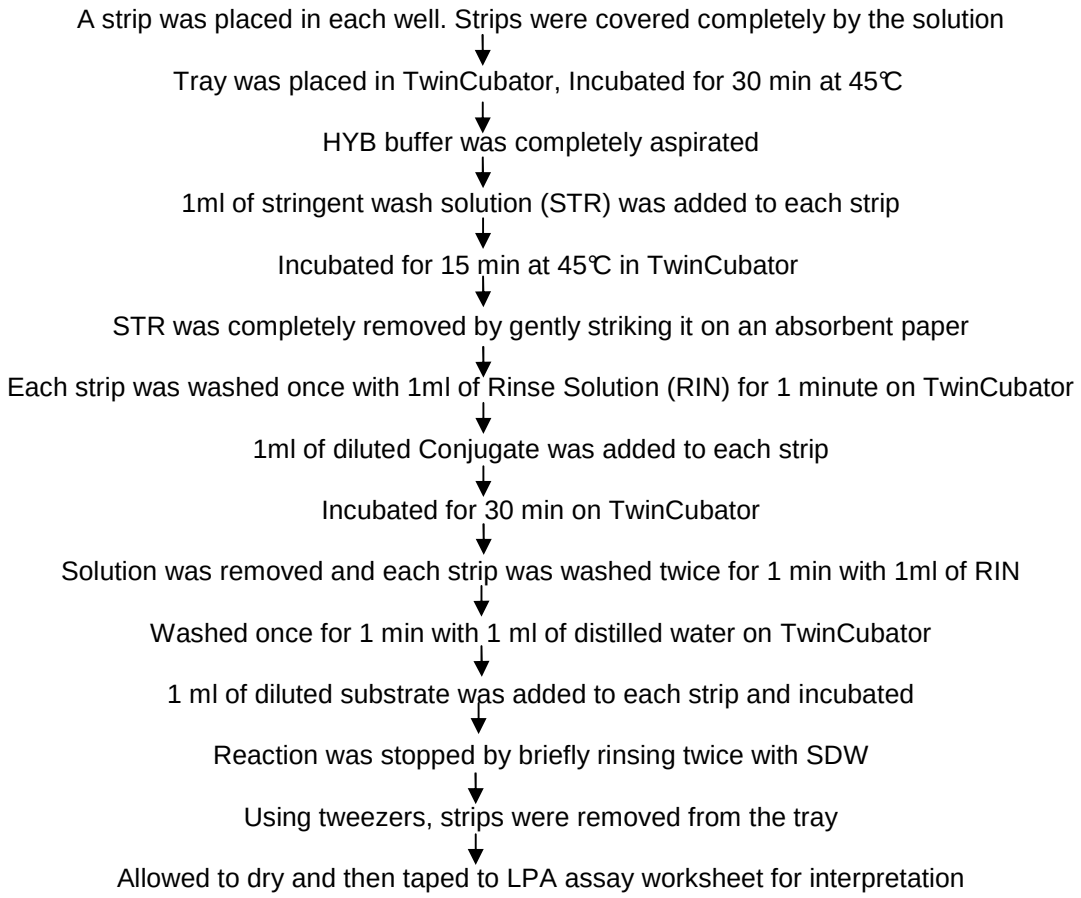
↓

1 ml of prewarmed Hybridization Buffer (HYB) buffer was added to each well.

↓

Tray was gently shaken until the solution has a homogenous color

↓



2.4 Repeat Testing and Discrepant Analysis

Samples with invalid results on LPA were retested using stored residual suspension. Band intensity had to be equal or greater than the Amplification Control (AC) band, for the test to be valid (according to product insert).

2.5 Data Analysis

All statistical tests were performed using SPSS version 17. P value <0.05 was considered statistically significant. Formal ethical review was obtained from ethical review committee.

