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Original Research Article

Rapid Detection of Drug Resistant Pulmonary Tuberculosis by Line Probe Assay at a Tertiary Care Centre in North Kerala

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Abstract

Background: Early detection of Isoniazid (INH) and rifampicin resistance is essential for treatment and control of multidrug resistant tuberculosis (MDR-TB). Conventional method will take about 8-10 weeks for culture and drug susceptibility tests (DST). MTBDR plus VER 2.0 Line probe assay (LPA) can be used for identification of *Mycobacterium tuberculosis* complex (MTB complex) and to detect drug resistance of INH and Rifampicin.

Materials and Methods: Cross sectional study was conducted in 100 smear positive patients with clinical features of pulmonary tuberculosis. After decontamination and concentration of sputum subjected to LPA and inoculated in parallel to LJ medium. Identification of MTB complex and resistance to INH, rifampicin were detected along with common mutations. Drug susceptibility test from LJ isolates were done by proportional method.

Result: Detection rate of MTB complex by LPA in comparison with conventional DST was determined. Sensitivity, specificity, PPV and NPV of LPA was 100%, 97.47%, 77%, 100% for detection of rifampicin mono resistance and 92.86%100%,100%,98.6% for INH mono resistance. Common mutation detected in rpoB gene was S531L.and S315T1 in katG gene. Mean turnaround time for LPA was 3.47 days and for conventional method it was 49.6 days.

Conclusion: Direct LPA from smear positive sputum is sensitive and specific diagnostic method for detection of MDR TB along with INH and rifampicin mono resistance. Shorter turnaround time with LPA helps in initiation of treatment earlier and preventing spread of MDR TB.

Keywords: Multidrug Resistant Tuberculosis, Line Probe Assay, Lowenstein Jensen Medium, Drug Susceptibility Test.

Introduction

Diagnosis, detection of drug resistance and treatment of tuberculosis (TB) are remaining as challenges. The spread of drug resistant strains of *M. tuberculosis* and the management of drug resistant cases are some of the difficulties faced by National tuberculosis control programme. India accounts for more than 25% of the world's total cases of TB. [1]

Important risk factors for drug resistance are previous inappropriate treatment with anti-tubercular drugs and contact with a person who has drug-resistant tuberculosis. Globally about 3.3% of new TB cases and 20% of previously treated cases of

multidrug resistant tuberculosis (MDR-TB) were present according to WHO global tuberculosis report 2015. [2] In India primary drug resistance observed was about 2.2% and acquired drug resistance about 15% according to Revised National Tuberculosis Control Programme (RNTCP) annual status report 2015. [3] Early detection of rifampicin (RIF) and isoniazid (INH) resistance are essential for efficient treatment and control of MDR-TB. The delay in proper treatment may adversely affect treatment outcome and contribute to the transmission of drug resistant TB. [4] Treatment of MDR-TB is more challenging requiring the use of costlier second line drugs which are having severe side effects and to be taken for up to 2 years. But only small fraction of MDR-TB is identified and treated appropriately.

Culture and drug sensitivity testing by proportional method using Lowenstein Jensen medium (LJ medium) is considered as the gold standard. But conventional drug susceptibility test procedure involve culture following which sensitivity testing will be done. [5] By conventional method due to the slow growing nature of the bacillus isolation of *M. tuberculosis* will take about 4-8 weeks and drug sensitivity tests another 4-6 weeks. [6] During this time patients may be inappropriately treated; drug resistant strains may continue to spread and the magnitude of resistance increases. This time lag is a threat to patient, community and health care workers. Newer technologies for rapid detection of drug resistance therefore should be of main concern. [7] The World Health Organization (WHO) has approved molecular line probe assay (LPA) for diagnosis of tuberculosis and detection of drug resistance. [8] For drug susceptibility testing (DST) of first-line drugs as per RNTCP protocol, rapid molecular tests like LPA and Cartridge Based-Nucleic Acid Amplification test (CB-NAAT) are the preferred DST methods. Genotypic or molecular methods utilise the fact that resistance in tuberculosis arise due to random mutation. Detection of mutations enable early diagnosis of drug resistance which help in initiating therapy. [9] Molecular methods do not require growth of organisms and can be performed directly on clinical specimens. By CB-NAAT test results can be obtained within short time but only rifampicin resistance can be detected by this method and not INH resistance. [10] MTBDR plus VER 2.0 Line probe assay (LPA) is a qualitative in vitro test for the identification of MTB complex. It recognises mutations in the rpoB gene for detection of rifampicin resistance, mutations in the katG gene for high-level INH resistance, and mutations in the promoter region of inhA gene for low levels INH resistance. [11] While resistance to INH is mainly associated with mutations in the katG, inhA and ahpC genes, resistance to rifampicin is mainly due to mutations in the rpoB gene. Results of LPA will be obtained within about 2-3 days compared to time of 12-14 weeks with conventional culture and DST tests. [12]

