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COMPARISON OF FLUORESCENT STAIN WITH TRUENAT AND LINE PROBE ASSAY OF SPUTUM SAMPLE FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS AT A TERITIARY CARE HOSPITAL, WARANGAL.

Clinical Microbiology

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ABSTRACT

Introduction: Tuberculosis (TB) a highly infectious disease. India is one among the high burdened countries responsible for more than 80%in the world prevalence of tuberculosis. Tuberculosis persists to be a one of the notifiable diseases with high mortality and morbidity. The need for fast, precise diagnostic tests to identify active tuberculosis is essential, mainly in endemic nations such as India. An automated real-time Polymerase Chain Reaction (PCR) method for pulmonary and extrapulmonary tuberculosis (TB) detection known as TrueNat and Line probe assay shows great promise as a complement to conventional sputum microscopy techniques **Aim:** To compare sensitivity, specificity of fluorescent stain with Truenat and Line probe assay for detecting Mycobacterium tuberculosis and drug susceptibility pattern in sputum samples **Material and Methods:** A prospective comparative study on patients with suspected pulmonary TB was conducted from January 2023 to December 2023 for a period of 1 year in a tertiary care hospital at Kakatiya medical college Warangal along with DTC,Warangal, Telangana. The sensitivity, specificity, diagnostic accuracy for the diagnosis of tuberculosis were calculated for fluorescent stained smear microscopy, TrueNat, and Line probe assay and compared with each other. **Results:** In our study a total of 4000 pulmonary samples were collected with clinical signs and symptoms and all samples were subjected to fluorescent smear microscopy, Truenat and line probe assay. Out of 4000 sputum samples, 357(8.9%) were positive for fluorescent smear microscopy ,452 (11.3%) samples were positive for Truenat among them 431(95.3%) Rifampicin sensitive and 21(4.87%) Rifampicin resistant. In Line probe assay 452(11.3%) were positive for Mycobacterium tuberculosis, among them 431(95.3%) Rifampicin sensitive and 21(4.87%) Rifampicin resistant and 433(95.1%) isoniazid sensitive ,19(4.2%) isoniazid resistant. **Conclusion:** TrueNat can be essential in sample positive patients as a diagnostic modality, for early diagnosis and medication. It is less time consuming, but it can detect only rifampicin resistance. Genotype MTBDRplus LPA VER 2.0 is a rapid diagnostic method for TB detection in sputum specimen and promotes accurate diagnosis of both RMP and INH resistance, susceptibility to drugs within a short turnaround time (4 hours). It provides rapid information to opt for early treatment and contain disease spread.

KEYWORDS

Mycobacterium tuberculosis (MTB), Rifampicin (RMP), Isoniazid (INH), Line probe assay (LPA)

INTRODUCTION:

