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Role of line probe assay in detection of extra-pulmonary tuberculosis/multidrug-resistant tuberculosis: The experience from Kerala state, India

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Abstract:

BACKGROUND: There are limited data on use of line probe assay (LPA) for detection of TB and Rifampicin resistance among extra-pulmonary tuberculosis (EPTB) patients under specific TB control programme settings. Our aim was to compare the positivity of LPA with Lowenstein–Jensen (LJ) and Bactec MGIT and to test its utility in faster testing and in patients on treatment.

METHODOLOGY: The data of all 387 EPTB samples processed in two years by the state-level accredited laboratory in 2013–2014 were selected for the study. The laboratory used standard N-acetyl-L-cysteine – sodium hydroxide processing method for all samples except for cerebrospinal fluid and urine samples before conducting Ziehl-Neelsen smear microscopy, LPA and culture on both LJ and MGIT. Those samples with a negative LPA result if culture positive was subjected to a second LPA as per laboratory protocol. Doubtful Rifampicin resistant (RR) results were cleared by phenotypic testing by MGIT. Anti-TB treatment (ATT) duration was correlated with positivity of these tests later.

RESULTS AND DISCUSSION: LPA done on 321 processed samples out of 387 EPTB samples identified eight RR cases among 136 positive results within two to four days. Performing LPA on culture positive isolates identified an additional seven RR cases, thus reducing turn-around-time by two to eight weeks for the susceptibility results. It also demonstrated far better positivity in smear negative (15.2%) and in smear positive samples (74.3%) compared to culture methods as the test demonstrated clearly, better positivity among patients on ATT for more than two months.

CONCLUSION: LPA in association with culture is an excellent combination with very good results in rapid identification of EPTB along with detection of Rifampicin resistance. LPA gives far superior positivity compared to MGIT and solid cultures among patients under ATT.

Keywords:

Extra-pulmonary tuberculosis, line probe assay, multidrug-resistant tuberculosis, MGIT culture and Lowenstein–Jensen culture

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Introduction

With an estimated 2.6 million tuberculosis (TB) cases and 62,000 notified multidrug-resistant/rifampicin-resistant (MDR/RR) TB cases, India is considered as the TB capital of the world.[1] The Revised National TB Control Program (RNTCP), India, had concentrated more on pulmonary TB (PTB) control over the years, justifiably as it is infectious; acting as the source of TB in the society. However, extra-pulmonary TB (EPTB) constitutes 10%–20% of immune-competent TB cases

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worldwide (>50% in HIV positives) and often causes more morbidity and mortality depending on the site involved.[2-6]

Bacteriological diagnosis of EPTB is often difficult because of the reduced sensitivity due to the lower bacterial load, difficulty in obtaining samples/biopsies[5] and to a certain extent due to the labour intensive processing of samples.

The introduction of commercial and well-validated nucleic acid amplification tests (NAATs) such as line probe assay (LPA) (GenoType MTBDR+, Hain Life Sciences, Nehren, Germany) and later cartridge-based NAATs such as GeneXpert (Cepheid, Sunnyvale, USA) had made dramatic changes to the programme for identification of MDR/RRTB, thus easing out the time-consuming and demanding growth-based culture tests. The WHO approved and even recommended over other tests, the use of GeneXpert (Xpert) for bacteriological diagnosis of EPTB (and RR EP cases) based on published studies.[7] However, LPA which was introduced earlier lacks data regarding its role in diagnosis of EP/EP MDR TB cases despite being used widely for identification of pulmonary MDR TB cases by TB control programmes worldwide. RNTCP, for example, has 50 certified LPA facilities running in India.[1] LPA performs very well against smear-positive pulmonary samples and culture isolates from pulmonary or EP samples.[8-11]

Kerala, a South Indian state, with 34 million population started accepting EP samples from EPTB patients, primarily for identification of MDR/RREPTB as part of the programme from 2012. The RNTCP state laboratory (IRL, Trivandrum) entrusted with the role has quality-assured facilities for both solid and MGIT culture drug and susceptibility testing (DST) and LPA, certified by the WHO Supranational Reference Laboratory, National Institute for Research in Tuberculosis, Chennai (formerly TB Research Centre).

The study aims to assess the additional yield in identifying EPTB/RR EPTB cases along with other benefits if routine LPA testing is introduced to all processed EP samples, based on the culture records available in the laboratory.

