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COMPARATIVE ASSESSMENT OF THE CONVENTIONAL PROCEDURE AND RAPID MOLECULAR LINE PROBE ASSAY (LPA) FOR DIAGNOSIS OF MULTIDRUG RESISTANCE *Mycobacterium tuberculosis* CLINICAL ISOLATES FROM WESTERN PART OF INDIA

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Abstract- *Background*: India has the uppermost TB burden in the world and about one fifth of occurrence of TB cases occurs in India. Since conventional diagnostic procedures have limitations, definitive and rapid diagnosis of tuberculosis particularly extrapulmonary tuberculosis is demanding to provide better treatment outcomes and reduces the transmission of MDR-TB. *Methods*: 100 clinical specimens from suspected tuberculosis were received in microbiology laboratory during 1st June 2012 to 30th June 2013. Line probe assay (LPA GenoTypeMTBDR*plus* VER 2.0 was compared to the "Gold Standard" of combined culture and clinical diagnosis. Bact/Alert 3D MB-Bact (BioMerieux Durham, North Carolina,USA) rapid automated system and L.J. media were used for culture. Positive growths in either media were identified using standard conventional methods and subjected to susceptibility testing. *Results*: 43 specimens were Lowenstein Jenesen culture positive for *M.tb* and 47 specimens were LPA positive for *M.tb* complex. Two specimen were smear positive and culture negative and but positive by LPA. 19 samples were culture positive for non tuberculous mycobacteria (NTM) and further analyzed as possible NTM by LPA. For LPA, overall sensitivity, specificity, positive predictive value (PPV), negative predictive values (NPV) were 95.74%, 100, 100, and 96.36% respectively. Detection of the mutations in the *rpoB*gene of *M. tuberculosis* has been reported to be an accurate predictor of rifampicin resistance. The most frequently observed mutation was Ser-513-leu in *rpoB* gene. *Conclusion*: LPA performs uniformly well, provided results approximately within 48 hrs. in direct detection and provides susceptibility results along with mutations in nine genes which will be significant in understanding the genetic makeup of diverse *M.tb* strains and in boosting the development of new diagnostics and vaccines. In the early stages, detection of MDR-TB provides better treatment outcome and reduces the transmission of MDR-TB.

Keywords- MDR tuberculosis, rapid diagnosis, Line probe assay

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Introduction

Tuberculosis (TB) is one of the leading infectious diseases in the world and is responsible for more than 9 million new cases and 2 million deaths annually. Tuberculosis control in the South-East Asia Region Annual Report published in 2012 by World Health Organization (WHO) estimated that India is the highest TB- burden country in the world in terms of absolute numbers of incident cases that emerge each year and it contributed one fourth of the estimated global incident TB cases in 2010 [1-3]. A combination of rifampicin (RMP), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) with or without streptomycin, is recommended for the standard or first-line treatment of TB. MDR-TB (multidrug-resistant tuberculosis) is defined as TB that is resistant at least to RMP and INH, the two more important first-line anti-TB drugs [3]. Resistance to these drugs has been linked to mutations in at least nine genes; *ropB* for RMP, *katG, inhA, aphC, ksaA* for INH resistance *rpsL* and *rrs* for streptomycin resistance, *embB* for EMB resistance and *pncA* for PZA resistance [4,5]. Emergence and spread of MDR- TB is the foremost medical and public problem. A preventive measure to control spread of MDR- TB in community is imperative. Since the slow growth rate of the causative agent *Mycobacterium tuberculosis (M.tb)*, isolation, identification and drug susceptibility testing of this organism can take several weeks or longer. In the last few years, there have been considerable technological advances in the area of diagnosis of TB and MDR- TB. In the early phase of clinical infection of TB, detection of TB and MDR-TB provides better treatment outcome and reduces the transmission of MDR-TB. Several molecular methods have been developed for direct detection, species identification, and drug susceptibility testing of Mycobacteria. These methods can potentially reduce time from weeks to days or hours.

Molecular line probe assay (LPA) is available *i.e.* GenoType MTBDR*plus* VER 2.0 (HainLifesciences GmbH, Nehren, Germany) [6,7].