Tuberculosis (TB) a highly infectious disease. India is one (WHO, World Health Organization) among the high burdened countries responsible for more than 80%in the world prevalence of tuberculosis (16). Every year ten million people develop the disease, and 1.4 million TB patients die (WHO, 2020). Africa and Asia are the two regions with the largest burden of tuberculosis (TB) and affected for 95% of new TB cases, according to the World Health Organization (WHO, 2020). Because of the high rapid spread of pulmonary TB, which accounts for more than 80% of all TB infections, it is critical to diagnose and treat the disease as early as possible. Although pulmonary TB accounts accounts for the majority of cases, extrapulmonary tuberculosis (EPTB) also contributes significantly to the burden of disease (15). According to the WHO global tuberculosis report, EPTB represented 16% of the 7.1 million TB cases notified in 2019, ranging from 8% in the WHO Western Pacific Region to 24% in the Eastern Mediterranean Region. India is among the countries which has the highest percentage of extrapulmonary cases among TB cases (30%) and the rate of EPTB among all TB cases is increasing probably due to the rise of immunosuppressive populations (2,7). In the same year, 480,000 cases of multidrug-resistant TB (MDR-TB) and 100,000 cases of rifampicin monoresistant TB were reported (5). The emergence of MDR-TB defined as TB caused by strains resistant to at least two first-line drugs, INH and RIF; and extensively drug-resistant tuberculosis (XDR-TB) defined as TB caused by strains resistant to the two above-mentioned drugs; to at least one fluoroquinolone; and to at least one of three injectable second line drugs (amikacin, kanamycin and capreomycin) makes for a worrisome situation. The diagnosis of pulmonary tuberculosis should be suspected in patients with relevant clinical manifestations (cough >2 to 3 weeks duration, lymphadenopathy, fever, night sweats, weight loss) and relevant epidemiologic factors (history of prior TB infection or disease, known or possible TB exposure, and/or past or present residence in or travel to an area where TB is endemic (10). As per National Tuberculosis elimination Program (NTEP) or World Health Organization (WHO), an individual with at least one sputum smears positive for AFB or culture positive for tubercle bacilli is labelled to be suffering from pulmonary tuberculosis. Most of the TB cases are diagnosed based on sputum smear microscopy. Worldwide sputum smear microscopy is the most common method for diagnosing pulmonary TB (8). In a clinical set-up, the sensitivity of direct smear microscopy is low, while specificity is high. World Health Organisation (WHO) endorses liquid culture system and molecular line probe as the current gold standard for rapid detection of Multidrug Resistance (MDR) TB (3). But growth takes about two to six weeks to yield results and requires a highly specialised, controlled laboratory set-up and highly trained people. Microscopy and culture are notably less sensitive than automated methods and molecular markers like PCR or real-time PCR. For the rapid identification of Mycobacterium tuberculosis in clinical specimens of pulmonary and extrapulmonary TB cases, some Nucleic Acid Amplification (NAA) methods have been developed (9). Though widely distributed in the Indian health care system, the performance of these two assays have not been evaluated to determine their performance in the detection of TB and associated drug resistance. The standard Drug susceptibility testing (DST) test is a twostep procedure involving culture and sensitivity testing. This takes a long time, first to isolate a culture and then to perform drug susceptibility testing (indirect DST). If DST could be set up at the same time as when a processed specimen is inoculated in solid and or liquid medium (direct DST), it could save significant time for the detection of drug resistance (11). In this study we accessed the sensitivity and specificity of fluorescent staining,TrueNat and Line probe assay in sputum samples for diagnosing pulmonary tuberculosis.

MATERIALS AND METHODS:

It was a prospective comparative study done for a period of 1 year from January 2023 to December 2023 at Kakatiya medical college, Warangal, Telangana.

Inclusion criteria: All the patients with informed consent who were referred to the DTC, TB & Chest Hospital and Department of Microbiology for a Mycobacteriology study by fluorescent staining and molecular detection of pulmonary TB were included in this study.

Exclusion criteria:

1) Patient who didn't give consent were excluded in this study
2) Samples other than sputum samples, macroscopically resembling saliva were excluded.
3) Samples that were obtained without a clinical history were excluded.
4) A patient who has a history of a fungal infection or lung disease.

Specimen collection, processing and transportation:

For initial diagnosis of pulmonary tuberculosis, collect a series of two sputum specimens 8-24 hours apart, at least one of which is an early morning specimen. Optimally, specimens are collected before drug therapy is started as even a few days of treatment may inhibit growth and prevent isolation of M. tuberculosis complex (MTBC) (17). Decontamination of all the specimens was done by the 5.0mL of NaCl solution (N-acetyl L-cysteine), sodium hydroxide method (NaOH-NALC). After holding (15 min) at RT (Room Temperature), the specimens were neutralized with phosphate buffer saline (PBS) at pH 6 and placed in a cold centrifuged for 20 min at 10°C at 4500 rpm. Sterile phosphate buffer (1.5 ml) was used to suspend the pellets and collected for further analysis (12). In the case of a delay, they were kept at 4°C for no longer than 24 hours before even being processed immediately. All samples were handled in a class II A2 biosafety cabinet (1).

