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Comparative analysis of line probe assay and conventional method of drug susceptibility testing for the diagnosis of multi-drug resistant tuberculosis

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Abstract
Context: Prevalence of multi-drug resistant tuberculosis (MDR-TB) is an alarming public health problem. Delay in diagnosis hinders effective treatment. Molecular methods have led to the development of rapid and reliable diagnostic and drug susceptibility tests (DST).
Aim: To Compare Line Probe Assay (LPA) and Conventional Method of DST for the Diagnosis of MDR-TB.
Methods and Material: Total 105 sputum samples were collected from clinically suspected TB patients and screened for TB using Ziehl Neelsen (ZN) staining. Further all samples were cultured on Media. DNA was extracted from the NALC-NaOH decontaminated sputum samples and was subjected to LPA. DST of culture positive samples was performed against Rifampicin (RIF) and Isoniazid (INH) by the Proportional method.
Results: Out of 105 sputum samples, 82% were found to be positive for Acid Fast Bacilli (AFB) by initial ZN staining. 80% were found to be culture positive and 95% were LPA positive. DST detected lesser (18%) samples positive for MDR-TB, than LPA (24%), from the TB positive samples. However, in case of susceptible and mono-resistant strains both methods gave similar results. In addition, conventional biochemicals and LPA identified 27% and 34% samples as Mycobacteria Other Than Tuberculosis (MOTT) respectively.
Conclusion: LPA is a reliable, rapid, sensitive and easy to perform protocol for detection of MDR TB which strongly facilitates rapid initiation of appropriate treatment for MDR-TB patients, long before the results of conventional DST are available. It can be used for detection of MOTT strains. Though, LPA serves as an early guidance of therapy; it is recommended to be followed by a phenotypic DST for confirmation.

Keywords: Drug Susceptibility Testing, Line Probe Assay, Multi-Drug Resistant, *M. tuberculosis*

1. Introduction
Tuberculosis (TB) is the leading cause of death in the world. In 2014, there were an estimated 9.6 million new TB cases and 1.5 million deaths due to TB. It is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (MTB). TB is the only disease ever declared a global emergency by the WHO [1]. Paradoxically, although we count on effective and proven cost-effective interventions for its control, TB continues to cause great mortality and suffering, especially in poor and less-developed countries [2]. For diagnosis of TB a large number of tests are available, each one having its advantages and disadvantages. The acid-fast bacilli (AFB) staining is simple, convenient, inexpensive and rapid. However, it requires a higher bacterial load, cannot distinguish between dead and live bacteria and is unable to identify different species of MTB. A large number of methods are available including the rapid cultures but they are time consuming. Serology has limited utility in diagnosis of TB due to variable antibody response and may not differentiate between present and past infections. Likewise, tuberculin test has limited utility in high prevalence areas and also likely to give false positive and false negative results [3]. The rise of MDR-TB has drawn attention to the need for rapid diagnosis of drug resistant TB. Early detection and appropriate management are critical in the prognosis of any chronic illness; hence, rapid and accurate diagnosis of TB is critical for the control of this serious disease. Delay in diagnosis can hinder effective surveillance and treatment. Remarkable progress has recently been made by upgrading the speed and quality of Mycobacteriology diagnostic services.

In the present study comparative analysis of conventional method and molecular method like LPA for diagnosis of MTB and detection of MDR TB is carried out.

2. Subjects and Methods

2.1 Collection and Processing of sputum samples: Hundred and five sputum samples were collected from suspected cases of TB from Sewree TB hospital, Mumbai, a tertiary care centre. All the samples were decontaminated using standard NALC-NaOH method [4, 5]. After decontamination, all the sputum samples were neutralized using Phosphate Buffered saline (PBS) (pH-6.8) and centrifuged at 3,500 rpm for 15 minutes. The pellet obtained was re-suspended in 1.5ml – 2.5 ml PBS and stored at -20 ˚C.

2.2. Microscopic analysis: All the samples were analyzed microscopically for presence of Acid-Fast Bacilli (AFB) by Ziehl Neelsen Carbol Fuchsin (ZNCF) staining method [4, 5].Decontaminated sputum samples were also analysed microscopically. AFB grading was done according to WHO guidelines [4].

2.3. Culture: All the decontaminated sputum samples were cultured on Lowenstein- Jensen medium (LJM). Each sample was inoculated in two LJM slants and was incubated at 37 ˚C.

2.4. Para-nitro benzoic acid (PNB) Test: Samples which were culture positive were subjected to *para*-nitro benzoic acid (PNB) test. In this test all the samples which gave growth on LJ medium were cultured on LJ slants containing *p*-Nitro Benzoic acid (PNB) for the confirmation of Mycobacterium tuberculosis complex and Mycobacteria Other than Tuberculosis (MOTT) [6].