3. RESULTS AND DISCUSSION

Out of total 92 smear positive sputum samples, 86 samples were identified as MTB by ICT test, remaining 6 samples were identified as *Atypical Mycobacteria*. Among 86 MTB strains, 71 (82.55%) were males and 15 (17.44%) were females. Highest numbers of males (57) were in the age group of 40-49 yrs and highest numbers of females (13) were in the age group of 20-29 yrs. All 86 MTB strains were tested for DST by Proportion method on MBBacT and were then run on LPA. 86 specimens gave interpretable LPA results, including repeat testing results which was performed in few isolates. Interpretation of the results on

LPA was strictly done as per the product insert, which states “only those bands whose intensities are about as strong as or stronger than that of the Amplification control zone are to be considered”.

3.1 Interpretation of Results

In MBBacT, 39 (45.34%) were resistant, 47 (54.65%) were sensitive to RIF and 48 (55.81%) were resistant, 38 (44.18%) were sensitive to INH. Susceptibility pattern on LPA showed that 36 (41.80%) were resistant, 50 (58.13%) were sensitive to RIF and 34 (39.53%) were resistant, 52 (60.46%) were sensitive to INH (Table 1).

Table 1. Susceptibility pattern on MBBacT and LPA

Drug	Susceptibility pattern	MBBacT	LPA
RIF	Resistant	39 (45.34%)	36 (41.80%)
	Sensitive	47 (54.65%)	50 (58.13%)
INH	Resistant	48 (55.81%)	34 (39.53%)
	Sensitive	38 (44.18%)	52 (60.46%)

Conventional method (MBBacT) is considered as gold standard. From all 39 RIF resistant strains, 36 could be correctly detected by LPA as well as 47 out of 47 susceptible strains, resulting in a sensitivity and specificity of 92.31% and 100%, respectively. But LPA detected 3 strains as sensitive to RIF which were reported as resistant on MBBacT (Table 2).

From all the 48 INH-resistant strains, 34 could be correctly detected by LPA, as well as 38 out of 38 susceptible strains, resulting in sensitivity and specificity of 70.83% and 100%, respectively. But in addition LPA detected 14 strains as sensitive to INH which were actually resistant on MBBacT.

Out of 86 strains, 19 (22.09%) were detected as MDR by MBBacT and 18 (20.93%) were detected as MDR by LPA (Table 2).

Table 2. Comparision of LPA result with MBBacT for RIF and INH resistance, multi drug resistance (n=86)

Tests for MDRTB detection		MBBacT	
		INH Res	**INH Sensitive**
LPA	**INH Res**	34	0
	INH Sens	14	38

Sensitivity: 70.83%, Specificity: 100%, NPV: 73.08%, PPV: 100%

Tests for MDRTB detection		MBBacT	
		RIF Res	**RIF Sensitive**
LPA	**RIF Res**	36	0
	RIF Sens	3	47

Sensitivity: 92.31%, Specificity: 100%, NPV: 94%, PPV: 100%
PPV – Positive predictive value, NPV – Negative predictive value.

3.2 Banding Patterns

The patterns of mutations associated with rifampicin and isoniazid resistance is shown in Table 3.

Table 3. Patterns of gene mutations by LPA

rpoB mutations	Frequency	Percentage (%)	*katG* muataions	*inhA* mutations	Frequency	Percentage (%)
S531L	26	72.22	S315TI	WT	20	66.66
D516V	2	5.55	WT	C15T	2	6.66
H526D	2	5.55	S315T1	C15T	2	6.66
Unknown	6	16.66	WT	Unknown	2	6.66
			Unknown	WT	2	6.66
			Unknown	C15T	1	3.33
			S315T1	T8C	1	3.33

4. DISCUSSION

By molecular analysis most common mutations have been detected and genotypic methods target these mutations and identify the resistance pattern. LPA's are highly sensitive (>=97%) and specific (>=99%) for the detection of rifampicin resistance, alone or in combination with isoniazid (sensitivity >=90%; specificity >=99%), on isolates of M. tuberculosis and on smear-positive sputum specimens. For detection of MDRTB the overall accuracy was 99% [1].