Fluorescent staining:

Fluorescence Microscopy Staining Procedure. The smears were flooded with filtered 0.1% auramine for at least 20 minutes. They were then rinsed with water and drained. Acid alcohol decolorizing solution (0.5%) was applied on the smear for 30 to 60 seconds, rinsed with water, and drained. They were then flooded with 0.5% potassium permanganate counterstain for a maximum of 1 minute and rinsed with water. The smears were allowed to air dry and examined microscopically using the dry (40x) objective lens of an LED illumination-based fluorescence microscope Figure.1.

TrueNat MTB testing:

DNA extraction was done using Trueprep-MAG kit instructions. According to the manufacturer's instructions, fresh specimens (sputum) from untreated individuals were processed, with a starting volume of 500 µL added to the sample pretreatment tube. The Trueprep-MAG Sputum and TrueNAT Mycobacterium tuberculosis kits contain only proprietary master mixes for PCR as well as all buffers and reagents essential for nucleic acid extraction (13).

Real-time PCR based on chip:

A preprogrammed profile on the TrueNat Mycobacterium tuberculosis microchip was used to conduct real-time PCR with 5 µL of extracted DNA and lyophilised master mix. The screen displayed the results. Unique primers and a Mycobacterium tuberculosis-specific probe were included in the lyophilised master mix (13) and Figure 2.

Line probe assay (LPA):

It is based on strip technology was used to diagnose TB and detect RIF as well as Isoniazid (INH) resistance due to mutations in rpoβ, and both inhA and katG genes. The test was performed according to the manufacturer's protocol (Hain Life Science GmbH, Nehren, Germany). The method involved three processes: DNA extraction, multiplex PCR amplification, and reverse hybridization. The DNA strip was removed from the tube and marked as per the number of samples. It was then added to each well containing 20 µl of corresponding amplified DNA sample with coloured part facing up. The well was placed in the twincubator and hybridization procedure was initiated. Hybridization occurred by pre-warming the hybridization buffer to 45 °C in water bath for 15 min in the twincubator machine (Hain Life Science GmbH, Nehren, Germany). Denaturing solution, 20 µl was pipetted to each of the tray that was used, and then 1 ml of rinse solution added per well and incubated for 1 min. The well was removed and rinsed with a rinse solution. One millilitre of the conjugate was added into each well, then incubated for 30 min, removed and washed with rinse solution. Finally, 1 ml of the substrate was added into the well and incubated for 10 min and then washed twice with distilled water. The strips were then left to dry and results scanned and interpreted by the Hain Life Science GmbH, Nehren, Germany machine as either Mycobacterium tuberculosis detected, resistant, sensitive or invalid (20) and Figure 3

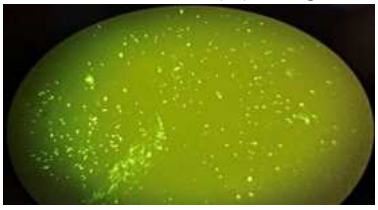

Figure.1 Auramine O Staining of sputum sample of Pulmonary tuberculosis.

Figure.2 Truenat Print Report of sputum sample of Pulmonary tuberculosis.

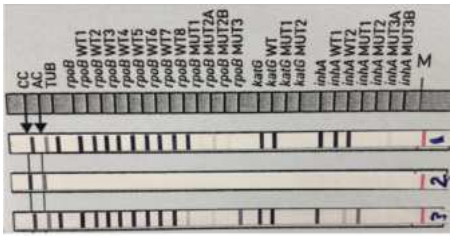

Figure.3 Line probe assay strip showing sensitivity and resistant pattern of Rifampicin and Isoniazid

Strip 1 showing valid positive Mycobacterium Tuberculosis complex and sensitivity pattern for Rifampicin and Isoniazid
Strip 2 showing Valid but negative for Mycobacterium Tuberculosis complex result
Strip 3 showing valid positive Mycobacterium Tuberculosis complex and resistant pattern for Rifampicin and Isoniazid

RESULT:

In our study a total of 4000 pulmonary samples were collected with clinical signs and symptoms and all samples were subjected to Fluorescent smear microscopy, Truenat and line probe assay. Out of 4000 sputum samples, 357(8.9%) were positive for Fluorescent smear microscopy ,452 (11.3%) samples were positive for Truenat among them 431(95.3%) Rifampicin sensitive and 21(4.87%) Rifampicin resistant. In Line probe assay 452(11.3%) were positive for Mycobacterium tuberculosis, among them 431(95.3%) Rifampicin sensitive and 21(4.87%) Rifampicin resistant and 433(95.1%) isoniazid sensitive ,19(4.2%) isoniazid resistant **Table.1-2**.

