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DRUG RESISTANCE PATTERN OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATED FROM PULMONARY AND EXTRA PULMONARY SAMPLES

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ABSTRACT

To understand the pathogenesis, diagnosis, treatment, and prevention of tuberculosis (TB), *Mycobacterium tuberculosis* (MTB) needs to be molecularly characterized. There is, however, a paucity of knowledge regarding the molecular traits and drug-resistant patterns of MTB in individuals with pulmonary and extra pulmonary tuberculosis. The objective of this study was to identify the levels and patterns of resistance of MTB isolates to two important anti-TB medications, rifampicin and isoniazid, as well as the types of mutations in the isolates' drug resistance genes, rpoB, katG, and inhA. This retrospective analysis aimed to comprehend and record our experience using Line probe assay (LPA) for *M. tuberculosis* complex detection and MDR-TB diagnosis under programmatic circumstances. 317 samples from individuals with a variety of presentations were analyzed overall in the current study. A direct smear with Acid Fast Bacilli was made for the samples. Using the Fluorescent Microscopy procedure, all of the smears were stained. 206 samples had favourable results from smear microscopy examination. LPAs showed overall high accuracy for the identification of (Rifampicin) RIF resistance in patients with pulmonary and extrapulmonary tuberculosis. LPAs showed good sensitivity and high specificity for detecting INH resistance. When applied to smear-positive specimens, LPAs have a high level of accuracy for the detection of *M. tuberculosis*. For the detection of MTB and drug-resistant TB, particularly RIF-resistant, INH-resistant, and MDR-TB, LPAs have good diagnostic performance.

Keywords: *Mycobacterium tuberculosis,* RIF-resistant, INH-resistant, MDR-TB and Line probe assay

INTRODUCTION

The biggest public health issue and the leading cause of death from an infectious disease is tuberculosis (TB) **[1]**. According to estimates, *Mycobacterium tuberculosis* infects one-third of humanity. According to the 2019 Global TB Report, there will likely be 10.0 million new cases of TB. Ethiopia is one of the 30 countries with the highest rates of TB, TB/HIV, and MDR-TB. According to the World Health Organisation (WHO), Ethiopia has a TB incidence rate of 151 per 100,000 people. In addition, the rate of RR/MDR-TB was 0.71% in new cases and 16% in those that had already been treated **[2]**. Based on the anatomical areas where the disease manifests itself, there are two different forms of TB. These are extra-pulmonary (EPTB) and pulmonary (PTB) TB. Extrapulmonary TB is a significant clinical issue since it accounts for roughly 5-20% of the TB burden and has a higher frequency in HIV-coinfected patients **[3]**.

To comprehend the dynamics of transmission and drug resistance pattern, Mycobacterium tuberculosis Complex (MTBC) strains' molecular characterisation and drug susceptibility testing are crucial. Deoxyribonucleic acid (DNA) fingerprints have been used to generate a variety of molecular typing approaches that are used to identify and classify MTBC **[4]**. The quality of PCR amplification is compromised in extrapulmonary TB specimens due to a number of inhibitors, including host proteins, blood, and eukaryotic DNA, which results in low sensitivity and false-negative results **[5]**. Spoligotyping is a vital tool for analysing the distribution of different MTB genotype strains in environments with a lack of resources, nevertheless. The MTBC is detected and typed using a PCR-based technique based on polymorphism in a direct repeat region of the mycobacterial chromosome. It has many beneficial features, such as genotyping strains from clinical samples that have a distinctive hybridization pattern. The best way to learn about the MTB strains that are prevalent in a community is by genotyping **[6, 7]**.

Line probe assay (LPA) was developed in response to the pressing requirement to identify medication resistance in TB patients and the widespread prevalence of MDR-TB. LPA is a quick method based on polymerase chain reaction (PCR) that is utilized by the Revised National Tuberculosis Control Programme (RNTCP) of India to identify Mycobacterium tuberculosis (MTB) complex as well as drug sensitivity to rifampicin (RPM) and isoniazid (INH). Under appropriate circumstances, it is utilised to diagnose drug-resistant TB. Line probe assay (LPA) **[8]** only tests the sputum samples that are smear positive for acid-fast bacilli (AFB). Even when samples test positive for AFB, MTB complex may still go undetected by LPA in some circumstances. Here, we discuss our experiences with programmatic MTB complex detection and MDR-TB diagnosis by LPA in a tertiary care facility and hospital in central India **[9, 10]**.