The study aimed to estimate the prevalence of MDR TB, rifampicin and INH resistance along with common mutations by line probe assay. Comparison of MTBDR plus VER 2.0 Line probe assay with conventional method (LJ medium) were also done.

Materials and Methods

A cross sectional study was conducted in patients with clinical features suggestive of pulmonary tuberculosis attending out patient department of Pulmonary Medicine of a tertiary care centre in North Kerala after getting approval of the Institutional Ethical Committee. Study was conducted from January 2013 to December 2013. AFB smear positive patients were included in the study and AFB smear negative patients were excluded from the study.

Sputum samples from suspected pulmonary tuberculosis patients collected were sent to Microbiology laboratory. They were subjected to microscopy by Ziehl Neelsen technique. The smears were graded as per Revised National Tuberculosis Control Programme (RNTCP) guideline of India. 100 AFB positive patients were selected for the study. Sputum specimens were decontaminated and concentrated by N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method. The sediment was suspended in 1 ml of phosphate buffer and 500 µl of this suspension was used for DNA extraction for line probe assay.

Conventional culture in Lowenstein Jensen medium (LJ medium)

A loopful of suspension was inoculated into two slopes of (L-J) media simultaneously and incubated at 37^0C. Presence of growth was checked weekly. All isolates were identified as *M. tuberculosis* by their colony morphology, inability to grow on L-J medium containing p-nitrobenzoic acid and negative heat stable catalase tests. The slants in which growth was not detected were kept for 8 weeks before discarding as negative.

Line probe assay was carried out using Genotype MTBDRplus version2.0 (Hain Lifescience GmbH, Nehren, Germany) [13]

DNA extraction: DNA extraction was done using Genolyse kit (Hain Life science, Nehren, Germany).500 µl of the suspension was centrifuged at 13,000g for 15 minutes and the supernatant was discarded.100µl of Lysis buffer was added and incubated at 95^0C for 5 minutes.10 µl of Neutralization buffer was added and vortexed. This was followed by centrifugation at 13,000g for 5 min. 5 µl of the DNA supernatant was used for multiplex PCR.

Multiplex PCR amplification: 45 µl amplification mix was prepared in a PCR tube and 5 µl of the DNA supernatant was added to it. After loading the tubes in thermal cycles PCR cycles were run.

Reverse hybridisation: Using amplification products reverse hybridisation was done using incubator. After addition of 20ml denaturation solution, amplification product was added followed by 1ml hybridisation buffer. DNA strip was placed into the wells. Conjuate and substrate were added with washing in between. Strips were taken out and sticked to the evaluation sheet.

Detection: By LPA *M. tuberculosis* complex was identified when TUB band could be detected. Resistance to both isoniazid and rifampicin were detected. Mutation in the relevant gene was signified by either absent wild type band and /or presence of mutant band within the regions of *rpoB, kat G* and i*nhA* which was indicative of rifampicin, high level and low-level INH resistance respectively. The presence of wild band without mutation band was interpreted as sensitive.

Conventional Drug Susceptibility Testing: It was carried out in LJ medium by proportional method. Rifampicin and INH were tested with concentrations of 40mg/ml and 0.2 mg/ml respectively. Any strain with 1% (the critical proportion) of bacilli resistant to any of the two drugs – rifampicin and INH were considered as resistant to that drug. If any strain was found to be resistant to both INH and rifampicin it was considered as multidrug resistant [14]. Turnaround time was calculated for both LPA and conventional method. Results obtained by LPA were compared with conventional method considering it as gold standard.

