Utility of Line Probe Assay for the Early Detection of Multidrug-Resistant Pulmonary Tuberculosis

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ABSTRACT

Background: Despite endorsement of the line probe assay (LPA) for the diagnosis of drug-resistant pulmonary tuberculosis patients, there is limited data available on the performance of LPAs in India, especially from high burden states like Maharashtra, for the early diagnosis and detection of drug resistance, in order to initiate timely and appropriate treatment. **Objective:** To evaluate the utility of the line probe assay (LPA) for the early diagnosis of drug-resistant pulmonary tuberculosis as compared to the 'Gold standard' 1% proportion method (PM). **Materials and Methods:** A total of 687 patients suspected of pulmonary tuberculosis were screened. One hundred samples (95 sputum and 5 BAL), positive for Acid Fast Bacilli (AFB) by Ziehl Neelson (ZN) smears, were included in the study. Digested and decontaminated specimens were subjected directly to the LPA (Genotype MTBDR@ plus assay) and were processed in parallel using the conventional culture on the Lowenstein-Jensen (LJ) medium followed by drug susceptibility testing (DST) using the PM. **Results:** All the 100 samples gave interpretable results on LPA with a turnaround time of 24-48 hours as opposed to six to eight weeks taken by the 1% proportion method. Sensitivity for the detection of rifampicin, isoniazid, and multidrug resistance (MDR) was 98.1, 92.1, and 95%, respectively, with a specificity of 97.8% for rifampicin and 98.33% for MDR detection. It also had the additional advantage of allowing a study of mutation patterns. **Conclusions:** High performance characteristics and a short turnaround time makes LPA an excellent diagnostic tool, for an early and accurate diagnosis, in a high MDR- TB-prevalent region, as reflected from our data.

Key words: Genotype MTBDR plus, pulmonary tuberculosis, multidrug resistance, heteroresistance

INTRODUCTION

Globally, tuberculosis (TB) cases continue to be on the rise despite aggressive public health interventions. India bears a quarter of world's tuberculosis burden and has the highest number of newly diagnosed cases annually.[1] Drug-resistant TB is the most important factor that threatens to disrupt the gains achieved in tuberculosis control. Multidrug resistant (MDR) tuberculosis is defined as resistance to both rifampicin and isoniazid. MDR TB needs to be treated with a different regimen, with second-line anti-TB drugs.

A prolonged time of diagnosis of six to eight weeks taken by the conventional culture and drug susceptibility testing (DST) leads to the patients being treated with an inappropriate drug regimen, which results in the selection of drug-resistant mutant strains and their continuous spread in the community.[2] Many alternate rapid methods have been devised to address this issue.[3] Genotypic or molecular methods exploit the fact that multidrug resistance arises due to random mutations, predominantly found in genes *rpoB, katG*, and *inhA,*[2] and detection of these mutations enables the early diagnosis of resistance and institution of the appropriate therapy. What is more, nucleic acid amplification tests (NAATs) do not require the growth of an organism, and can be performed directly on the clinical samples. They carry a lesser biohazard risk and have the feasibility for automation.[3]

The line probe assay (LPA) is a promising rapid diagnostic tool, based on a combination of the multiplex polymerase chain reaction followed by the DNA strip reverse hybridization assay.[4] Although the World Health Organization (WHO) has endorsed the incorporation of

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LPAs in screening and diagnostic algorithms for pulmonary tuberculosis patients, especially in high-burden countries,[4] limited data is available on the performance of LPAs in India, especially from the Western region where MDR touches 53%.[5]

This study was, therefore, undertaken, (i) to evaluate the performance of LPA for early detection of multidrug resistance, as compared to the conventional 'Gold standard,' that is, the 1% proportion method (PM) on Lowenstein-Jenson solid culture medium and (ii) to study the frequency of mutations in *M. tuberculosis* in Western Maharashtra.

MATERIALS AND METHODS

Study design and setting: This cross-sectional analytical study was carried out at the Department of Microbiology, B.J. Government Medical College and State TB Training and Development Centre (STDC), Intermediate Reference Laboratory (IRL), over a period of 15 months, from January 2011 to June 2012. The study was approved by the Institutional Ethical Committee of the Hospital.