Sample collection

A total of 317 clinical specimens were received from various districts in Karnataka during the period of January 2022 to October 2022 at IRL, Bangalore were used for this study. 240 pulmonary and 77 extra pulmonary specimens for drug susceptibility testing, including treatment patients and presumptive patients were processed using standard methods. Pulmonary like sputum and extrapulmonary specimen like gastric aspirates, pleural fluid, bronchial lavage/ wash, pus, tissue, biopsy, ascetic fluid, lymph node, cervical fluid, endometrium, peritoneal fluids, pericardial fluid, synovial fluid and Cerebrospinal Fluid (CSF) will be included during the study period. Specimens will be centrifuged at 3000 g for 20 mins at 4^{o}C using a refrigerated centrifuge and decontaminated by NALC – NaOH method. Same procedure was used for tissue samples after homogenization by cutting into small pieces and grinding with tissue grinder. CSF can be directly taken for further testing whereas it requires decontamination in case of turbid (visual appearance) CSF sample. Samples were processed in STDC-IRL, Bangalore, Karnataka, India. This study was conducted after the approval of the Institutional Ethical committee with ethical clearance certificate number 008/12/2021/IEC/SMCH.

Smear preparation

Fluorescent Microscopy (Auramine O) staining was used for the bacteriological investigation after decontamination and digestion by NALC-NaOH method. 3-5ml of samples were collected in a falcon tube at the district and sent to STDC IRL, Bangalore in triple layer packing in cold chain method. When obtaining fine needle aspirations (FNAs) from patients who were thought to have extra-pulmonary tuberculosis (EPTB), a skilled pathologist exercised the utmost safety and prudence. The Fluorescent Microscopy

(Auramine O) smear method was then employed to confirm its positivity. For the purposes of this investigation, suction samples of about 3-5ml of samples were collected in a falcon tube at the district and sent to STDC IRL, Bangalore in triple layer packing in cold chain method. The boxes will be opened and the clinical samples will be subjected to Decontamination and digestion by using NALC- NaOH method. The starting concentration of NaOH is 4%, the final concentration in the sputum specimen is 1%. The inclusion of the mucolytic agent, N-acetyl L-cysteine (NALC), enables a more rapid digestion of sputum and the decontaminating agent, sodium hydroxide, to be used at a lower NALC loses activity rapidly in solution so it has to be made freshly daily **[11-13]**.

Line probe assay

In order to diagnose tuberculosis (TB) and identify RIF as well as isoniazid (INH) resistance brought on by mutations in the rpoB, inhA, and both katG genes, the line probe assay (LPA), based on strip technology, was adopted **[14]**. According to the manufacturer's instructions (Hain Life Science GmbH, Nehren, Germany), the test was carried out. Testing culture isolates (indirect testing, e.g., Genoscholar PZA-TB II), acid-fast bacilli smear microscopy-positive specimens (first-line LPA), and both smear-positive and smear-negative sputum specimens (second-line LPA) are all done by direct testing. The procedure included three steps: reverse hybridization, multiplex PCR amplification, and DNA extraction **[15]**.

Extraction is used done by Genolysis. 500µl decontaminated clinical specimen was taken and centrifuged for 15mins at 10,000 X g in a centrifuge with aerosol tight rotor. Supernatant was discarded, followed by added 100µl Lysis Buffer [A-LYS] and resuspend. Incubated for 5 min at 95° C. 100µl Neutralizing Buffer [A-NB] was added and vortexed. Centrifuged for 5 min at 10,000 X g in a centrifuge with aerosol tight rotor. Transferred the supernatant (DNA solution) into a new tube (Store at -80° C for a longer storage)

By determine the number of samples to be analyzed along with a positive control (H37RV standard strain), negative control and master mix control were added. Pipetted out 10µ of AM- A first and then 35µl of AM- B in a cryovial and transferred 45 µl master mix into each PCR tube. Bring the PCR tubes out of the Amplification room 5 µl extracted DNA solution were added and placed in the PCR machine.

Programed the Thermocycler as follows:

Time and Temperature	Culture samples	Direct patient material
15 min 95°C	1 cycle	1 cycle
30 sec 95°C 2 min 65°C	10 cycles	20 cycles
25 sec 95°C 40 sec 50°C 40 sec 70°C	20 cycles	30 cycles
8 min 70°C	1 cycle	1 cycle

For FL LPA GenoType MTBDRplus V2.0 and GenoType MTBDRsl V2.0 assay kits were used.