Statistical analysis

The data was entered in Microsoft excel spread sheet and analysed using Statistical Package for the Social Sciences (SPSS) software. Continuous data were presented as mean ±SD. Qualitative variables were expressed as frequency and percentage.

Comparison of nominal data was performed by paired T test. Test performance was assessed by determining sensitivity, specificity positive predictive value and negative predictive value. Agreement between the tests were assessed by Kappa value and P value of <.05 was considered as statistically significant.

Results

Total 100 smear positive specimens were processed. Out of 100 specimens 80 were from male patients and 20 from females with male to female ratio 4:1. Mean age±SD was 49.83±14.74. Maximum number of study population belonged to age group 41-50 in both males and females followed by 51-60 and 61-70 (Fig 1).

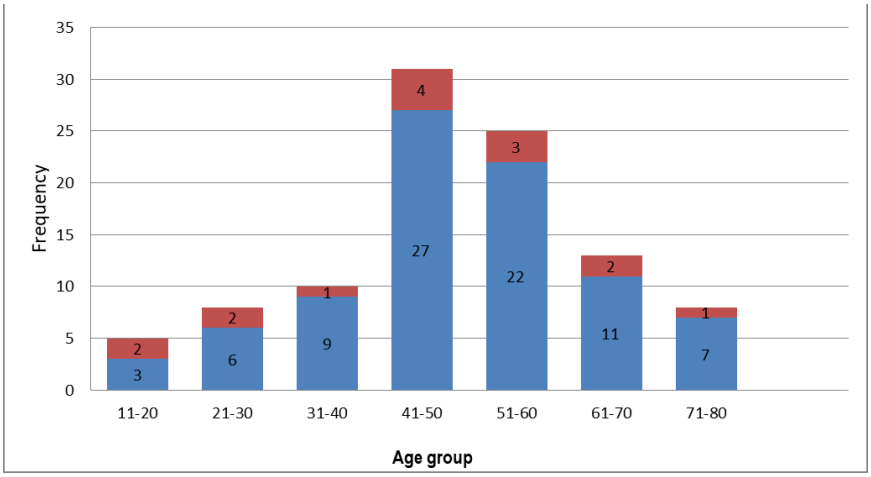

Figure 1: Age and Sex distribution of study population

By Ziehl Neelsen staining six sputum samples were found to be scanty positive, 33 were of grade 1, 39 were grade 2 and 22 grade 3. By LPA valid result were obtained for 92(92%) cases out of 100 (Fig 2). MTB complex was not detected from 8 specimens. 6 specimens from which MTB complex could not be detected by LPA showed scanty bacilli by Zeihl Neelsen staining. The other two samples from which MTB could not be detected were identified as Nontuberculous Mycobacteria (NTM).

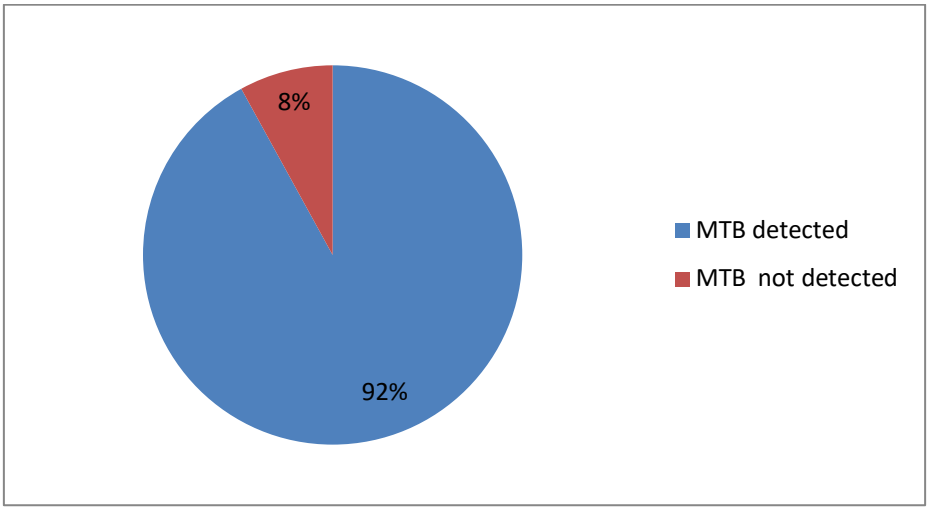

Figure 2: Detection rate of MTB by LPA
MTB-Mycobacterium tuberculosis

Out of 92 specimens from which MTB was detected, 16(17.39%) showed drug resistance by LPA. 6 (6.52%) were MDR TB, seven (7.61%) showed INH mono resistance and three (3.26%) rifampicin mono resistance. 76(82.61%) were sensitive to both INH and rifampicin, (Table 1).