Patient selection: Patients attending the Outpatient Department and admitted to the Inpatient Ward, with symptoms and signs suggestive of pulmonary tuberculosis, were referred to our TB laboratory.

Inclusion criteria: Pulmonary specimens positive for AFB by ZN smears

Exclusion criteria: Pulmonary specimens negative for AFB and extrapulmonary specimens.

Processing and culture: All specimens were subjected to digestion and decontamination by the N-acetyl L-cysteine and sodium hydroxide (NaLC-NaOH) method.[6] The sediment was reconstituted in 1-2 ml of phosphate buffer pH 6.8. Two loopfuls each were inoculated into the LJ medium and the remaining sediment was stored in screw-capped vials. This sediment was directly subjected to the line probe assay after DNA extraction and polymerase chain reaction (PCR). The LJ medium slopes were followed for a period of six weeks and any growth was confirmed to be an *M. tuberculosis* complex by slow growth, niacin accumulation, and nitrate utilization tests, as also the inability to grow in the LJ medium incorporated with para nitrobenzoic acid.

Conventional drug susceptibility testing: A one percent economic variant of the proportion method on the LJ medium was done by the standard method.[6] The concentrations of Isoniazid, Rifampicin, Ethambutol, and Streptomycin were 0.2 μg/ml, 40 μg/ml, 2 μg/ml, and 4 μg/ml, respectively. The standard strain of *M. Tuberculosis,* H37Rv, was used as a positive control. A strain is considered resistant to a particular drug, if greater than 1% of the population of bacilli (critical concentration) is able to grow on the drug-containing medium.

The line probe assay: LPA was carried out using the commercially available Genotype MTBDR plus assay (Hain Lifescience, GmbH, Nehren, Germany), according to the manufacturer's instructions.

DNA extraction: 500 μl of the decontaminated sample was centrifuged at 10,000 g and the sediment was resuspended in 100 μl of sterile distilled water. Heat killing was done at 95°C for 20 minutes followed by sonication for 15 minutes. The samples were then centrifuged at 13,000 g for 5 minutes and 5 μl of the supernatant containing DNA was used for the PCR.

The amplification mixture consisted of 35 μl of the primer nucleotide mix, 5 μl of the PCR buffer with 20 mM MgCL2, 1 U of HotStar Taq DNA polymerase from Qiagen, 3 μl of molecular biology grade water, and 5 μl of the supernatant containing DNA in a final volume of 50 μl. Amplification was done in a thermal cycler (Applied Biosystems) using the amplification profile: Denaturation of 15 minutes at 95°C, 40 seconds at 53°C, and 40 seconds at 70°C, and the extension step of 8 minutes at 70°C.

Hybridization was done using a preprogrammed Twincubator (Hain life Science, GmbH, Nehren, Germany). After denaturation, the biotin-labeled amplicons were hybridized to single-stranded membrane-bound probes. After stringent washing, a Streptavidin-Alkaline phosphatase conjugate was added to the strips and an alkaline phosphatase mediated staining reaction was observed as bands, where the amplicon and the probe had hybridized.

Each LPA strip has 27 reaction zones (bands) — six control bands included conjugate, amplification, *M. tuberculosis* complex (TUB), rpoB, katG, and inhA, eight rpoB wild-type (WT1-WT8), four mutant probes (rpoB MUT 1 D516V, rpoB MUT 2 H526Y, rpoB MUT 3 H526D, and rpoB MUT4 S531L), 1 katG wild-type and two mutant probes (katG MUT 1 S315T1 and katG MUT 2 S315T2), and two inhA wild-type and four mutant probes (inhA MUT1 C15T, inhA MUT2 A16G, inhA MUT3A T8C,

inhA MUT3B T8A). Interpretation was done according to the manufacturer's guidelines as follows [Figures 1a-d]:

- Susceptible: Presence of wild-type bands and absence of mutant bands.
- Resistant: Absence of wild-type bands and / or presence of mutant bands.
- Heteroresistance: Presence of bands due to hybridization of both wild-type and the corresponding mutant probes indicates a heterogeneous population or mixed infection of a sensitive and a resistant strain.