2.5. Drug Susceptibility Test (DST): DST was carried to all the culture positive samples by Standard Proportion method [7]. Two drugs were used for DST, namely Rifampicin (RIF) and Isoniazid (INH) for MDR detection. Both the drugs were incorporated in the LJM at specific concentrations and plain LJM was used as control. Growth from LJM slant was scraped with a loop and suspension was made in sterile distilled water, vortexed and its turbidity was matched with McFarland opacity tube No. 1. Dilutions of 10^{-2} and 10^{-4} were made from the suspension. The initial suspension and the diluted suspension were inoculated on the control as well as drug containing media and were incubated at 37 ˚C. The first reading was taken after 28 days of incubation and the second on 42nd day. They are based on the estimation of growth or no growth of an MTB strain in the presence of a single critical concentration of one drug. The critical concentration of an anti-tuberculosis drug represents the lowest concentration of the drug in the medium that indicates clinically relevant resistance if growth is observed. Susceptible wild-type strains are inhibited by this concentration. Resistance is defined if over 1% of the bacterial population of a strain is able to grow.

2.6. Line Probe Assay (LPA): LPA was performed according to the manufacturer's (Hain Lifescience, Nehren, Germany) instructions. WHO approved Geno Type MTBDR plus kit was used. Three steps for LPA included. DNA extraction, Multiplex Polymerase Chain Reaction (PCR) amplification and reverse hybridization [8].

1) *DNA Extraction:* Mycobacterial DNA was extracted according to manufacturer's instructions. 500 µl of decontaminated sputum sample was centrifuged at 10,000 rpm for 15 min, the supernatant was discarded and the pellet was resuspended in 100 µl sterile distilled water. The specimen was then heat killed at 95 ˚C for 20 min in water bath. This was followed by sonication for 15 min and centrifugation at 14,000 rpm for 5 min. Supernatant containing the DNA obtained was transferred in sterile vial which was used for PCR.
2) *Multiplex PCR:* The amplification protocol consisted of 15 min of denaturation at 95 ˚C, followed by 10 cycles comprising denaturation at 95 ˚C for 30 sec and 58 ˚C for 2 min. This was followed by 20 cycles comprising 95 ˚C for 25 sec, 53 ˚C for 40 sec and 70 ˚C for 40 sec and a final extension at 70 ˚C for 8 min. Master mixture for PCR consisted of 35 µl primer provided with kit, 5 µl of 10X PCR buffer with 15 mM $MgCl_2$, 2 µl of 25 mM $MgCl_2$, 0.2 µl (1U) of HotStarTaq DNA polymerase,3 µl nuclease-free molecular grade water and 5 µl of DNA supernatant in a final volume of 50 µl
3) *Reverse hybridisation:* Hybridization was performed with the automatic machine- twincubator. After hybridization and washing, strips were removed, fixed on paper and results were interpreted. Each strip of LPA had 27 reaction zones (bands), including six controls (conjugate, amplification, *M. tuberculosis complex (TUB),* rpoB, katG and inhA controls), eight rpoB wild-type (WT1–WT8) and four mutant probes (rpoB MUT D516V, rpoB MUT H526Y, rpoB MUT H526D, and rpoB MUT S531L), one katG wild-type and two mutant probes (katG MUT S315T1 and katG MUT S315T2), and two inhA wild type and four mutant probes (inhA MUT1 C15T, inhA MUT2 A16G, inhA MUT3A T8C, inhA MUT3B T8A). Either missing of wild-type band or the presence of mutant band was taken as an indication of a resistant strain.

Ethical clearance was taken from the Institutional Ethics Committee for this study.

3. Results

Table 1: Ziehl Neelsen Carbol Fuchsin (ZNCF) staining Number of samples: 105

Sr No.	AFB status	Number of samples (%)
1	Positive	82
2	Negative	18

82% of the sputum samples were found to be positive for AFB by ZNCF Staining.

Table 2: Comparison of conventional method and LPA for detection of MTB complex and MOTT Number of samples: 105

Sr No.	Sample	Conventional methods (%)	LPA (%)
1	MTB complex	53	61
2	MOTT	27	34
3	Negative	20	5

Conventional method showed 53% MTB complex, 27% MOTT whereas 20% samples were negative for MTB. In LPA, 61% were MTB complex positive, 34% were MOTT whereas 5% samples were negative for TB.