Table 1: Detection rate of drug resistance by LPA

Susceptiblity	Number	Percentage (%)
INH mono resistant	7	7.61
Rifampicin mono resistant	3	3.26
INH + Rifampicin resistant	6	6.52
INH + Rifampicin sensitive	76	82.61
Total	92	100

All MDR strains showed rpoB mutations along with katG mutations. INH mono resistance was detected in 7 strains out of which 2 showed inhA promotor region mutation only. Others showed katG mutation. Common mutation detected in rpoB gene was S531L in 5 cases of MDR strains and two rifampicin mono resistant cases. D516V mutation was detected in one MDR and one rifampicin mono resistant case. S315T1 mutation was detected in katG gene in 5 MDR cases and 5 INH mono resistant case. S315T2 mutation in one MDR case and C15T mutation detected in inhA promotor region was seen in 2 INH mono resistant cases (Table 2).

Table 2: Distribution of common mutations detected by LPA

Gene	Absent WT band	codons	Mutation band	Mutations	MDR TB	INH mono resistance	Rifampiciin monoresistance	Frequency	Percentage
rpoB	rpoB WT8	530-533	*rpo*B MUT3	S531 L	5	-	2	7	77.8
	rpoB WT3	513-519	rpoB MUT1	D516V	1	-	1	2	22.2
katG	katGWT	315	katGMUT1	S315T1	5	5	-	10	76.9
	katGWT	315	katGMUT2	S315T2	1	-	-	1	7.69
inhA	inhAWT1	15	inhAMUT1	C15T		2		2	15.4

By LJ medium only 86 (86%) isolates could be obtained when compared to LPA (Table 3), 86 patients from whom MTB could be detected by both LPA and LJ medium were only considered in for drug susceptibility studies.16 drug resistant strains were detected by LPA compared to 15 by LJ medium. Seven INH mono resistant strains were detected by LPA whereas 8 by LJ medium. LPA could detect three rifampicin mono resistance whereas one only by LJ medium. Six MDR-TB cases were detected by both methods (Table 4).

Table 3: Comparison of detection of MTB by LPA and LJ medium

LPA	LJ medium culture for MTB			
	Positive	Negative	Total	Percentage
MTB positive	86	6	92	92
MTB negative	0	8	8	8
Total number	86	14	100	100
Percentage	86	14	100	100

Table 4: Comparison of drug resistance by LPA and LJ medium

	LJ Medium				
LPA	**INH R Rif R**	**INH S Rif R**	**INH R Rif S**	**INH S RIF S**	**Total**
INH R Rif R	6	0	0	0	6
INH S Rif R	0	1	0	2	3
INH R Rif S	0	0	7	0	7
INH S Rif S	0	0	1	69	70
Total	6	1	8	71	86

Sensitivity, specificity, positive predictive value and negative predictive value were 100%, 71%, 96.50% and 100% respectively for LPA in detection of MTB complex. Kappa value was 0.696 and P Value was <0.001. Sensitivity, specificity positive predictive value and negative predictive value of LPA was100%, 97.47%, 77%, 100% for detection of Rifampicin resistance with kappa value of 0.490 and P value <0.001 and 92.86%, 100%, 100%, 98.6% for INH resistance with Kappa value 0.926 and P-value <0.001. For MDR TB sensitivity, specificity, PPV and NPV were 100%. Kappa value was 1 and P-value <0.001 (Table 5).

Table 5: Performance of LPA in comparison with LJ medium

Detection	Sensitivity %	Specificity %	PPV %	NPV %	kappa value	P value
MTB	100	71	96.5	100	0.696	<0.001
Monoresistance to rifampicin	100	97.47	77.77	100	0.490	<0.001
Mono resistance to INH	92.86	100	100	98.63	0.926	<0.001
Drug resistance rifampicin+INH	100	100	100	100	1	<0.001

Mean turnover rate was 3.47 days for LPA whereas mean time for isolation plus detection of drug resistance is about 49.92 days for LJ medium. By paired T test P value was found to be <0.001 (Table 6).