Data analysis

A 2 × 2 contingency table was used to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the Kappa coefficient of the line probe assay in comparison to the conventional 1% proportion method, for detection of rifampicin and isoniazid resistance, as also multidrug-resistant tuberculosis. The pattern and frequency of mutations were also analyzed.

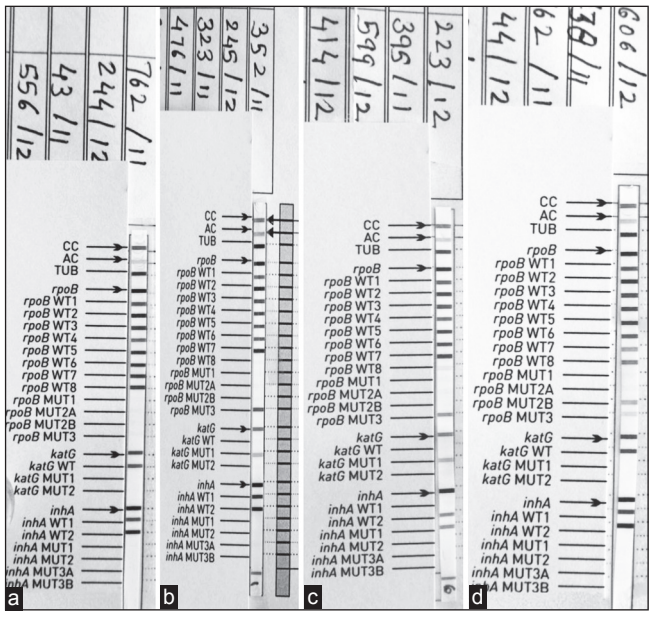

Figure 1: (a) Sensitive strain: All Wild-Type Bands for Rifampicin (rpo B) and Isoniazid (inhA and katG) present (b) Strain resistant to both Rifampicin and Isoniazid (Multidrug resistant): rpo B Wild-Type Band (WT8) absent and corresponding mutation (MUT3) present. katG Wild-Type 1 (WT) absent and corresponding (MUT 1) present. (c) Strain resistant to both Rifampicin and Isoniazid (Multidrug resistant): rpo B Wild-Type Band (WT8) absent and corresponding mutation (MUT3) present. katG Wild-Type 1(WT) absent and corresponding (MUT 1) present. inhA Wild-Type Band(WT1) absent and corresponding mutation(MUT1) present. (d) Heteroresistance: All rpo B Wild-Type Bands present and mutation bands MUT2B and MUT3 present, which reflects the presence of both sensitive and resistant strains in the same sample (patient). Proportion method showed resistance to Rifampicin

RESULTS

A total of 687 patients suspected of pulmonary tuberculosis were screened. Of them, 100 sputum and bronchoalveolar lavage (BAL) specimens, positive for acid fast bacilli on a ZN smear examination, were included in the study. Of the 97 sputum samples studied, 16 were +1, 37 were +2, and 44 were +3 by the Revised National Tuberculosis Control Program (RNTCP) grading for the bacillary load of AFB positive smears.

By the proportion method, of the 100 cases, 26 (26%) were susceptible to all the first-line drugs (isoniazid, rifampicin, ethambutol, and streptomycin), 51 (51%) were resistant to Isoniazid, 53 (53%) were resistant to rifampicin, 40 (40%) strains were multidrug-resistant (MDR), that is, resistant to both isoniazid and rifampicin.

By the line probe assay, all 100 samples gave valid interpretable results.

Concordance between LPA and PM

Taking into consideration the 1% proportion method as the 'Gold Standard', LPA accurately identified 52 of the 53 rifampicin-resistant strains, showing a sensitivity of 98.1%, specificity of 97.8%, and a kappa coefficient of 0.96. Forty-seven of the 51 isoniazid strains were identified correctly, with a sensitivity of 92.1%, a specificity of 97.9%, and a Kappa coefficient of 0.90. Thirty-eight of the 40 MDR were identified with a sensitivity of 95%, specificity of 98.33%, and a kappa coefficient of 0.93 [Table 1]. Discordant results for detection of resistance to rifampicin, isoniazid, and MDR were obtained for two, five, and three strains, respectively [Table 2].