The twincubator and GT Blot machine (twincubator is a manually operated device and GT Blot is an automated machine) was used for the hybridization process.

The DNA strip was taken out of the tube and labeled according to the quantity of samples. The HYB and STR solutions (Hain Life Science GmbH, Nehren, Germany) was pre-warmed at 45°C in a water bath for 15 minutes before starting the hybridization process. 20 µl of the denaturing solution was pipetted into each well of the tray and 20 µl of the amplicon and was mixed well by pipetting up and down for 5 times, the labeled strip was inserted to each well with coloured half facing up. Followed by 1 ml of pre-warmed HYB solution and gently moved the trough to and fro to homogenously mix HYB solution with amplicon. Incubated the tray at 45 °C for 30 min. HYB solution was completely aspirate using transfer pipettes. 1ml of Pre-warmed STR solution was added to each trough using a repeat pipette and incubated for 15 min at 45 °C. Followed by 1 ml of the rinse solution was added and minute of incubation. After being taken out, the well was rinsed with a solution. Each well received 1ml of the conjugate (10 µl CON-C +990 µl CON – D - dilute conjugate concentrate 1:100 with conjugate diluent), which was then added, incubated for 30 minutes, removed, and then washed with rinse solution. The well was then filled with 1 ml of the substrate ((10 µl SUB-C +990 µl SUB–D - dilute substrate concentrate 1:100 with substrate diluent), which was then incubated for 10 min before being twice rinsed with distilled water. The strips were then stuck to the interpretation sheet and interpreted the data as either Mycobacterium tuberculosis identified, resistant, sensitive, or invalid **[16]**.

RESULT AND DISCUSSION

Additionally prohibited is the diagnosis of extrapulmonary cases, which continues to be a challenge for doctors. Although extrapulmonary TB represents a sizeable portion of TB patients in several countries, it is often underreported because of diagnostic difficulties. Sputum smear microscopy is frequently used to identify pulmonary TB patients even though it lacks sensitivity **[17, 18]**. Furthermore, it prohibits the diagnosis of extrapulmonary cases, which is still a challenge for doctors.

Extrapulmonary TB is often underestimated because of diagnostic difficulties, despite the fact that it accounts for a large portion of TB patients in several countries **[19]**.

The low isolation rates in this investigation can be due to sample washing with 4% NaOH or prior anti-tubercular medication **[20]**. Only 84% of the 100 lung samples with positive smear results displayed growth on the LJ medium. 206 mycobacterium samples that had positive smear results were cultured on Lowenstein-Jensen (LJ) media in the current experiment. Mycobacterial species have a beige colour, an uneven or smooth appearance, and a glossy or thick feature while they are growing on solid culture media.

TUB band was not seen in 77 (5.95%) of the 317 LPA tests that were performed on such materials. Our research showed that at least 0.77 percent of sputum samples with positive AFB smear results had been misclassified by LPA as lacking any MTB complex members. To get the best outcomes, it's also crucial to assess the decontamination processes, particularly centrifugation and the duration of reagent exposure **[21-22]**. However, it may not be totally accurate to rely simply on molecular assays for the diagnosis of MTB infection given that 0.77 percent of samples that were negative for MTB by LPA were culture positive. Since smear microscopy was performed on concentrated sputum samples following decontamination, we were unable to relate this to the grading of sputum samples. Using LPA for TB, it has been shown that 97% of smear-positive specimens yield interpretable findings in 1-2 days.

The World Health Organisation (WHO) has approved both molecular tests, the cartridge-based nucleic acid amplification test (NAAT) and the ligand-binding assay (LPA), however it is unclear whether test is better. These powerful tests are now more widely accepted in clinical practise in India's primary health centres because to the free testing offered by the Revised National Tuberculosis Control Programme (RNTCP). The requirement for a primary culture of sputum samples and subsequent drug sensitivity testing (DST) of the mycobacterial isolates has been greatly diminished by the two molecular techniques. A mycobacteriological culture is still necessary for smear-negative specimens, as stated in the WHO policy statement on LPA, and conventional culture and DST are not completely replaced by LPAs **[22, 24]**.