Methodology

All the records of EP samples received at IRL, Trivandrum, between January 1, 2013 and December 31, 2014 were included in the study. The laboratory had followed a routine of processing by standard N-acetyl-L-cysteine – sodium hydroxide (NALC-NaOH) method for both culture and LPA except for cerebrospinal fluid (CSF) and urine samples, where unprocessed sample (due to low volume and otherwise sterile nature) and acid decontamination method, respectively, were used. Entire first morning urine samples for three days were used for suspected urinary tract TB cases.

The processed (homogenised and centrifuged at 3000 G for 20 min) samples were subjected to smear microscopy by Ziehl-Neelsen (ZN) staining followed by LPA (LPA1) and culture on Lowenstein–Jensen (LJ) medium and BD BACTEC MGIT (Becton Dickinson, Maryland, USA). All samples without valid Rifampicin susceptibility results from LPA1 and with positive culture results were subjected to a second LPA (LPA2) performed on the cultures. Only the samples with Rifampicin susceptibility results still unclear were confirmed with MGIT DST [Figure 1].

As the sensitivity of LPA in identifying INH (Isoniazid) resistance is lesser compared to RIF as documented in various studies[9,10] and considering the status of Rifampicin resistance as a surrogate marker for INH resistance, INH susceptibility testing was not included in this study.

The samples were stratified based on the duration of anti-TB treatment (ATT) and the positivity of each test method was compared with others.

Results

A total of 387 EP samples were received from all districts of Kerala state during the period. Samples from private hospitals were 62 (20%) out of the total 318 cases, where data were available. Most of the samples from districts other than Trivandrum (42% of total) were transported by ordinary courier services using standard three-layer packing. The rest were transported by hand by the patient's relatives. Cold packs were only occasionally used. The mean transportation times ranged from less than one to 5.5 days with a median of 1.6 days [Table 1].

The data of the samples were incomplete in certain parameters but included in the study as could be expected in retrospective studies based on laboratory data. Maximum number of samples was received from 16 to 30 age group followed by 31–45 age group among both sexes [Table 2]. Median age of females showed a relative shift to the younger side apparently due to a large number of lymph node samples received from females in 16–30 years age group (65 females/29 males).

Out of 384 samples with data, 185 (50.8%) were from lymph nodes; both biopsies and aspirates, followed by

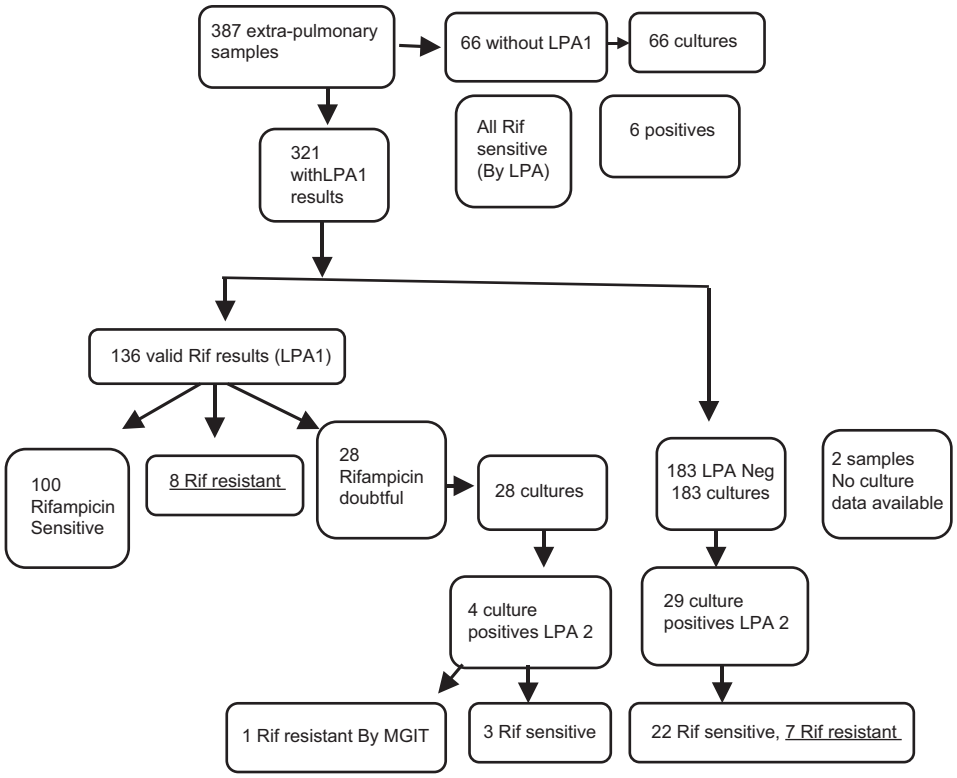

Figure 1: Work flow of extra-pulmonary tuberculosis specimens received by the intermediate reference laboratory of Kerala state, 2013–2014