Table 1: Comparison of results by the proportion method and line probe assay

Drug	Resistant by both methods	Discordant results		Percentage of discordant results (%)
		Sensitive by proportion method but resistant by line probe assay	Resistant by proportion method but sensitive by line probe assay	
R	52	1	1	2
H	47	1	4	5
Both H and R (MDR)	38	1	2	3

Table 2: Statistical comparison of results of LPA with the proportion method

Drug	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa coefficient
R	98.1	97.8	98.1	97.8	0.96
H	92.1	97.9	97.9	92.3	0.90
H + R (MDR)	95	98.33	97.4	96.7	0.93

Turnaround time to results

Including the time of sample processing, DNA extraction, and line probe assay, the time taken by this genotypic test, for providing drug susceptibility testing results was only 24-48 hours, as opposed to 10-12 weeks taken by the conventional susceptibility testing.

The pattern and frequency of mutations analyzed for rpoB, katG and inhA

The TUB band, which bound the amplicons to the *M. tuberculosis* complex, was present in all 100 strains, which confirmed them to be the *M. tuberculosis* complex. Among the 53 rifampicin-resistant strains, the mutations seen were: Missing rpoB WT8 in 83.01% and presence of the S531L mutation in 47.19% of the strains [Table 3].

Among the 48 isoniazid-resistant strains, katG mutations occurred in 100%: 87.5% had the S315T1 mutation and 2% had the S315T2 mutation. katG WT bands were missing in all strains (100%). Eleven MDR and two monoresistant strains showed absence of inhA WT band. In our study only two strains showed heteroresistance: One had all the WT bands along with the MUT2A and MUT3 band and the other had all WT bands with MUT 1 and MUT 3 bands. They were resistant to rifampicin by PM [Figure 1].

Table 3: The Pattern and frequency of mutations analyzed for *rpoB, katG, and inhA*

Gene	Band	Gene region or mutation	MDR (39)	Inh monoresistant (9)	Rif monoresistant (14)
rpo B	WT1	506-509	39	9	14
	WT2	510-513	39	9	14
	WT3	513-517	36	9	13
	WT4	516-519	37	9	14
	WT5	518-522	39	9	14
	WT6	521-525	39	9	14
	WT7	526-529	37	9	14
	WT8	530-533	8	9	1
	MUT1	D516V	5	0	0
	MUT2A	H526Y	1	0	1
	MUT2B	H526D	1	0	0
	MUT3	S531L	24	0	1
katG	WT	315	0	1	14
	MUT1	S315T1	34	8	0
	MUT2	S315T2	1	0	0
inhA	WT1	−15/-16	28	7	14
	WT2	−8	39	0	14
	MUT1	C15T	8	2	0
	MUT2	A16G	0	0	0
	MUT3A	T8C	2	0	0
	MUT3B	T8A	0	0	0
			0	0	0

DISCUSSION

Rapid diagnosis of tuberculosis, especially multidrug-resistant TB is vital for the national TB program as India is a major high TB burden country.[1] The Sassoon Hospital, Pune is the prime tertiary care center in western Maharashtra, with an accredited tuberculosis laboratory and cares for a large number of drug-resistant pulmonary tuberculosis cases from this entire high-prevalence region of Maharashtra.

Our data show that the LPA performed very well, with a sensitivity of 98.1% and specificity of 97.8% for detection of rifampicin resistance, and 92.1% sensitivity and 97.9% specificity for the detection of isoniazid resistance. These data correlate well with earlier reports from South Africa,[7] Vietnam,[8] and India.[9] For the detection of MDR, a significant sensitivity of 95% and specificity of 98.33% also correlate well with the other published studies.[10] In addition, a high Kappa coefficient demonstrates a very strong agreement between the results of both the tests.

The minor discordant results could be due the fact that the strains that did not show any mutation on LPA, were resistant by the proportion method, which was perhaps due to other less common mutations like ahpC, kas A, and so on, that were not detected by the test probes or that may have occurred in the genes, whose products were involved in drug permeation or metabolism.[11] However, the strains that were resistant by the proportion method, but sensitive by the LPA, could possibly be due to a mutation that was not phenotypically expressed, that is, a silent mutation.[12] These could form areas for future research.