Table.1 Comparison of results of Fluorescent staining, Truenat and Line probe assay

Total number of samples	Fluorescent staining		Truenat		Line probe assay	
	Positive	Negative	Positive	Negative	Positive	Negative
4000	357	3643	452	3548	452	3548
Percentage	8.9 %	91 %	11.3%	88.7%	11.3 %	88.7%

Table.2 Comparison of Drug Susceptibility pattern in Truenat and Line probe assay

Total number of positive samples	Truenat		Line probe assay			
	Rifampicin		Rifampicin		Isoniazid	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
452	431	21	431	21	433	19
Percentage	95.3%	4.87%	95.3%	4.87%	95.1%	4.2%

DISCUSSION:

Appliance of molecular methods in routine for diagnosing in developing country like India depends on diverse factors like high cost, rapid and accessibility of skilled personnel to perform the test. Although economical and extensively available, microscopy was nonspecific in our study population and less sensitive than Nucleic acid amplification Test (NAAT) and thus not satisfactory for rapid diagnosis. In the current study, the diagnostic accuracy of Fluorescent smear staining to detect Mycobacterium tuberculosis in pulmonary specimens was evaluated and compared with TrueNat and Line probe assay. The drug susceptibility of Rifampicin was done by using Truenat and 1st Line drug susceptibility testing, Rifampicin and Isoniazid was done by using Line probe assay.

Fluorescent staining: Fluorescent stain is superior to that of Ziehl-Neelsen's stain in the presence of a low bacterial load as seen in smears with diagnostic cytomorphological featured tuberculosis, in problem areas like AIE (acute inflammatory exudates) alone or with occasional granuloma. Using fluorescent staining, the tubercle bacilli when examined under ultraviolet illumination, the bacilli appeared as a bright rod against a dark background. Since there was a contrast, the bacilli were readily seen, and therefore in very less time, a large area could be examined. Images were then captured with the digital camera and enhance through imaging processing techniques. The fact that fluorescent stain has difficulty in detecting paucibacillary can be attributed to the fact that only a loop-full of sputum sample was taken to make the smear slides. In present study 357(8.9%) were smear positive and in a study done by Padmaja GV et al the smear positive rate was 77 (40.9%) out of 188 samples (10). In contrast a study done by Isaac Dadzie et al out of 200 samples analyzed 71 (35.5%) was yielded under fluorescent smear microscopy (18). Therefore, the introduction of LED-FM would be time-saving and allow for quality microscopy.

TrueNat: Compared to the above techniques, TrueNat has the advantages of less turn-around time (two hours) for detecting drug resistant TB, high sensitivity of detection of TB with simultaneous assessment of rifampicin resistance and thus has potential to replace the gold standard culture method. In present study detection rate of Mycobacterium tuberculosis bacilli is 452 (11.3%) and among them 431(95.3%) Rifampicin sensitive and 21(4.87%) Rifampicin resistant. In a study done by Penn-Nicholson A, Gomathi SN, Ugarte-Gil C et al, out of 1807 enrolled participants with TB signs/symptoms, 24% were culture-positive for Mycobacterium tuberculosis, of which 15% were RIF-resistant (6). In terms of patient-important outcomes, quicker turnaround from testing to treatment can be expected when testing is conducted at primary healthcare centres. Overall, the Truenat assays have been estimated to be cost-effective in India compared with microscopy and Xpert (14).