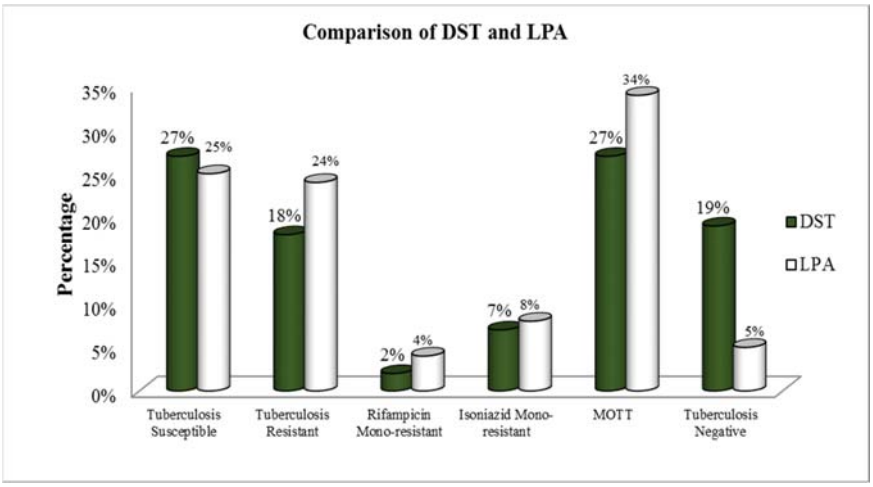

Fig 1: Comparison of DST and LPA

18% and 24% samples were found to be MDR-TB cases by DST and LPA respectively from the TB positive samples.

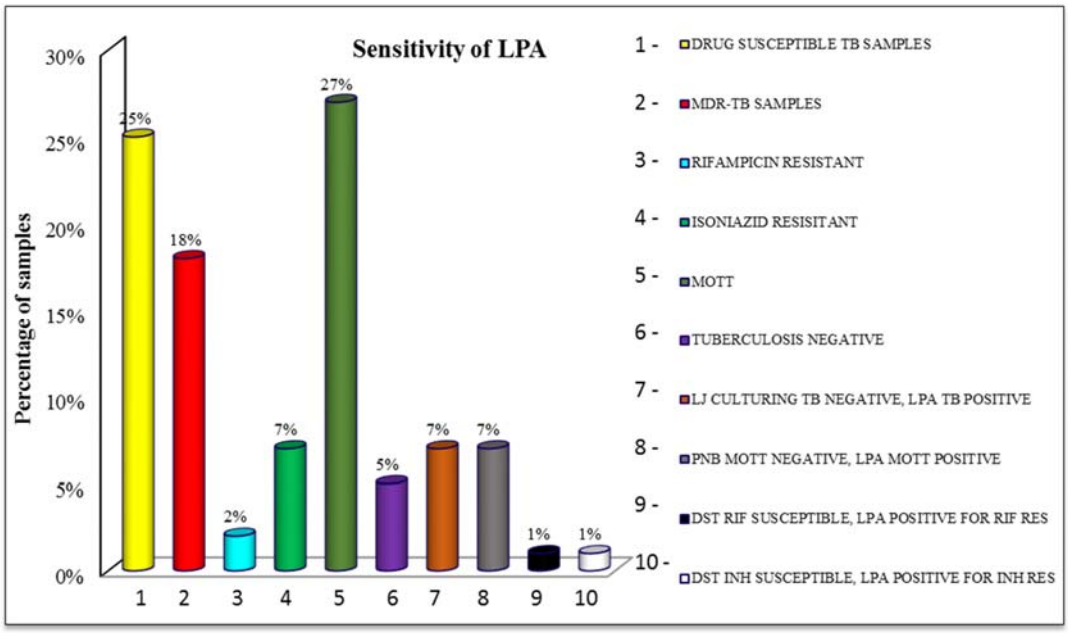

Fig 2: Sensitivity of Line Probe Assay (LPA)

All the samples were analyzed, 18% of all the samples were found to be MDR, 25% and 27% were found to be susceptible and MOTT strains respectively by both the methods. 7% samples were positive for TB and MOTT, but were negative by conventional method.

Table 3: Comparison of conventional method and LPA for Diagnosis of MDR-TB

	Drug Susceptibility Testing (Conventional Method)	**Line Probe Assay**
Time For Reporing (Approx.)	42 Days	12 Hours
Cost (Approx.)	Rs. 150 to 200/-	Rs. 1200/-
Infra-Structure	Bio-safety	Bio-safety, Centrifuge, PCR, Twincubater
Man-Power	Skilled laboratory Personnel required	Skilled laboratory Personnel required

4. Discussion

In the recent years, a major importance has been given on rapid diagnosis and quick initiation of accurate treatment for MDR TB. Precise and early diagnosis of MDR-TB is extremely beneficial as it interrupts further transmission of the disease and avoids addition of life-saving drugs and consequently increases of drug resistance. It also avoids unnecessary cost of administration and occurrence of serious side - effects of second line anti-tuberculosis drugs in case one is dealing with drug sensitive MTB strains.