Table 6: Turn-around time of LPA and LJ

Pair 1	mean	N	Std	td error mean				
LPA turnaround time	3.47	86	0.502	0.054				
LJ turnaround time	49.92	86	0.939	0.975				
Paired sample test								
Paired differences								
				95% confidence interval of the difference		t	df	sig 2 tailed
Pair 1	mean	std deviation	std error mean	lower	upper			
LPA turnaround time. LJ turnaround time	46.453	9.071	0.978	48.398	44.509	47.494	85	0.000

Discussion

Prevalence of MDR TB is increasing in India every year. Early diagnosis of MDR TB helps in initiation of treatment. It will interrupt transmission of disease and reduce the number of XDR TB cases also. Molecular methods have reduced the time for diagnosis of tuberculosis and detection of drug resistance considerably. By line probe assay both INH (high level and low

level) and rifampicin resistance can be detected within short time.

The present study was conducted on 100 smear positive patients with clinical feature of pulmonary tuberculosis. Of the 100 smear positive sputum samples 80% were from males and 20% from females with male to female ratio 4:1. Similar results with higher male to female ratio were observed in different studies conducted in Maharashtra in 2017 and Nepal in 2020 [3,15].

Male predominance may be due to increased exposure risk of males and difference in access to medical care of both males and females and women are less likely to seek medical advice for early symptoms than males. This may be due to socio-cultural reasons associated with TB-related stigmas [16]. In this study 75% of study population belonged to 41-70 age group. The results obtained were comparable with that of other studies [17,18]. Higher prevalence in elderly may be due to latent tuberculosis, comorbidities like diabetes and immunosuppression occurring at a higher rate among them.

Rate of detection of MTB was 92% by LPA. A multisite validation study by Neeraj et al and another study from Jammu Kashmir showed high rate of detection of MTB (94%) by LPA [19,20]. In another study conducted in Rajasthan by Mukesh Sharma et al rate of detection of MTB was found to be 89.74% by LPA [21]. There was definite relationship between smear positivity and LPA positivity. MTB could be detected by LPA from all smear positive cases excluding two which were identified as NTM and six scanty smear positive samples. Previous studies also have shown that there is relationship between smear positivity and LPA positivity [14,19]. In low bacillary load specimens' detection of MTB may be missed.

Among this 18.6% were drug resistant with 8.14% INH mono resistance, 3.49% rifampicin mono resistance and 6.97% MDR resistance. In a study in Uttar Pradesh rate of detection of MDR TB by LPA were higher whereas rifampicin and INH mono resistance detected were almost same as our study. [14] Lower rate of MDR TB detected in our study may be due to the fact that almost half of study population were new patients.

All mutations associated with rifampicin resistance were related with rpoB gene. Most common mutation associated with rpoB gene was S531L. Similar findings were observed in studies by Raizada et al, Syed Beenish Rufai et al in 2014, by Sara Siddiqui et al in 2019 and another study conducted in Andhra Pradesh in 2019.[19,22-24]. High level INH resistance associated with katG was detected in 84.61% cases. Mutation associated with katG was S513T1 in 90.9% like study done in Pakistan in 2019.[23] Low level INH resistance associated with inhA promotor region was detected in two cases (15.39%) which was associated with C15T mutation (100%). Study by Sara Siddiquiet al in 2019 and study conducted by Hossein kazemian in Iran in 2019 showed similar findings. [23,25] In patients with high level INH resistance high dose INH cannot be given since most of them had history of treatment with INH. But in low level INH resistance high dose of INH can be given. INH mono resistance can be detected rapidly by LPA. Rifampicin sensitive strains always may not be INH sensitive. This is very important in starting the treatment. Rifampicin is considered as surrogate marker for MDRTB [26,15] but Rifampicin resistant strains need not be INH resistant. To detect rifampicin mono resistance in short time LPA can be used.