In the present study, we compared "Gold Standard" diagnostics methods with rapid molecular diagnostic test. We used LPA which is targeting *rpoB, katG, inhA* gene mutaion and its detection is

based on nucleic acid amplification technology which allows for rapid detection of *M.tb* Complex. This is followed by reverse hybridization along with rifampicin (RMP) resistance and/or isoniazid(INH) resistance in smear –positive and smear-negative sputum samples or in culture isolates. Species included in TB causing *M.tb* Complex are *M. tuberculosis, M. africanum, M. bovis subsp. Bovis, M. bovis subsp. Caprae, M. bovis BCG, M. microti, M. canettii, and M. pinnipedii.* The identification of RMP resistance was facilitating by the detection of the most significant associated mutation of the *rpoB* gene (coding for β-subunit RNA polymerase). Detection of *katG*gene (coding for the catalase and peroxidase) and *inhA*gene (coding for NADH enoyl ACP reductase) identified INH resistance. With the purpose of that the test is indicated as an aid for the diagnosis and intended for use in clinical laboratories for rapid detection of MDR-TB.

Materials and Methods

The study was carried on 100 clinical specimens from clinically suspected cases of pulmonary and extrapulmonary tuberculosis from Dr. D. Y. Patil Medical College, Hospital and Research Centre Pimpri Pune-a tertiary care hospital in India.

Specimens were collected in sterile container according to the revised National Guidelines [8] and received in microbiology laboratory during 1st June 2012 to 30th June 2013.

Both acid fast smear positive and smear negative samples from these specimens were analyzed. LPA was compared to the "Gold Standard" of combined culture and clinical diagnosis. GenoType MTBDR*plus* VER 2.0 (HainLifesciences GmbH, nehren, Germany) was used targeting *rpoB, katG, inhA* genes. Sputum samples were processed in class II biosafety cabinet in a biosafety level (BSL)-3 laboratory. Samples were decontaminated by N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method [5]. After decontamination they were neutralized with phosphate-buffered saline (0.067 M, pH 6.8) and centrifuged at 3,500 × *g* for 20 min. The pellet was suspended in 1 ml of phosphate-buffered saline out of which 0.5 ml of the processed specimen was inoculated into MB/BacT bottles and L.J. (Lowenstein-Jenson) medium each. Specimens collected from sterile sites were concentrated by centrifugation without prior decontamination. 500µl of the processed sample was used for DNA isolation in a screw capped tube.

Media and Culturing Methods

The MB/BacT system consists of a bottle containing 10 ml of modified Middlebrook 7H9 broth enriched with casein, bovine serum albumin, and catalase. Before inoculating specimen, bottles were supplemented with 0.5 ml of MB/BacT MAS supplement (amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and vancomycin) which was reconstituted with 10 ml of MB reconstituting fluid according to the manufacturer's instructions. Bottles were placed inside the BacT Alert 3D instrument (Bio Merieux Durham, USA) and incubated at 37°C for 6 weeks. Any bottle which displayed as positive was taken out of the instrument. The L.J. bottles were incubated at 37°C for 8 weeks and were read weekly and identified as soon as sufficient growth was visible. L.J. bottles failing to show any growth after 6-8 weeks were discarded as negative [9-11].

Microscopy

Any growth obtained on the bottle was stained by ZN (Ziehl - Neelsen) for detection of acid fast bacilli.

Conventional Drug Susceptibility Testing

Proportion Method

The proportion method is currently the method of choice in the majority of laboratories in the world. DST (Drug susceptibility testing) was performed on LJ media containing anti tubercular drugs with streptomycin (4µg/mL), isoniazid (0.2µg/mL), rifampicin (40µg/mL), ethambutol (4µg/mL). Standardization of inoculum: Inoculum was matched to 1 Mc.Farland solution and inoculated on drug containing L.J. media [8].

Incubation and Reading

The inoculated slopes were incubated at 37°C and were examined every week. The contaminated bottles were discarded and the sensitivity testing repeated using the original purified growth. The reading was recorded at 28th and 42nd day of incubation as per RNTCP (Revised National Tuberculosis Control Programme) guidelines [8].

Quality Control

Standard strain of *M. tuberculosis* H37Rv was tested from time to time for quality assurance.

Line Probe Assay

Molecular LPA is based on nucleic acid amplification technology which allows for rapid detection of *M.tb* Complex along with resistance to RMP and INH.

A total of 100 specimens were assessed, of that 61 specimens were from extrapulmonay infections and 39 were from pulmonary infections. All specimens were screened by staining with ZN technique. Bact/Alert 3D (BioMerieux Durham, North Carolina,USA) rapid automated system and L.J. media were used for culture. Positive growths in either media were identified using standard conventional methods and subjected to susceptibility testing. The performance of line (LPA) was assessed.