In the present study we have performed a comparison of the conventional method of Drug Susceptibility Test and a newer molecular method that is Line Probe Assay for the detection of MDR TB from the sputum samples. In this study, samples were directly subjected to LPA rather than doing it from culture as carried out usually. The results of LPA were then compared to the DST, which is still a gold standard.

Hundred and five sputum samples were analyzed for MDR - TB by DST and LPA by targeting two drugs for its resistance namely RFM and INH. It was found that 18% and 24% samples were found to be MDR-TB cases by DST and LPA respectively from the TB positive samples. Also, 19% of the samples were found to be negative for TB by conventional method whereas only 5% were found to be negative by LPA. The percentage of MOTT strains also varied i.e. 27% and 34% of samples was found to be MOTT strains from conventional method (PNB test) and LPA respectively. Each sample was analysed individually, it was found that 18% of the samples were found to be true positive for MDR-TB, 27% were found to be true positive for MOTT strains and 5% were found to be true negative. 7% of the samples were found to be culture negative but TB positive by LPA and 7% of the samples were negative for MOTT by PNB test, but positive for MOTT by LPA. Therefore the percentage of TB negative samples identified by conventional methods rose up to 19% in comparison to LPA which only showed 5%. This may be due to DNA extraction protocol of LPA. In LPA, DNA extraction was done directly from the sputum samples; hence even the DNA of dead bacilli may have contributed to it. Whereas only those samples which were found to be positive by conventional method and confirmed MTB complex group, were considered for DST Therefore, higher percentage of TB positive in LPA may be attributed to the limitation of the technique that cannot differentiate between live and dead bacilli.

The Sensitivity of Line Probe Assay was found to be 100% as it identified the MDR-TB cases accurately compared to DST. The Specificity was found to be around 94%. Some previous studies done by D. I. Ling *et al.* and F. Bwanga *et al.* found the sensitivity of LPA around 95% - 99% and 96% - 99% respectively [9, 10]. In case of mono resistant cases, the Sensitivity and Specificity for each RFM resistant and INH resistant sample was found to be 100% and 98%. These results for mono resistance were similar to the study done at New Delhi, India [11].

The major advantage of LPAs is that the drug susceptibility results are available within 12 hours as this technique can be performed directly using sputum samples. Many laboratories have now replaced phenotypic DST with the LPAs as the primary method for DST [12]. The disadvantages of LPAs are that they are labor intensive and require highly trained personnel and dedicated laboratory space and equipments [13]. This study also proved that performing LPA directly from sputum samples might give false positive results and may lead to higher percentage in resistance which was mainly due to its limitation to differentiate between live and dead bacilli.

Phenotypic DST methods for MTB are inexpensive and accurate but time consuming. Genotypic DST methods for MTB are rapid and accurate but expensive. Thus molecular methods have certain benefits as they can play an important role in scaling-up programmatic management of drug-resistant TB, in particular with regard to speed, standardization of tests, reduction in turnaround time, potential for high throughput, and reduced Biosafety needs [Table 3] [14]. Also such molecular tests helps in rapid identification and isolation of the patients infected with MDR isolates and, thus, minimizes transmission of MDR-TB [15].

5. Conclusion

Laboratory diagnosis of TB still involves the smear examination and culture confirmation for MTB. Conventional culture confirmation using LJM has the disadvantage as the time required to observe the growth is high, since the TB bacilli takes around four weeks to grow. This contributes in delay of treatment, spread of the disease and leads to unnecessary treatment of non-specific pulmonary infections. Nevertheless, conventional method still remains the gold standard for culture and DST of TB. It is useful for early confirmation of viable TB bacilli, observing response to treatment in MDR-TB. Significant advances have been made in the rapid and accurate diagnosis of MDR-TB. The use of molecular tests can reduce the need for invasive diagnostic procedures, which are both costly and pose an added risk to the patient. One of the means for diagnosis of MDR-TB is a molecular-hybridization method, LPA. This test is a reliable, rapid and easy to perform for the simultaneous detection of RFM and INH resistance in MTB. With high sensitivity and high specificity obtained in this study with respect to detection of MDR-TB, we can conclude that this test strongly assists in adequate treatment of MDR-TB, long before the results of conventional DST are available. Such molecular methods contributes in many ways; significant reduction in the time to diagnose MDR-TB, the earlier initiation of appropriate therapy and the potential to prevent transmission of drug-resistant strains constitutes the major advantages of such methods. These methods require skilled operators, good infrastructure and relatively high expenses.The LPA test serves as an early guidance of therapy, which can be followed by a phenotypic DST confirmation for all suspected MDR-TB patients.

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