Table 1: Samples received: District wise and corresponding mean transportation times

Name of the districts	Total number of samples	Mean transportation time in days	Number of samples from government sector	Number of samples from private sector	Mode of transport: By courier	Mode of transport: By-hand	Sample with cold packs	Sample without cold packs
Thiruvananthapuram	129	<1	112	16	1	128	1	128
Kollam	26	<1	23	3	6	19	0	26
Pathanamthitta	14	1.2	8	6	4	10	0	14
Alappuzha	14	1.8	10	4	8	3	0	14
Kottayam	21	1.3	17	3	17	2	0	20
Ernakulam	17	2.6	4	13	11	6	2	15
Idukki	5	1	4	1	5	0	0	5
Thrissur	16	1.6	9	7	15	0	0	15
Malappuram	10	2.2	10	0	10	0	3	6
Kozhikode	23	3.2	20	3	19	4	10	13
Palakkad	8	1.8	7	1	7	0	0	8
Wayanad	2	5.5	1	0	2	0	1	1
Kannur	25	1.6	22	3	24	0	0	25
Kasargod	2	1	2	0	2	0	0	2
Not given	75	<1	7	2	9	25	2	70
Total	387	<1-5.5	256	62	140	197	17	347

91 samples of body fluids (21.6%) including cerebrospinal fluid, pleural fluid and peritoneal fluid and 66 samples of Aspirated pus from other sites (15.9%).

Of 247 patients with relevant data present, 39 (16%) had PTB also, either concomitant or within the past two years. Another 13 patients gave a history of PTB more than two years back.

Performance of line probe assay compared to culture in extra-pulmonary tuberculosis samples

Lymph node samples had the best LPA positivity rates (95/163, 58.3%) followed by pus (25/51, 49%) and other solid tissues (33.3%). Body fluids including CSF, pleural fluid and peritoneal fluid had very poor positivity (8.7%) [Table 3].

Table 2: Lymph node samples/other extra-pulmonary samples: Age and sex distribution

Age group	Male	Female
0-15	0/8	6/16
16-30	29/23	65/26
31-45	19/33	27/23
46-60	9/29	11/7
>60	3/20	3/6
Age not recorded	7/4	4/6
Total	184	200

Sex of the patient was not recorded for three samples

Out of 384 samples, only 299 had complete data regarding ZN microscopy (done after NALC-NaOH processing), LPA, culture on LJ medium and culture on MGIT 960 [Table 4].

Among patients not exposed or exposed to ATT for less than two months, direct LPA (LPA1) on processed samples yielded 18.2% positivity compared to smear microscopy (28.3%) and culture (26.3%). Patients who were under treatment for more than two months and for longer durations of ATT, LPA1 showed very good positivity rates (54.8%–59%) against culture positivity, which was generally poor (7.7%–15.1%), with MGIT yielding slightly better results than LJ culture [Tables 4a and b].

On direct comparison between ZN microscopy (done on NALC-NaOH processed sample) and LPA, LPA1 gave positive results in only 110 (74.3%) out of 148 smear positive samples with available data, wherein 66% (98 out of 148) of the smear positives were scanty positives (<10 bacteria in 100 oil immersion fields). LPA1 also gave 26 positives out of 171 (15.2%) smear negative samples.

In addition, LPA1 gave a positive result for MTBC but with invalid susceptibility results for 15 more samples, 11 of that were smear-negative while the remaining four scanty positive.

Rifampicin resistance detection

Of the 321 samples where LPA1 results were available, 136 (42%) gave MTBC positive with valid Rifampicin susceptibility results. Out of this, 8 (6%) were found to be resistant to Rifampicin and 28 (20.5%) had a doubtful Rifampicin susceptibility result. All the eight cases found RR on direct testing of samples were originally smear-positive for acid-fast bacilli on ZN staining, but four of them turned out to be culture negative.

Twenty-nine of the 183 LPA1 negatives and four of the 28 Rifampicin doubtful susceptible (LPA1) were culture positive (culture data missing for two cases) and further underwent additional LPA testing (LPA2) which yielded 7 more RR cases. One of the samples which gave doubtful Rifampicin susceptible results on LPA1 and LPA2 was subsequently found to be RR by MGIT 960. In total, 15 (5%) RR cases were detected by LPA. One of the RR cases detected by LPA1 was eventually found to be a case of 'Extensively drug resistant' TB.

No Rifampicin resistance were detected among new cases (103 nos.) where treatment is yet to start and those under two months of ATT. Nine out of 39 retreatment cases not responding to three or more months of ATT and chronic cases with repeated ATT courses were detected RR [Table 5].