Procedure

DNA from the decontaminated specimen was extracted using the chemical cell lysis method. DNA extraction kit named GenoLyse® HAIN Lifesciences was used for the same. The extracted DNA was then subjected to PCR (Polymerase Chain Reaction) followed by LPA by reverse hybridization method for the analysis and diagnosis of TB. PCR was carried out using primers specific for *M.tb* complex gene loci and genes associated with resistance to RMP and INH. An initial denaturation at 95°C for 15min was carried out to allow complete separation of the two templates. Further the amplification profile was as follows; denaturation at 95°C for 25 sec, annealing at 50°C for 40 sec and extension at 70°C for 40 sec. A hold of 70°C for 8 min was done, in order to complete the final extension. A total of 30 cycles with the above profile were performed. The amplified product was then analyzed by "Reverse Hybridization" technique using the DNA strip technology. The strips provided are pre-attached with 27 different probes (bands) including six controls (conjugate, amplification) [12]. *M. tb 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 *katGMUT S315T2*), and two inhA wild type and four mutant probes (*inhA MUT1 C15T, inhA MUT2A16G, inhA MUT3A T8C, inhA MUT3B T8A).* Conjugate control **(CC)** line must develop in this zone, documenting the efficiency of conjugate bind-

ing and substrate reaction. Amplification control **(AC):** When the test is performed correctly, a control amplicons will bind to Amplification Control zone. When only CC and AC bands developed, this represents valid negative result. *M. tuberculosis* complex **(TUB)**, this zone hybridizes, with amplicons generated from all members of *M. tb complex*. Locus control (*rpoB, kat G, ingA*), this zones detect a gene region specific for the representative locus. Wild type probes comprise the most important resistance regions of the respective genes. When wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. This indicates that the strain tested is sensitive for the representative antibiotic. Mutation probes detect some of the most common resistance-mediating mutations [Fig-1].

Complementary to MTB complex gene loci and the mutant products of the *rpoB, katG* and *inhA* genes that are involved in imparting resistance to RMP and INH drugs. The banding profile seen was indicative for the presence of *M. tb* complex and 1st line drug resistance. Either missing of wild-type band or the presence of mutant band was taken as an indication of a resistant strain. Incomplete amplification of RIF and/or INH genes was considered as an inv*al*id result.

Quality Control for LPA

In order to validate the correct performance and implementation of kit constituents, each strip includes 5 control zones i.e. CC- conjugate control zone to check the binding of the strip and a correct chromogenic reaction, AC- amplification control zone to check successful amplification reaction and three locus control zones (*rpoB, katG and inhA*) to check optimal sensitivity of the reaction for each of the tested gene loci.

Results

The culture and LPA were evaluated for their abilities to detect *M. tb* complex in 100 patients those were suspected of having pulmonary and extra pulmonary mycobacterial infections. 62 specimens were culture positive for mycobacterial infections of which, in 43 specimens *M.tb* were isolated and while in 19 specimens NTM were isolated by conventional method. Total 66 specimens were LPA positive of which 47 were *M.tb* complex and 19 were NTM. Two specimens were smear positive and culture negative and positive by LPA [Fig-1].

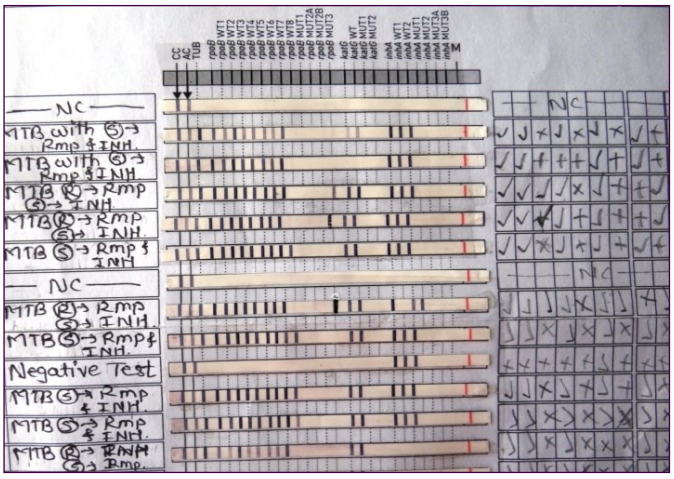

Fig. 1- Banding pattern of DNA strip with respect to RMP and/or INH resistance.

NTM were analyzed as possible NTM by LPA as well as by "Gold Standard" conventional method. For LPA, overall sensitivity, specificity, positive predictive values (PPVs), negative predictive values (NPVs) were 95.74%, 100, 100, 96.36% respectively. For culture, overall sensitivity, specificity, PPVs, NPVs were 91.48%, 100, 100, 92.98% respectively.