In present study, first line LPA was carried out for 317 pulmonary and extra pulmonary samples. 102 (32.17%) samples both Rif and INH are sensitive, 26 (8.2%) Rif resistance, 27 (8.5%) INH low level resistance, 41 (12.9%) INH high level resistance, 1(0.3%) Kat G-R & Inh A-R, 61(19%) Rif resitance and INH high level resistance were detected. 4(1.2%) Rif resistance and INH low level resistance, 3 (0.9%) Rif resistance and INH both high and low level resistance was found. No TB band was observed in 41 (12.9%) samples. In 11 (3.4%) samples, bands did not develop clearly. Second line LPA was done for 317 pulmonary and extra pulmonary samples. 210 (66%) samples both FL and SL are sensitive, 30 (9.4%) FLQ resistance and SLID sensitive, 6 (1.8%) FLQ sensitive and SLID resistance, 6 (1.8%) FLQ resistance and SLID low level sensitive were observed. No TB band was observed in 47 (14.8%) samples. In 18 (5.6%) samples, bands did not develop clearly hence inoculation is done.

Table 1: Line probe assay for detection of *Mycobacterium tuberculosis* complex

Line probe assay	Sensitive/ Resistance	Number	%
First Line LPA	Both Rif & INH Sensitive	102	32
	Rif Resistance	26	8
	INH low level Resistance	27	8.5
	INH high level Resistance	41	12.9
	Kat G-R & Inh A - R	1	0.3
	Rif Resistance and INH high level Resistance	61	19
	Rif Resistance and INH low level Resistance	4	1.2
	Rif Resistance and INH both high and low level Resistance	3	0.9
	NO TB Band	41	12.9
	Bands did not developed clearly hence incoulation is done	11	3.4
	Total	317	100
Second Line LPA	Both FLQ & SLID are Sensitive	210	66
	FLQ Resistance and SLID Sensitive	30	9.4
	FLQ Sensitive and SLID Resistance	6	1.8
	FLQ Resistance and SLID low level Resistance	6	1.8
	NO TB Band	47	14.8
	Bands did not developed clearly hence incoulation is done	18	5.6
	Total	317	100

Rif - Rifampicin, INH- Isoniazid, FLQ- Fluoroquinolones SLID- Second Line Injectable

CONCLUSION

A total of 317 samples from patients with various presentations were looked at. A direct smear with Acid Fast Bacilli was made for the samples. Using the Fluorescent Microscopy procedure all of the smears were stained. 206 samples had favourable results from smear microscopy examination. The identification of RIF resistance by LPAs in patients with pulmonary and extrapulmonary tuberculosis showed great overall accuracy. LPAs showed good sensitivity and high specificity for detecting INH resistance. When applied to smear-positive specimens, LPAs have a high level of accuracy for the detection of *M. tuberculosis*. For the detection of MTB and drug-resistant TB, particularly RIF-resistant, INH-resistant, and MDR-TB, LPAs have good diagnostic performance. The latest WHO policy recommendations were based in part on these findings.

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Conflict of interest

No conflict of interest

REFERENCES

[1] World Health Organization. Global Tuberculosis Report. Geneva: WHO press. 2018.

[2] World Health Organization. Global Tuberculosis Report. Geneva: WHO press;. 2019.

[3] Lee JY. Diagnosis and treatment of extrapulmonary tuberculosis. *Tuberc Respir Dis (Seoul)*. 2015;78(2):47–55. pmid:25861336

[4] Kulchavenya E. Extrapulmonary tuberculosis: are statistical reports accurate? *Ther Adv Infect Dis*. 2014;2(2):6170. pmid:25165556

[5] Purohit M, Mustafa T. Laboratory Diagnosis of Extra-pulmonary Tuberculosis (EPTB) in Resource-constrained Setting: State of the Art, Challenges and the Need. *J Clin Diagn Res*. 2015;9(4): EE01–6. pmid:26023563

[6] World Health Organization. Global Tuberculosis Report. Geneva: WHO press 2014.

[7] Kumar A DA, Jena PP, Rongpharpi SR, Gur R. Zeihl Neelsen Stain: Still a Reliable Option for EPTB Diagnosis in Resource Constraint Settings. *Journal of Infectious Diseases and Medicine*. 2018;03(01).