Discussion

Lymph node samples were the most common (50.7%) samples received with a particular predilection toward young females much in consistence with other studies from India/world.[4-7,11-14]

The lower than expected positivity rates of smear microscopy, LPA1, culture on LJ and culture by MGIT in the untreated and in those patients on less than two months of ATT (4/47, all tests combined) were probably due to numerical dilution by samples of body fluids with very poor positivity in that category and by samples from 'presumptive' EPTB cases, where a diagnosis of TB was not established at the time of collection of samples. The final TB statuses of these patients are not known.

The comparatively high rates of positivity by microscopy and LPA1 (64.1% and 59% respectively) among those on two to four months of ATT, further points to the fact that a good proportion of those untreated 'presumptive TB cases' was investigated probably to rule out TB. The poor positivity of LJ and MGIT cultures (7.7%–11% and 10.3%–15.1%, respectively) compared to the high LPA positivity in greater than two months ATT groups [Figure 2] were probably due to the loss of viability of mycobacterium caused by multiple factors including the inhibitory effects of multiple anti-TB drugs taken for a sufficiently long time.

The difference between testing by LPA and testing by culture methods in TB patients on treatment more than two months is statistically significant ($P < 0.0001$). LPA positives are 112 compared to 29 MGIT positives. The difference can be seen in Figure 2, very obviously.

The other general factors would be time taken for sample transportation without a proper cold chain and perhaps, more importantly, the processing of samples including the homogenisation of solid tissues with high speed motor driven pestles resulting a considerable generation of heat in the process although care was taken to control it in.

Table 3: Line probe assay positivity on different (processed) samples

	Lymph node samples	Aspirated pus	Body fluids	Solid tissue	Urine	Others	Total
Pos/total (%)	95/163 (58.3)	25/51 (49)	6/69 (8.7)	7/21 (33.3)	0/6 (0)	3/11 (27.3)	136/321 (42.4)

Table 4: Positivity of Ziehl-Neelsen microscopy, line probe assay, Lowenstein-Jensen culture and MGTT culture on samples in relation to treatment duration (299 samples with all the data available)

Treatment duration	Total number of samples	ZN microscopy positives (%)	LPA direct positives (%)	LJ culture positives (%)	MGIT culture positives (%)
Presumptive TB cases and TB cases on ATT less than two months	99	28 (28.3)	18 (18.2)	26 (26.3)	26 (26.3)
Two to four months ATT	39	25 (64.1)	23 (59.0)	3 (7.7)	7 (10.3)
ATT failures	88	47 (53.4)	49 (55.7)	8 (9.1)	11 (12.5)
Relapses on ATT	73	39 (53.4)	40 (54.8)	8 (11)	11 (15.1)
Total	299	139 (46.5)	130 (43.5)	45 (15.1)	55 (18.4)

ZN: Ziehl-Neelsen; LPA: Line probe assay; LJ: Lowenstein-Jensen; MGTT: (BD bactec) Mycobacterial growth indicator tube; ATT: Anti-tuberculosis treatment; TB: Tuberculosis

Table 4a: Line probe assay versus MGIT among presumptive tuberculosis cases and tuberculosis cases on antituberculosis treatment for less than two months: Total cases 96 nos

	LPA		Total
	Positive	Negative	
MGIT positive	12	14	26
MGIT negative	6	64	70
Total	18	78	96

McNemar Chi-square (corrected) *P*=0.1175. OR (95% CI)=2.3333 (0.8967-6.072). LPA: Line probe assay; CI: Confidence interval; OR: Odds ratio; MGIT: (BD bactec) Mycobacterial growth indicator tube

Table 4b: Line probe assay versus MGIT among tuberculosis patients on antituberculosis treatment for greater than two months: Total 200 nos

	LPA		Total
	Positive	Negative	
MGIT positive	20	9	29
MGIT negative	92	79	171
Total	112	88	200

The difference between testing by LPA and testing by culture methods in TB patients on treatment more than two months is significant (*P*<0.0001). LPA positives are 112 compared to 29 MGIT positives. McNemar Chi-square (corrected) *P*<0.0001. OR (95% CI)=0.0978 (0.0493-0.194). LPA: Line probe assay; CI: Confidence interval; OR: Odds ratio; MGIT: (BD bactec) Mycobacterial growth indicator tube; TB: Tuberculosis; LPA: Line probe assay

