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Original Article

Microbiology Section

Study on Drug Resistant Pulmonary Tuberculosis using Line Probe Assay in a Tertiary Care Hospital from Eastern India

SHIVENDRA KUMAR SHAHI, ROHIT KUMAR, ANIMA XESS, PRASHANT GOSWAMI, BALKRISHNA MISHRA, RAKESH KUMAR, SHAILESH KUMAR, RAJ KISHORE SHARMA

ABSTRACT

Introduction: Pulmonary Tuberculosis (PTB) is a major public health issue globally, all age groups in community are at high risk to get infected. Multidrug Resistant (MDR) and extensively drug resistant *Mycobacterium tuberculosis* is increasing day by day, which is an alarm for policy maker to improve the policy. Success of any Tuberculosis (TB) control programs depends on availability of accurate data regarding TB.

Aim: To know the resistant profile of *Mycobacterium tuberculosis* causing PTB by using Line Probe Assay (LPA).

Materials and Methods: It is a laboratory based observational study conducted in Department of Microbiology, IGIMS and TBDC, Bihar, India between January 2016 to December 2016. Sputum specimens were collected from all clinically suspected cases of TB. All specimens were subjected to smear microscopy, conventional culture and LPA. Standard operating protocols were followed for all the techniques.

Results: Totally 1772 sputum samples were collected during study period, positive for smear microscopy, conventional culture and LPA were 54%, 66% and 72% respectively. All smear positive isolates were positive by LPA, whereas only 840 by conventional culture. Among the smear negative isolates 309 were positive by LPA and 336 by conventional culture method. In LPA negative isolates 73 cases were positive by conventional culture methods. Out of 1272 LPA positive isolates 249 patient were diagnosed as PTB due to resistant strains. Among resistant isolates 60.64% were identified as both rifampicin and isoniazid resistant, whereas 21.68% and 17.67% was rifampicin and isoniazid mono resistant respectively.

Conclusion: We found that LPA can be a good diagnostic tool for early diagnosis of mono-resistant as well as MDR TB. To control the transmission of TB in community, control programs should be followed and early diagnosis of drug resistant TB must be ensured.

Keywords: Conventional culture, Isoniazid, Smear microscopy

INTRODUCTION

Pulmonary Tuberculosis (PTB) is an infectious disease caused by *Mycobacterium tuberculosis* infection is acquired by inhalation of Acid Fast Bacilli (AFB) [1]. AFB can cause pulmonary infection as well as Extra Pulmonary Tuberculosis (EPTB). EPTB refers to Tuberculosis (TB) involving organs other than lungs e.g., pleura, lymph nodes, abdomen, spleen, genitourinary tract, skin, joints and bones, or meninges [2]. TB remains a major cause of high morbidity and mortality compare to other infectious disease globally. As per WHO, about 2 million of death occurs annually due to TB in that 90% occurs alone from developing countries [3]. Multi Drug Resistance (MDR) and Extensively Drug Resistance Tuberculosis (ExDR-TB) are important factors causing death of patient with TB [4]. According to WHO global Tuberculosis report 2016, there are an estimated 10.4 million new (incident) TB cases reported from worldwide, of which 5.9 million (56%) are men, 3.5 million (34%) are women and 1.0 million (10%) are children. India, Indonesia, China, Nigeria, Pakistan and South Africa together contribute to 60% of the new cases alone [5]. The disease caused by *Mycobacterium tuberculosis* resistant to two primary anti-tubercular drug i.e. Rifampicin (RIF) and Isoniazid (INH) is known as MDR-TB. In India, MDR-TB is found to be 2.3% in new cases and 17.2% in previously treated cases [6]. Early diagnosis is essential to reduce the rate of transmission of TB. Chest X-ray is a useful diagnostic tool but it is not a specific test. Whereas community acquired pneumonia and PTB cannot be solely differentiated by chest X-ray alone [7]. Culture of AFB is considered to be the gold standard method for the definitive diagnosis of TB. The generation time of AFBs are high, therefore culture is time consuming and laborious. Whereas sputum smear microscopy by Ziehl-Neelsen (ZN)

staining is a rapid, simple, and inexpensive tool for diagnosis of Pulmonary TB, but it has low and variable sensitivity [8]. Recent molecular based method like GenoType MTBDRplus test have advantages over conventional phenotypic methods in terms of accuracy and turnaround time.