Correlation of DST on LPA performed directly from sputum smear positive cases and that performed on MBBacT was very high as reported by other studies [13]. Error in DNA extraction process may be the reason for initial invalid results. Interpretable results were obtained by repeat testing.

In this study low resistance rates for INH and RIF was noted in LPA when compared to MBBacT. INH resistance in 14 isolates was not detected by LPA which could be due to inability of the test to detect mutation in other genes *(ahpC-oxyR and ndh)* conferring INH resistance. Some cases of resistance may be caused by mutations not detectable with LPA, e.g., in other regions of *rpoB, katG*, or unknown genes [14]. Discrepant results may also be caused by a nosocomial strain during the patient stay in the hospital. The phenomenon of the nosocomial transmission of MDR/ XDR-TB between patients being determined by the investigation of M. tuberculosis DNA diversity using DNA fingerprinting in TB hospitals from Moldova was demonstrated in earlier studies (V. Crudu et al., presented at the Annual Congress of the European Respiratory Society, Barcelona, Spain, 18 to 22 September 2010).

In this setting performance of LPA was similar to that reported previously, with high specificity for detection of RIF and INH resistance, high sensitivity for detection of RIF resistance and somewhat lower sensitivity for INH resistance [9,15].

RIF resistance was noted to be highly associated with mutation in S531L of *rpoB* gene as reported in other studies [16,17,18]. Specific mutation could be detected in 30 of 36 (78.94%) RIF resistant isolates on LPA. Of these, 26 (72.22%) had mutation in codon S531L, 2 (5.55%) in D516V and 2 (5.55%) in H526D. In 6 (16.66%) of 36 RIF resistant

isolates, one or more wild type probes were missing with no gain in mutant probes (Table 3).Bands were missing in 16.66% of RIF resistant strains. Among these maximum isolates had missing WT8-10. In a study from South Vietnam, 66.7 per cent isolates did not have any known mutation [19].

Our study showed high sensitivity and high specificity for RIF resistance detection by LPA in comparison to MBBacT. High sensitivity for RIF resistance detection has been observed in other studies like 97.6% [20], 98.1% [15] and 100% [9,21]. Ling et al. [15], Albert H et al. [9] and Huyen et al. [19] reported similar specificity for RIF resistance detection.

Most common mutation site conferring INH resistance was noted in *katG* gene S315T, as observed in other studies [22]. Among 34 INH resistant isolates, *katG* mutation occurred in 26 (76.47%) of isolates. Specific mutations in codon S315T1 of *katG* gene was found in 23 (84.46%) isolates. Remaining 3 had missing wild type with no gain in mutant probes. Mutations in *InhA* gene occurred in 8 of 34 (23.52%) INH resistant isolates. Specific *InhA* mutations were found in 6 of 8 (75%) INH resistant isolates, out of which 5 had mutations in codon C15T and 1 had muatatioin T8C (Table 3). In remaining 2 isolates, no specific mutation band could be detected. The mutation pattern obtained was similar to other studies in this regard [9]. Studies conducted in different countries showed variable association of INH resistance with mutations in *katG* or *inhA* [1].

Low sensitivity for INH resistance detection in our study is in concordance with other studies like 83.3% [20], 84.3% [15], 80.8% [9] and 67% [21]. However high sensitivity for INH resistance was found in other studies as 92.6%, 93%, 95.3%. [12,19,23]. The difference in sensitivity rates for INH resistance in various studies is because of small numbers of isolates sampled from populations, lack of DNA fingerprinting defining the relationship between isolates in population samples, limited number of genotypically distinct isolates, restriction of investigation to MDRTB isolates, and limited analysis of sensitive isolates.

In our study, LPA could detect 18 out of 19 (94.73) MDR strains. Thus LPA is on par with conventional methods for detecting MDRTB but with very short turnaround time of less than 48 hrs. Similar findings were observed in other studies [24].