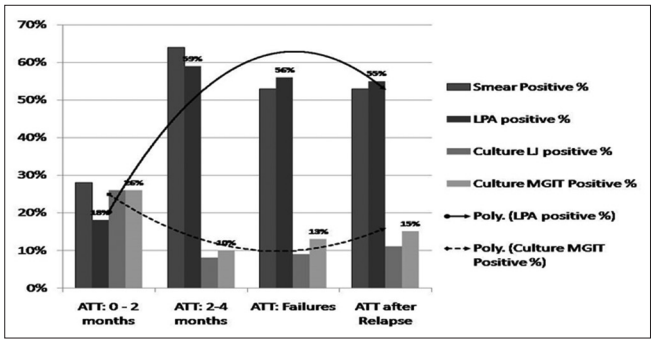

Figure 2: Performance of culture based methods compared to line probe assay and microscopy depending on tuberculosis treatment status

LPA could rapidly identify a good number of RR cases; about half of them were identified by direct testing of processed clinical specimens within two to four days, thus providing the advantage of rapid turnaround for patient management. Even among the rest, LPA detected Rifampicin resistance on culture isolates thus saving time of two to six weeks in comparison to liquid or solid media-based DST [Figure 1].

In a high TB prevalent country like India, where it is not operationally feasible or sustainable to screen Rifampicin resistance for all cases before starting treatment, down selecting 'likely Rifampicin resistant' cases by defining criteria often based on treatment response could be the only workable option and indeed the method followed by RNTCP so far. Very much into that scenario, this study shows that NAATs such as LPA yields much better results than culture methods for bacteriological confirmation and Rifampicin resistance detection of presumptive EPTB cases under empirical ATT [Tables 4, 4a, b and Figure 2] for reasons described above. This trend was also noted by Vadwai *et al*.,[2] using GeneXpert, another NAAT, though both LPA and GeneXpert have poorer detection limits compared to culture among viable bacterial populations. The importance of LPA is evident from the finding that four out of the eight RR cases identified by LPA1 were culture negative and could have been missed otherwise.

The unusually higher numbers of 'RIF doubtful susceptible' results in this study could be due to the paucibacillary nature of EP samples or/and the presence of tissue inhibitors[13-16] to DNA amplification as culture and further LPA in those cases yielded three clear sensitive results out of a total four culture positives. The remaining doubtful was confirmed resistant by MGIT.

Our study had several strengths. First, to the best of our knowledge, this is one of the first studies providing data on performance of LPA in EPTB patients from programmatic settings in India and adds to limited evidence available

Table 5: Rifampicin resistance by line probe assay (processed sample or culture isolate) related to treatment duration

Treatment duration	RIF		Total (%)
	Sensitive (%)	Resistant (%)	
New cases			
Less than two months ATT	103 (100.0)	0	103 (100.0)
Greater than two months ATT	113 (96.6)	4 (3.4)	117 (100.0)
Relapse cases	43 (97.7)	1 (2.3)	44 (100.0)
Retreatment ATT greater than three months or greater than two courses of ATT in the last three years	30 (77)	9 (23)	39 (100.0)
Unknown duration of ATT	16 (94.1)	1 (5.9)	17 (100.0)
Total	305 (95.3)	15 (4.7)	320 (100.0)

ATT: Antituberculosis treatment; RIF: Rifampicin

globally. Second, the study had large numbers with samples coming from a large geographic area. Third, the tests were performed in a quality-assured reference laboratory accredited by a WHO supranational reference laboratory, thus making the results valid and reliable.

There were a few limitations. First, the test samples received may not be a representative sample of all EPTB patients in the state and the final TB status of those patients with 'presumptive TB' at the time of sending of the sample is not known. Hence, the rate of Rifampicin resistance found in the study may not be generalisable. This requires future prospective studies using well-defined criteria and consecutive enrolment of EPTB patients. Second, some samples were excluded from analysis given the lack of data in the registers, reflecting the operational nature of the study.

Our study provides encouraging results for RNTCP as there are sufficient certified laboratories with LPA and culture facilities across the country (50 and 26, respectively[1]) with reserve capacities, which may need only additional trained workforce to start accepting EP samples for identification of TB along with Rifampicin and INH resistance screening.

Conclusion

LPA was successful in rapidly providing bacteriological confirmation and detecting Rifampicin resistance among EPTB patients as it is fast and accurate with proper interpretation. It gave far better positivity in smear-positive and smear-negative samples compared to LJ and MGIT cultures together where samples are received usually after the patients are exposed to various duration of ATT. However, simultaneous cultures are strongly advisable, both for increasing the yield and for confirmation as doubtful RR cases are fairly common among LPAs done directly on processed samples.

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Conflicts of interest

There are no conflicts of interest.

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