Table 1- Drug Profiling Of Tot*al* Suspected Specimens By MTBDR plus V2 assay (n=47)

Sr. No	Drug profile	Frequency	Percentage(%)
1	MTB with RIF & INH sensitivity	30	63.82
2	MTB with RIF monoresistance	10	21.27
3	MTB with INH monoresistance	2	4.25
4	MDR-TB	5	10.63
	Total 19 NTM were detected by PLA- MTBDR plus V2		

Of the 47 LPA positive *M.tb* strains, 30(63.82%) strains were RMP and INH sensitive. 10(21.27%) strains were RMP monoresistant and 2(4.25%) strains were INH monoresistant. 5(10.63%) strains were MDR-TB. Of the 5 MDR-TB results by conventional DST method, LPA detected 5 as a MDR-TB. Conventional DST methods also detected similar results in 7 strains with RMP monoresistance and 2 were resistant to INH, while 30 strains were RMP and INH sensitive [Table-1]. Overall concordance between genotypic LPA test and phenotypic conventional DST was 98% (47/45). NTM were detected in 19 strains which were also confirmed by GenoType®Mycobacterium CM VER1.0 (HAIN LIFE SCIENCES) assay and these results were in agreement of conventional culture method.

Mutation Patterns in LPA

7 RIF monoresistant strains had a mutation in *rpo B* S531L (MUT 3 band). Other mutations associated with RIF resistance in MDR-TB strains included *rpo B* H526D (MUT2B band), *rpo B* D516V (MUT 1 band) and H526Y (MUT 2Aband) however these mutations were not seen in RIF monoresistant strains. The most frequent mutation found in INH monoresistant strains was *kat G* S315 T2 (MUT 2 band). Mutation in MDR-TB strains were *inh*AT8C (MUT 3A) and *inh*AT8A (MUT 3B) [Table-2].

The turnaround time of LPA assay was 48 hours whereas; it was 70 -75 days for phenotypic DST *i.e.*25-30 days for conventional culture growth and another 30-40 days for DST.

Discussion

In the recent times major importance has been given for precise and early diagnosis of the MDR-TB, which is extremely advantageous to disrupt further transmission of the disease. In view of the fact that there are many report globally regarding rapid diagnosis on *M.tb* infections. In the present study, first time we have evaluated performance of LPA test for detection of *M. tb* Complex along with RIF and INH resistance in direct sputum samples or in culture isolates of clinically suspected tuberculosis patients. Subsequently, genotypic LPA and phenotypic L.J proportion DST results were compared. We observed that LPA test results had a good concordance with the conventional DST with a added advantage of a shorter turnaround time. In the present study, overall sensitivity, specificity, PPVs, NPVs for LPA were 95.74%, 100, 100, and 96.36% respectively. For culture, overall sensitivity, specificity, PPVs, NPVs were 91.48%, 100, 100, 92.98% respectively are in agreement with results of meta-analysis done by Ling *et.al* [13]. Sensitivity (97%)

and specificity (100%) for detection of MDR-TB in the present study corroborated with a previously reported study by Anek-vorapong *et.al* [14] and Raveendran R. *et.al* [15] from Thiland and India respectively and these findings suggest that performance of LPA is similar to conventional DST in a quality assured TB laboratory.

In the present study, MDR-TB were detected in 10.63% strains while MTB with RIF monoresistance were detected in 14.89% strains and MTB with INH monoresistance were detected in 4.25% strains. Joel Bazira *et.al* (2010) study from Uganda- showed 4.8% resistant to INH, 3.2% resistant to RIF while 1.6% to both INH and RMP (MDR) [16]. Maurya *et.al* [17] reported 4.7% INH resistant and RIF is 4.2% which was similar to other responds from Germany, Italy, Finland, France, Denmark, Turkey, Vietnam and Taiwan. The sensitivity of Genotype ® MTBDR plus assay for detection of MDR-TB was 97.7%. Umubyeyi AN *et.al* [18] reported 6.2% INH resistant and 3.9% MDR from East African countries. Kibiki *et.al* [19] reported 9.9% resistant to INH & 2.7% resistant to RIF while 2.7% MDR from Northern Tonzania. MDR-TB prevalence is estimated to be 2.3% among new cases and 12-17% among re-treatment cases. However, due to the size of population and number of TB cases reported annually, India ranks second among the 27 MDR-TB high burden countries worldwide after China.