The GenoType MTBDRplus assay is a commercially available Line Probe Assay (LPA) that allow specific gene markers associated with RIF and INH resistance to be detected. It is designed to detect the most important mutations of *rpoB* genes for RIF resistance and *inhA* and *katG* genes for INH resistance in *Mycobacterium tuberculosis* within 8 hours [9]. Mutation in 81 base pair region of *rpoB* gene is targeted in phenotypic RIF resistant identification and same mutation genes is targeted in LPA molecular method [10]. Mutation in *S531L* is the most frequent mutation in RIF mono-resistant isolates [11], whereas the specific mutation *S315T1* in *katG* gene account for commonest resistance mechanism in INH. However, mutation in the *inhA* gene accounts low-level of resistance to INH [12]. Overall sensitivity and specificity of LPA for detection of RIF resistance is high at 96% and 99% respectively, sensitivity and specificity to detect INH resistant is 72% and 97% respectively [13,14]. The aim of the present study was to know the resistant pattern of *Mycobacterium tuberculosis* causing PTB, using LPA from Eastern part of India.

MATERIALS AND METHODS

This was a laboratory based observational study, conducted at Department of Microbiology, Indira Gandhi Institute of Medical Science (IGIMS), Patna, Bihar and State Tuberculosis Demonstration and Training Centre (TBDC), Agamkuan, Patna, Bihar, India, between the period of January 2016 to December 2016. The study was approved by Institutional Ethical Committees of IGIMS Patna, Bihar.

Inclusion criteria: i) Clinical feature associated with PTB like, cough more than two weeks, haemoptysis, weight loss, fever, and chest pain; ii) Patient of all age group; iii) Both sexes (male and female).

Exclusion criteria: Smear microscopy negative for Tubercle bacilli but ZN staining reveal *Nocardia* Species or Actinomyces species.

Specimen collection: Sputum sample were collected from PTB suspected patients, who were attending hospital OPD. Standard RNTCP protocol was followed for sample collection. The patients were provided a clean, dry, sterile wide-neck, leak-proof plastic container. Patients were educated to cough deeply to produce sputum specimen and how to collect sputum without contaminating the collection container.

Microscopic observation: Direct smear was prepared from specimen for ZN staining and it was examined under binocular bright field microscope, as per the standard procedure [15].

Bacteriological culture: Lowenstein-Jensen (LJ) medium was used for cultivation of AFBs. Specimens were firstly decontaminated by NALC-NaOH (N-acetyl L-cysteine-Sodium hydroxide) method and concentrated thereafter [16]. Three or four drop of deposit were inoculated on two slopes of LJ medium and was incubated at 37°C. They were examined within 3-5 days after inoculation for early detection of rapidly growing *Mycobacterium* and of contaminated cultures, followed by examination once a week for eight weeks. On the basis of colonies morphology on culture media culture was reported as positive and ZN staining reveal the presence of AFB [15].