Line probe assay: Molecular diagnostic tools for the diagnosis of MDR-TB effectively address the issue of the long turnaround time associated with culture and sensitivity testing though high cost has hampered wide applicability of these tests. With the introduction of LPA for the rapid diagnosis of drug-resistant TB, there has been a significant reduction in time to initiation of treatment in MDR suspect cases. In Line probe assay 452(11.3%) were positive for Mycobacterium tuberculosis, among them 431(95.3%) Rifampicin sensitive and 21(4.87%) Rifampicin resistant and 433(95.1%) isoniazid sensitive ,19(4.2%) isoniazid resistant. In a study done by Prabha Desikan et al, among 1217 samples positive, of which 232 (19.1%) were MDR, 130 (10.6%) were rifampicin monoresistant and 101 (8.3%) were INH monoresistant. 754(61.9%) strains were found to be pansensitive (19). Another study done by S. Jayanthi et al showed 216 (51%) Genotype MTBDRplus LPA (v2.0), detected 7 (3.2%) MDR, 27 (13%) resistant to RIF and 30 (14%) resistant to INH, 189 (88%) were susceptible to both INH and RIF (4).

CONCLUSION:

TrueNat is more sensitive and specific in detection of pulmonary TB when compared to Fluorescent smear microscopy. An additional feature of rifampicin resistance can also be detected by TrueNat. Routine investigation with TrueNat will detect early pulmonary TB as it can detect paucibacillary cases. It provides rapid information to opt for early treatment and contain disease spread as Turn around time is 2 hours .Line probe assay can be used for early and prompt detection of Mycobacterium tuberculosis along with Sensitivity pattern of Rifampicin and Isoniazid and to detect MDR stains . By using molecular methods accurate, early diagnosis can be made which prevents false negative cases by microscopy, transmission of disease and decrease in incidence and subsequent thorough control and eradication of disease.

Abbreviations:

MTB;Mycobacterium tuberculosis, LPA-Line probe assay, Rif-Rifampicin, Inh-Isoniazid, RTPCR-Reverse transcription polymerase chain reaction

Ethical approval and consent to participate:

The ethical approval for this study was taken from Kakatiya Institutional Ethics Committee, No. ECR/840/Inst/TG/2016/RR/20/61, Kakatiya medical college before sample collection and processing. Consent was obtained from participants.

Authors' contributions:

1.Goteti Venkata Padmaja: Concepts, Design, Statistical analysis, Manuscript preparation Manuscript editing and review, Data analysis.
2. Golla Eshwara Chandra: Concepts, Design, Literature search, Sample collection, Data acquisition, Statistical analysis, Manuscript preparation.
3.Chilakalapalli Ramya Harshita: Definition of intellectual content, Literature search, Data analysis, Manuscript editing and review, Clinical studies.
4. Hima Bindhu Kodida : Data acquisition , Data analysis and review