4.1 Advantages and Limitations of Genotypic LPA over Conventional DST Method

LPA's are rapid for detection of slow growing organism like MTB, for partially treated cases where the growth of the organism is inhibited, for detection of resistance directly in clinical specimens, and obviate the need for prior isolation of MTB by culture. LPA assess the genotype of the organism, whereas conventional techniques assess the phenotype under laboratory conditions. LPA do not carry the biohazard risk associated with cultivation of MTB in conventional methods [25,26].

Both the PCR technology and the reverse hybridization technique used for LPA are proved to be robust and reproducible, and interpretation of the results are easy and doesn't require the expert knowledge which is required for the interpretation of real-time PCR data or DNA sequencing data. LPA can easily be implemented in routine work flows and all tests can be run by using the same platform technology. On the other hand, LPA has the same limitations as other molecular tests used for the detection of antibiotic resistance, and therefore, it cannot totally replace conventional culture-based methods for DST. A variable proportion of resistant strains will not be detected because none of the molecular tests established targets

for all the possible genes or mechanisms involved in resistance. The second inherent limitation is the detection limit of ca. 10% mutant DNA in a mixture of wild-type and mutant DNA. If the proportion of resistant cells in an isolate is less than that amount, it can hardly be detected by molecular methods, whereas conventional DST testing might give a more sensitive test result in these cases [5]. Genotypic mutations may not always lead to phenotypic expression levels manifesting as drug resistance [26]. But it is important to remember that even the data generated by conventional in-vitro methods of drug susceptibility testing do not always correlate to clinical drug resistance [27].

For carrying out LPA in laboratory, it requires atleast 3 separate rooms – one each for DNA extraction, pre-amplification procedures, and amplification and post-amplification procedures. Amplicon contamination leading to false positive results can be avoided by restricted access to molecular facilities, uni-directional work flow, and stringent cleaning protocols [1]. Cost of LPA's under routine diagnostic algorithms varies between 30% and 50% when compared to conventional DST methods. The cost was still lower when LPA was used to test on smear positive specimens than on isolates from liquid primary culture [1].

Establishment of molecular laboratories, maintenance of BSL2, adequate laboratory staffing, supervision of the laboratory staff by a senior individual who has adequate training and experience in molecular assays, stringent laboratory protocols, standard operating procedures for LPA's, and internal quality control mechanisms and a programme for external quality assessment of laboratories involved in LPA's are demanding and should be developed as a matter of priority [1].

Conventional DST takes 2-4 months during which time a patient is often treated according to the standard regimen for drug-susceptible TB. This amplifies the drug resistance and treatment outcome is also adversely affected because of the resultant delay in proper treatment. But widespread implementation of culture-based DST may be challenging in many settings due to financial, infrastructural and human resource requirements [9].

Line probe assays are not a complete replacement for conventional culture and DST, as MTB culture is still required for smear-negative specimens while conventional DST is still necessary to confirm XDR-TB [1]. Nevertheless, LPA assay appears to be a valuable tool that allows the detection of resistant M. tuberculosis isolates within one working day. Considering the high MDR isolates in several parts of the world, LPA has the potential to complement and accelerate the variety of different measures in laboratory diagnostics that are necessary for improved tuberculosis control in the future [5].

LPAs are currently validated only for use directly from smear-positive specimens, although reasonable performance in a small sample of smear-negative specimens. Ongoing research into improved DNA extraction methods may enable LPAs to be performed directly from smear-negative sputum in future. However the cost-effectiveness of routine testing of smear-negative specimens would have to be carefully evaluated since the majority of specimens will be negative in most settings [9]. In order to facilitate widespread access for LPA testing for public health sector in high burden countries, FIND provides equipments and reagents at a lesser price.

5. CONCLUSION

In conclusion LPA is a rapid, reliable, sensitive and specific test for MDRTB screening. When compared to other improved diagnostic facilities, LPA implementation as an

appropriate tool can only impact on MDRTB patient care. Larger field trials are necessary to access the validity of the test, so that LPA can be deemed to play pivotal role in TB control at national level.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this article.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the JSS institutional board of study meeting and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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