Line Probe Assay: The test was performed according to the manufacturer's instruction [9]. Direct clinical specimen after decontaminant was used to perform LPA. The test is based on DNA strip technology and has three steps: DNA extraction, multiplex PCR amplification, and reverse hybridization [17]. The test was performed in three different rooms with restricted access and unidirectional workflow. In Biosafety level-3 laboratory *Mycobacterium* DNA was extracted using DNA extraction kit (Genolyse®- Hain Lifescience). Final volume of 50 µl master mixture consisting, 10 µl of amplification mix A, 35 µl of amplification mix B (provided with kit) and 5µl of DNA supernatant were used for amplification. An initial step of denaturation was carried out at 95ºC for 15 minute followed by 30 cycle at 95ºC for 25 second, annealing at 50°C for 40 second, extension at 70°C for 40 second and a final extension at 70°C for 8 minute. Finally the result of amplified product was analysed by 'Reverse Hybridization' technique using DNA strip technology. Each strip of LPA were pre-attached with 27 different reaction zones consisting of, six controls (conjugate, amplification, *Mycobacterium tuberculosis* Complex (TUB) gene loci, *rpoB*, *katG* and *inhA*), one *katG* wild-type and two mutant probes (*katG* MUT1 S315T1 and *katG* MUT2S315T2), two *inhA* wild type and four mutant probes (*inhA* MUT1 C15T, *inhA* MUT2 A16G, *inhA* MUT3AT8C, *inhA* MUT3B T8A), and eight rpoB wild-type (WT1–WT8) and four mutant probes (*rpoB* MUT1D516V, *rpoB* MUT 2A H526Y, *rpoB* MUT2B H526D, *rpoB* MUT3 S531L).

RESULTS

In present study a total of 1772 sputum were collected from clinically suspected cases of PTB. All specimens were subjected to microscopy, conventional culture on LJ medium and LPA. Gender distribution were as follows- 1294 (73%) males and 478 (27%) females.

Among the 1772 specimen, 1345 (76%) patients were diagnosed PTB positive using different diagnostic test. The distribution of PTB confirmed result by different methods are as follow- 963 (54%) positive by sputum smear microscopy, 1176 (66%) by conventional culture method and 1272 (72%) by LPA [Table/Fig-1].

Among smear positive isolates 840 samples were found to be positive by conventional culture methods, whereas LPA was

positive for all. Out of the 1272 LPA positive isolates, 1103 was positive by conventional culture methods. Whereas, in LPA negative isolates 73 was positive by conventional culture method [Table/Fig-2].

Test	Positive	Percentage (%)
Sputum Smear Microscopy	963	54%
Culture	1176	66%
GenoType MTBDRplus Assay	1272	72%

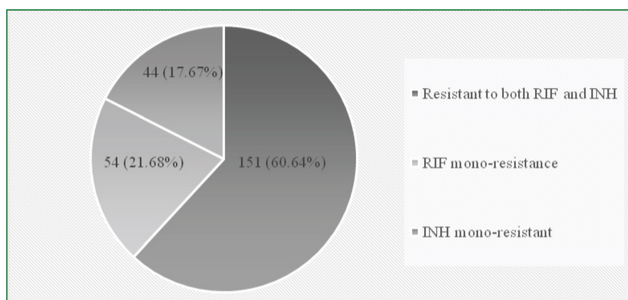
[Table/Fig-1]: Distribution of results according to test used.

a)	Smear Positive	Smear Negative	Total
Culture Positive	840	336	1176
Culture no Growth	27	436	463
Culture Contamination	96	37	133
Total	963	809	1772
b)	**LPA Positive**	**LPA Negative**	**Total**
Culture Positive	1103	73	1176
Culture no Growth	67	396	463
Culture Contamination	102	31	133
Total	1272	500	1772

[Table/Fig-2]: Two by two contingency tables comparing (a) sputum smear and; (b) LPA test with conventional culture method.

Interpretation of LPA results: Valid result were obtained by LPA test for 1272 (72%) specimen including results of repeat testing which was performed in 83 cases. The causes of repeat testing included no TUB band, rpoB band being very faint or absent and indistinct bands. Out of the 1272 specimens, 1023 (80%) patient were diagnosed as PTB due to sensitive strains, whereas 249 (20%) patient were diagnosed as drug resistant TB due to any type (RIF and INH both, RIF alone or INH alone). Among the resistant isolates 151 (60.64%) was MDR-TB i.e., resistant to both RIF and INH, whereas 54 (21.68%) and 44 (17.67%) had RIF mono-resistance and INH mono-resistant respectively [Table/Fig-3].