[8] Seung KJ, Keshavjee S, Rich ML. Multidrug-Resistant Tuberculosis and Extensively Drug-Resistant Tuberculosis. *Cold Spring Harb Perspect Med*. 2015;5(9):a017863. pmid:25918181

[9] Kumar R, Kushwaha RAS, Jain A, Hasan Z, Gaur P, Panday S. Tuberculosis Among Household Contacts of Multidrug-Resistant Tuberculosis Cases at a Tertiary Hospital in Lucknow, India. *International Journal of Life-Sciences Scientific Research*. 2018;4(2):1707–12.

[10] Zhang J, Abadia E, Refregier G, Tafaj S, Boschiroli ML, Guillard B, et al. Mycobacterium tuberculosis complex CRISPR genotyping: improving efficiency, throughput and discriminative power of 'spoligotyping' with new spacers and a microbead-based hybridization assay. *J Med Microbiol*. 2010;59(3):285–94. pmid:19959631

[11] Chakravorty S, Sen MK, Tyagi JS. Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology. *J Clin Microbiol*. 2005;43(9):4357–62. pmid:16145077

[12] Suzana S, Shanmugam S, Uma Devi KR, Swarna Latha PN, Michael JS. Spoligotyping of Mycobacterium tuberculosis isolates at a tertiary care hospital in India. *Trop Med Int Health*. 2017;22(6):703–7. pmid:28374900

[13] Mehta PK, Raj A, Singh N, Khuller GK. Diagnosis of extrapulmonary tuberculosis by PCR. *FEMS Immunol Med Microbiol*. 2012;66(1):20–36. pmid:22574812

[14] Sankar MM, Singh J, Diana SC, Singh S. Molecular characterization of Mycobacterium tuberculosis isolates from North Indian patients with extrapulmonary tuberculosis. *Tuberculosis (Edinb)*. 2013;93(1):75–83. pmid:23140853

[15] Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, et al. Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiol*. 2006;6:23. pmid:16519816

[16] Worku T, Mengistu Z, Semahegn A, Tesfaye G. Rehabilitation for cancer patients at Black Lion hospital, Addis Ababa, Ethiopia: a cross-sectional study. *BMC Palliat Care*. 2017;16(1):53. pmid:29145841

[17] Fantahun M, Kebede A, Yenew B, Gemechu T, Mamuye Y, Tadesse M, et al. Diagnostic accuracy of Xpert MTB/RIF assay and non-molecular methods for the diagnosis of tuberculosis lymphadenitis. *PLoS One*. 2019;14(9):e0222402. pmid:31525214

[18] Zhao P, Fang F, Yu Q, Guo J, Zhang JH, Qu J, et al. Evaluation of BACTEC MGIT 960 system for testing susceptibility of Mycobacterium tuberculosis to first-line drugs in China. PLoS One. 2014;9(9):e99659. pmid:25248118

[19] Owusu E, Newman MJ. Microscopic Observation Drug Susceptibility (MODS) Assay: A Convenient Method for Determining Antibiogram of Clinical Isolates of Mycobacterium tuberculosis in Ghana. *Med Sci (Basel)*. 2020;8(1). pmid:31991795

[20] Maningi NE, Malinga LA, Antiabong JF, Lekalakala RM, Mbelle NM. Comparison of line probe assay to BACTEC MGIT 960 system for susceptibility testing of first and second-line anti-tuberculosis drugs in a referral laboratory in South Africa. *BMC Infect Dis*. 2017;17(1):795. pmid:29282012

[21] Neonakis IK, Gitti Z, Petinaki E, Maraki S, Spandidos DA. Evaluation of the

GenoType MTBC assay for differentiating 120 clinical Mycobacterium tuberculosis complex isolates. *Eur J Clin Microbiol Infect Dis*. 2007;26(2):151–2. pmid:17256112

[22] Kamberek J, Schouls L, Kolk A, Van Agterveld M, Van Soolingen D, Kuijper S et al. Simultaneous Detection and Strain Differentiation of Mycobacterium tuberculosis for Diagnosis and Epidemiology. *Journal of Clinical Microbiology*. 1997;35(4):907–14. pmid:9157152.

[23] Kumar P, Balooni V, Sharma BK, Kapil V, Sachdeva KS, Singh S. High degree of multi-drug resistance and hetero-resistance in pulmonary TB patients from Punjab state of India. *Tuberculosis (Edinb)* 2014;94:73–80. [PubMed] [Google Scholar]

[24] Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME. Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *Am J Respir Crit Care Med.* 2008;177:787–92. [PubMed] [Google Scholar]