Conflict of interest: Nil

REFERENCES

1. Mtafya B, Qwaray P, John J, Sichone E, Shoo A, Gillespie SH, Ntinginya NE, Sabiiti W. A practical approach to render tuberculosis samples safe for application of tuberculosis molecular bacterial load assay in clinical settings without a biosafety level 3 laboratory. Tuberculosis (Edinb). 2023 Jan; 138:102275. doi: 10.1016/j.tube.2022.102275. Epub 2022 Nov 15. Erratum in: Tuberculosis (Edinb). 2023 Feb 15:102321. PMID: 36434867.
2. World Health Organization. Global tuberculosis report 2020. https://www.who.int/publications/i/ item/9789240013131 (accessed May 2022).
3. Zijenah LS. The World Health Organization Recommended TB Diagnostic Tools. In: Kayembe, J. N., editor. Tuberculosis [Internet]. London: IntechOpen; 2018 [cited 2022 Sep 04]. Available from: https://www.intechopen.com/ chapters/58595. Doi: 10.5772/intechopen.73070
4. Vijay DD, Meharaj SHS, Jayanthi S, et al. A Comparison Study of CBNAAT, Gene Xpert and Line Probe Assays in the Diagnosis of Tuberculosis in smear Negative Specimens. J Pure Appl Microbiol. 2022;16(3):1953-1963. doi: 10.22207/JPAM.16.3.42
5. Singh AV, Singh S, Yadav A, et al. Genetic variability in multidrug-resistant Mycobacterium tuberculosis isolates from patients with pulmonary tuberculosis in North India. BMC Microbiol. 2021;21(1):123. doi: 10.1186/s12866-021-02174-6
6. Penn-Nicholson A, Gomathi SN, Ugarte-Gil C, et al. A prospective multicentre diagnostic accuracy study for the Truenat tuberculosis assays. Eur Respir J 2021; 58: 2100526 [DOI: 10.1183/13993003.00526-2021].
7. Natali D, Cloatre G, Brosset C, et al. What pulmonologists need to know about extrapulmonary tuberculosis. Breathe (Sheff) 2020; 16: 200216
8. Pathrikar TG, Bansal VP, Mulay MV, Ghogare HS. Comparison of Ziehl Neelsen smear microscopy and AFB culture in a resource limited setting from various clinical samples. Int J Health Sci Res. 2020;10(4):46-51
9. Umair M, Siddiqui SA, Farooq MA. Diagnostic accuracy of sputum microscopy in comparison with GeneXpert in pulmonary tuberculosis. Cureus. 2020;12(11):e11383. Doi: 10.7759/cureus.11383. PMID: 33312784; PMCID: PMC7725198
10. Padmaja GV, Srujana K, Sadhana C. Comparison of Ziehl-Neelsen's stain, fluorescent stain with CBNAAT of sputum for the diagnosis of pulmonary tuberculosis. J NTR Univ Health Sci 2019; 8:238-43
11. Aricha et al. BMC Infectious Diseases (2019) 19:852, Comparison of GeneXpert and line probe assay for detection of Mycobacterium tuberculosis and rifampicin-mono resistance at the National Tuberculosis Reference Laboratory, Kenya https://doi.org/10.1186/s12879-019-4470-9.
12. Kazemian H, K-Y J, Bahador A, et al. Efficacy of Line Probe Assay In Detection Of Drug-Resistant Pulmonary Tuberculosis In Comparison With GeneXpert And Phenotypic Methods In Iran And Genetic Analysis Of Isolates By MIRU-VNTR. Infect Drug Resist. 2019; 12:3585-3593. doi: 10.2147/IDR.S222905
13. Dr. Vijayalakshmi, J., Dr. A. Surekha, Dr. A. Renuka devi and Dr. Uma devi, S. 2019. Truenat - A Novel Diagnostic Tool for Rapid Detection of Mycobacterium Tuberculosis and Rifampicin Resistance in Pulmonary Samples. Int.J.Curr.Microbiol.App.Sci. 8(10): xx-xx. doi: https://doi.org/10.20546/ijcmas.2019.810.xx
14. Lee DJ, Kumarasamy N, Resch SC, et al. Rapid, point-of-care diagnosis of tuberculosis with novel Truenat assay: cost-effectiveness analysis for India's public sector. PLoS One 2019; 14: e0218890
15. Ayed HB, Koubaa M, Marrakchi C, et al. Extrapulmonary tuberculosis: update on the epidemiology, risk factors and prevention strategies. Int J Trop Dis 2018; 1: 1-6
16. Dean T. Jamison, Hellen Gelband, Susan Horton, Prabhat Jha, Ramanan Laxminarayan Charles N. Mock, Major Infectious Diseases, Disease Control Priorities, Third Edition. 2017;6. doi: 10.1596/978-1-4648-0524-0
17. Kasper DL, Fauci AS, Hauser S, Longo D, Jameson JL, Loscalzo J. Harrison's Principles of Internal Medicine 19/E (Vol. 1 and Vol. 2). McGraw-Hill Education; 2015
18. Diagnostic Yield of Fluorescence and Ziehl-Neelsen Staining Techniques in the Diagnosis of Pulmonary Tuberculosis: A Comparative Study in a District Health Facility, University of Cape Coast, Cape Coast, Ghana
19. Prabha Desikan1, Nikita Panwalkar, Line probe assay for detection of Mycobacterium tuberculosis complex: An experience from Central India DOI: 10.4103/ijmr.IJMR_831_14.
20. https:/Hain Lifescience | Company Profile (hain-lifescience.de) /en/company/company-profile.html and https://IFU | Instructions for use from Hain Lifescience (hain-lifescience.de) /en/instructions-for-use.html