Interpretation of LPA results in comparison to male and female: In comparison to male and female resistant



[Table/Fig-3]: Diagnosis of drug resistant TB using LPA test.

pattern, MDR-TB were observed in 105 (42%) and 46 (19%) respectively, whereas RIF mono-resistant in 37 (15%) and 17 (7%) respectively, and INH mono-resistant in 31 (12%) and 13 (5%) respectively.

Out of the 249 resistant isolates, 74 (30%) patients including both sex belonging to young age groups (21-30 years) was infected by resistant strains, whereas none of the infant or children belonging to age groups (0-10 years) was found to be infected with resistant strains. In >60 year age group 3 (1.20%) female was identified as drug resistant TB and 17 (6.82%) male was found to be drug resistant TB [Table/Fig-4].

Age (in years)	Male			Female			Total
	RIF^R INH^R	RIF^R INH^S	RIF^S INH^R	RIF^R INH^R	RIF^R INH^S	RIF^S INH^R	
0-10	Nil	Nil	Nil	Nil	Nil	Nil	Nil
11-20	31	4	4	19	5	3	66
21-30	25	13	11	14	6	5	74
31-40	18	11	8	6	4	2	49
41-50	13	4	3	5	Nil	1	26
51-60	7	Nil	4	1	1	1	14
>60	11	5	1	1	1	1	20
Total	105	37	31	46	17	13	249

[Table/Fig-4]: Distribution of drug resistant Pulmonary Tuberculosis in comparison to age and sex.
*RIFR-RIF resistant, INHR-INH resistant, INHS-INH sensitive and RIFS-RIF sensitive.

Interpretation of LPA results in comparison to smear grading system: Among the 249 drug resistant isolate, 162 (65%) isolate was smear positive and 87 (35%) isolate was smear negative. Out of the 162 smear microscopy positive isolates 48 was 3+, 56 was 2+, 45 was 1+ and 13 was scanty. Among the 162 smear positive isolates 98 (60%) patient were diagnosed as RIF and INH both resistant, 25 (15%) and 39 (24%) patients were mono-resistant i.e. RIF and INH respectively. Whereas, in 87 smear microscopy negative isolates 53 (61%) patient were RIF and INH both resistant, RIF and INH mono-resistant was observed in 29 (33%) patient and 5 (6%) patient respectively. Out of the 13 scanty smear positive isolates 62% was diagnosed as MDR-TB and 31% was INH mono-resistant [Table/Fig-5].

Drug Resistance Pattern	Smear Grading System					Total
	3+	2+	1+	Scanty	Negative	
RIFR INHR	31	34	25	8	53	151
RIFR INHS	7	7	10	1	29	54
RIFS INHR	10	15	10	4	5	44
Total	48	56	45	13	87	249

[Table/Fig-5]: Proportion of drug resistant isolates of *Mycobacterium tuberculosis* in comparison to smear grading system.