Table 2- Mutational Patterns Associated With RIF & INH Resistant Genes In MTB Strains

Gene analyzed	Mutational pattern	Codons analyzed	Frequency	Percentage
rpo B gene-wild type banding	rpo B WT -1	505-509	40	90.9
	rpo B WT -2	510-513	44	100
	rpo B WT -3	510-517	42	95.45
	rpo B WT -4/wts	516-522	43	97.72
	rpo B WT -6	522-526	41	93.18
	rpo B WT -7	526-529	42	95.45
	rpo B WT -8	530-533	22	50
rpo B gene –mutational banding	rpo B MOT 1/	D 516V/D516 Y /de/515 /del 518/N518/S522L/S522Q	0	0
	rpo B MOT 2A	H526 Y	0	0
	rpo B MOT 2B	H 556D/ H526R/ H526N/ H526L/ H526S/ H526C	0	0
	rpo B MOT 3	S531L/S531W/L533P	6	13.63
kat G gene- Wild type banding	Kat G WT	351	40	90.9
	Kat G MUT 1	S315T 1	4	9.09
	Kat G MUT 2	S315T 2	0	0
Inh A gene- Wild type banding	Inh A WT -1	0.9375	39	88.63
	Inh A WT -2	-8	43	97.72
Inh A gene- Mutational banding	Inh A MUT 1	CIST	4	9.09
	Inh A MUT 2	A169	0	0
	Inh A MUT 3A	T8C	0	0
	Inh A MUT 3B	T8A	0	0

Among molecular tests, LPA provides a better DST profile as compared to Gene-Xpert, and offers additional advantage of deciding the drug regimen in patients with INH monoresistance. WHO recommends addition of ethambutol as a third drug in the continuation phase in settings where the level of isoniazid resistance among new TB cases is high [5]. Additionally, this test can also be useful for systematic surveillance of INH monoresistance in countries with high isoniazid resistance. The genetic basis of the resistance against the anti-tubercular drugs has been unraveled.

RMP resistance is most common due to point mutations and small insertions and deletions in the *rpoB* gene which code for B-subunit of RNA polymerase in *M.tb.* RMP is key drug in treatment of TB and is also a useful surrogate marker for MDR-TB. RMP resistance is known to be associated with mutations in 81 base pair region (codon 527 to 533) of the *rpoB* gene [13].

In the present study, the finding of dominant mutation for RMP resistance is *rpoB* S531L, similar to a previously published report by Mani C *et.al* and Mitto P *et.al* from India and Italy respectively [20,21]. One false RMP resistant strain with missing WT8 band was observed with the LPA test. The nature and frequency of mutation in *rpo B* gene of RMP resistance in clinical isolates of *M.tb* vary considerably according to geographical locations. Distribution of mutations of *katG* and *inhA* genes is known to vary in different geographical regions. Frequencies of *katG* gene, *inhA* gene and combined *katG* and *inhA* gene mutations in the present study 2.73% and 6.84% respectively are within the range of previously reported studies [18-19]. Finding of frequency of combined mutations of *KatG* and *InhA* in the present study is comparable to a recent study from Uttar Pradesh India [17]. Diagnosis of extrapulmonary tuberculosis is a challenge as clinical manifestations are indistinct and typical radiograph finding may not evident till late in the disease, as a result, rapid detection plays very important role in diagnosis and in the early hour's treatment. Hence development of rapid diagnosis such as LPA has become priority [22-23]. In the present study, of 47 LPA positive strains, 20 were from cases of extrapulmonary tuberculosis which were diagnosed early as compared to conventional results come out. Positive treatment outcome were seen in such cases.

The present study highlights the facts that conventional culture methods however "Gold Standard" but obligatory for time utilization as compare to LPA results. LPA detected *M.tb* in two samples which failed to grow on conventional cultures. LPA test minimizes chances of contamination which demonstrate superiority of LPA test. Limitations of Genotype MTBDR*plus* VER 2 assay include need for an appropriate infrastructure, adequately trained and trained laboratory workforce.

Conclusion

Our results reveal that LPA performs uniformly well with pulmonary and extrapulmonary tuberculosis samples and provided results approximately within 48 hrs. in direct detection as compared to conventional DST method. Present study established the finding that LPA test is highly sensitive and specific for rapid diagnosis of MDR-TB. It represents an important key aspect in smear negative samples as it is gravely important that any tuberculosis patient should not be overlooked. Additionally, the test also detects nonresistance to INH and RMP. More studies with large number of samples are essential to corroborate these preliminary findings, and describe the accurate place of this test in the diagnostic algorithm for MDR-TB under programmatic settings in higher TB burden countries like India.

Ethics Statement: Study protocols were approved by the institutional ethics committee.

Conflicts of Interest: None declared.

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