The LPA test offers an enormous advantage for the treating clinician and to the patients, as the test is directly performed on clinical samples, with a short turnaround time of 24-48 hours. It would be highly advantageous for high burden areas where one needs to rapidly screen for MDR in a large number of samples, in order to initiate early and appropriate treatment.[7] Moreover, the vexing problem of contamination of the solid culture media faced in phenotypic assays, which leads to loss of culture samples, can also be overcome by a rapid genotypic assay such as LPA. Despite the requirement of an expensive elaborate laboratory in a resource-poor setup, with the cost per test being approximately Rs. 2300, the LPA may well be cost-effective in the long-term, as the prompt diagnosis and treatment of drug-resistant patients could decrease further occurrence of new cases, reducing the overall financial burden on the government to treat them. Another necessary potential area for future research is

the validation of LPA for smear-negative pulmonary TB and extrapulmonary TB, as the atypical presentation of tuberculosis is also rising, owing to the HIV epidemic.[13]

By including the probes targeting rpoB in the assay, LPA had successfully detected 98.1% of the rifampicin-resistant strains (both monoresistant and MDR) , corroborating the observation that more than 95% of rifampicin resistance worldwide and in India could be accounted for by mutations in the 81 bp region of the *rpo B* gene.[12] Absence of the WT 8 band and presence of the corresponding S531L hotspot mutation were similar to the data reported from Vietnam.[8] However, other studies from China,[14] South Africa,[7] and Denmark[15] showed a higher frequency of hotspot mutations, suggesting epidemiological variation of the strains in different geographical areas. As mutations in *M. tuberculosis* are profoundly influenced by the genetic background of a strain and the ethnic origin of a patient, determining the frequency of a particular mutation could be a useful way of monitoring drug resistance. This would also evaluate the need to develop newer assays with additional probes targeting additional mutations.

A high prevalence of katG mutations has been reported, which accounts for Isoniazid resistance in countries with high TB prevalence.[16] The same was seen in the present study, wherein, katG mutations accounted for resistance in 100% isoniazid-resistant strains. The S315T1 mutation was the most common mutation, as reported by others also.[8,14] More significant was the detection of monoresistance, where katG mutation in eight and inhA mutation in two isoniazid monoresistant strains was observed. LPA had a significant advantage over the GeneXpert MTB/RIF, its current competitor, which only detected rifampicin resistance, presuming that rifampicin was a surrogate marker for MDR.[17] In high INH-monoresistance countries, such as India, it would be highly erroneous to presume that a rifampicin-sensitive strain would be sensitive to Isoniazid as well, considering how vital isoniazid was in the RNTCP drug regimens.[18]

Heteroresistance, namely, the presence of both sensitive and resistant strains in the same patient, can be detected by LPA as the presence of all wild-type bands corresponding to the sensitive strain and presence of mutation bands corresponding to the resistant strain. This has also been reported in similar studies from Mumbai,[19] France,[20] and Turkey.[21] Heteroresistance can occur due to infection from two different strains, usually seen in new patients or in a single strain segregating into sensitive and resistant, as seen in previously treated patients, due to the positive selection pressure of drugs. LPA applied directly to clinical samples enhances the chance of detection of heteroresistance,[22] which can serve as an indicator of the quality of anti-TB programs.[23] Heteroresistant samples on LPA eventually showed drug resistance by phenotypic drug susceptibility testing, that is, they corresponded to the mutated organism.[16] Therefore, if a clinical sample is detected to be heteroresistant in the early screening by LPA, it can be inferred that the patient is drug-resistant and must given a regimen for drug-resistant TB.

Although LPA detects the mutations well in time, making it an excellent component of the screening algorithm for pulmonary TB, it is recommended that the results be eventually confirmed by a phenotypic drug susceptibility test, always.

CONCLUSION

With performance characteristics at par with the conventional PM, a short turnaround time to diagnosis, and the ability to simultaneously detect rifampicin and isoniazid resistance, makes LPA an excellent and reliable tool for the early detection of multidrug-resistant pulmonary tuberculosis in a high-burden country such as India.

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