DISCUSSION

The level of drug resistant profile of TB from different part of country will help the policy makers to control the transmission of resistant bacteria in the community as well as success of TB control program in any country. By conducting the present study, an attempt was made to know the resistant profile of *Mycobacterium tuberculosis* causing PTB among different age groups in eastern part of India by using LPA. Present study indicate 69% of male account drug resistant TB, whereas other study reported 79% of male was infected with resistant strains [18]. Study by Kumar P et al., have shown that 43% were RIF and 33% INH mono resistance in India [19], whereas 13.5% mono resistant were reported from another high TB-burden country like South Africa and 13% mono resistant from low-TB-burden country like United States [20,21]. A study from Gulbarga, South India reported 9.80% of INH mono resistant among new cases of PTB [22]. Our study found 4.25% of RIF mono resistant and 3.46% of INH mono resistant using LPA, which is comparatively low. In this study we observed 11% of MDR-TB, which is less compare to similar study conducted in Lucknow [23]. A study from North Bihar conducted in 2015 reported prevalence of MDR-TB was 15%, which is higher compare to this study [18]. Researcher from AIIMS, Delhi reported 25.8% of MDR, 10.4% and 22.2% of INH and RIF mono resistant respectively using LPA, which is comparatively high compare to our study [24]. In clinical practice, it is still a topic of debate about the significance of INH mono resistance and effect on TB treatment outcomes. However, a study from South Africa and a meta-analysis both have reported poor outcomes of TB with INH mono resistant [25,26], whereas two different study suggest that early detection of INH resistance can lead to better outcomes with modification of treatment [27,28]. In this study, maximum number of drug resistant TB was diagnosed among 21-30 year age group, whereas similar result was observed in other study indicating 16-30 year age group [18]. Many studies from India suggested that LPA can be the important diagnostic tool for rapid diagnosis of drug resistant PTB, even few research mentioned it can be used in nation TB programme for testing of suspected cases of MDR-TB [13,29].

LIMITATION

One of the major limitation of this study was sample size and inadequate demographic information. We suggest that, larger, observational studies should be conducted to provide better results, so that guidelines can be formulated.

CONCLUSION

In present situation where drug resistant case is increasing day by day, LPA can be an important diagnostic tool for rapid screening of drug resistance TB. LPA has the potential to substantially reduce the turnaround time, which resulting early management of drug-resistant cases. WHO recommendations must be followed on infrastructure, training, quality assurance and other requirements to ensure high quality results.

The results obtained in this study suggest that the LPA is a rapid and reliable methods for identification and Drug Sensitivity Testing (DST) to INH and RIF. The availability of DST will guide the clinician to start appropriate treatment regimens, thereby improving treatment outcome and reducing transmission. Furthermore, research need to be conducted at large platform to assess the usefulness of the LPA in smear negative patients.

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AUTHOR(S):

1. Dr. Shivendra Kumar Shahi
2. Dr. Rohit Kumar
3. Dr. Anima Xess
4. Dr. Prashant Goswami
5. Dr. Balkrishna Mishra
6. Dr. Rakesh Kumar
7. Dr. Shailesh Kumar
8. Dr. Raj Kishore Sharma

PARTICULARS OF CONTRIBUTORS:

1. Professor and Head, Department of Microbiology, Indira Gandhi Institute of Medical Sciences (IGIMS), Patna, Bihar, India.
2. PhD Research Scholar, Department of Microbiology, Indira Gandhi Institute of Medical Sciences (IGIMS), Patna, Bihar, India.
3. Professor, Department of Microbiology, Indira Gandhi Institute of Medical Sciences (IGIMS), Patna, Bihar, India.
4. Microbiologist, Intermediate Reference Laboratory (IRL), State Tuberculosis Demonstration and Training Centre (TBDC), Agamkuan, Patna, Bihar, India.
5. Chief Medical Officer, Department of Health and Family Welfare, State Tuberculosis Demonstration and Training Centre (TBDC), Agamkuan, Patna, Bihar, India.
6. Assistant Professor, Department of Microbiology, Indira Gandhi Institute of Medical Sciences (IGIMS), Patna, Bihar, India.
7. Additional Professor, Department of Microbiology, Indira Gandhi Institute of Medical Sciences (IGIMS), Patna, Bihar, India.
8. Postgraduate Student, Department of Microbiology, Indira Gandhi Institute of Medical Sciences (IGIMS), Patna, Bihar, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Rohit Kumar,
Department of Microbiology, Indira Gandhi Institute of Medical Sciences (IGIMS), Patna-800014, Bihar, India.
E-mail: krrohit1982@gmail.com

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