Detection of MTB by LPA was 92% whereas isolation by LJ was only 86% .Sensitivity, Specificity, PPV, NPV of LPA over LJ medium for the detection of MTB complex is 100%, 71%, 96.5%, 100% respectively. Almost similar

findings were observed in a study conducted in Jammu and Kashmir with sensitivity, specificity, PPV and NPV of 100%, 78.6%, 98.9% and 100% respectively [20]. Kappa value was .696 which shows moderate agreement between the two and p value <.001 which is statistically significant . In present study LPA could detect MTB from all the samples from which isolation was obtained from LJ medium. Valid results were obtained from 6 samples by LPA which were culture negative. Negative LPA results in the present study were found in sputum specimens with lower bacillary load (scanty positive) so the direct use of LPA test is not ideal for scanty positive cases.

Drug resistant strains detected by LPA was sixteen (18.6%) and by proportion method it was fifteen (17.4%). LPA could detect seven INH mono resistance (8.14%) whereas conventional method detected eight (8.85%). Sensitivity, specificity, PPV, NPV of line probe assay in detecting INH mono resistance were 92.9%, 100%, 100%, 98.63%. Kappa value was .987 with excellent agreement between the tests and P value was <0.001 which is highly significant. By LPA three rifampicin mono resistance (3.49 %) and by LJ medium only one (1.16%) were detected. For rifampicin Sensitivity, Specificity, PPV, NPV were 100%, 97.47%, 77.8% and 100% respectively with kappa value 0.490 with good agreement and P value <0.001. Six MDR TB cases were detected by both methods (6.98%). Sensitivity, specificity, PPV and NPV of 100% were measured for detecting MDR-TB with kappa value of 1 which shows perfect agreement between two tests with P value <0.001, which is highly significant. In INH resistance, mutation in katG and inhA promotor gene only can be detected by LPA. INH resistance not detected in LPA could be detected by proportion method. Mutation might have occurred in other genes like ahpC-oxyR and ndh. It can also be due to silent mutation, which is not phenotypically expressed. Although Gene Xpert MTB/RIF is the most rapid test to detect drug resistance it detects only rifampicin resistance. So INH mono resistant cases will be missed especially in areas where more INH resistant cases are prevalent. 3 rifampicin mono resistance was detected by LPA. More than 95% of the drug resistance of rifampicin occur due to mutation of rpoB gene, which can be detected by LPA. Rifampicin is considered as surrogate marker for MDRTB [27,15]. So diagnostic methods like Gene Xpert MTB/RIF to detect rifampicin resistance are commonly used so that treatment can be started for MDRTB. Possibility of rifampicin resistance without INH resistance should be of concern because these cases will also be treated as MDR TB. Studies in South Africa are showing the increasing occurrence of rifampicin mono resistance.[28] By LPA 70(81.39%) were sensitive to both INH and Rif. In a study by Prabha Desikan in 2017 61.9% sensitivity to both INH and Rif by LPA could be detected. [29].

Mean turnaround time for LPA was about 3.47 days and several studies have shown similar results. [7,1,26,29,30] whereas by conventional LJ medium average time was around 49.6. Difference in turnaround time is statistically highly significant since P value is <.001. This may be because LPA do not require the growth of an organism, and can be performed directly on the clinical samples whereas in conventional method DST can be done only from the isolates. LPA test results had a good concordance with the conventional culture and DST. Line probe assay detect MTB along with MDR TB, INH low level and high-level resistance and rifampicin resistance within short time. In order to start the treatment for drug resistant TB LPA is highly useful in places where there is high prevalence rate of TB since large number of samples can be screened in a short time.

Conclusion

Rapid and precise diagnosis of tuberculosis along with detection of drug resistance is essential for early management of pulmonary TB. LPA test done directly from smear positive sputum samples is highly sensitive and specific for rapid detection of MDR TB, INH and rifampicin resistance. They require shorter turnaround time as compared to conventional DST method. Longer turnaround time for culture and sensitivity testing is a main drawback of conventional method. Primary culture of smear positive sputum samples and subsequent DST of the isolates is not necessary in LPA. Reduction in time for diagnosis of drug resistant cases helps in earlier initiation of treatment also. In 92 per cent of smear-positive samples interpretable results are obtained within 2-3 days using LPA. Common mutations associated with INH and rifampicin resistance can be detected by LPA. But LPA is not a complete replacement for conventional culture and DST and culture is still required for smear negative and scanty smear positive sputum specimens where direct detection of drug resistance by LPA is not much effective.

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