Diagnostic Utility of Fluorescent Microscopy vis-a-vis GeneXpert MTB/RIF in Extra Pulmonary Tuberculosis: A Retrospective Analysis

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ABSTRACT

Introduction: Tuberculosis (TB) kills more people in India and Southeast Asia than any other infectious disease. In developing countries like India, the percentage of Extra Pulmonary Tuberculosis (EPTB) is between 15-20% which has increased to more than 50% among HIV co-infected patients.

Aim: To evaluate the frequency of EPTB and diagnostic efficacy of GeneXpert MTB/RIF in comparison to fluorescent microscopy.

Materials and Methods: A retrospective analysis of EPTB cases was done during April 2018-January 2019 and a total of 239 cases were enrolled. Pus, CSF, Pleural fluid, Ascitic fluid was screened for TB by GeneXpert MTB/RIF and fluorescent microscopy and data was statistically analysed.

Results: Out of the 239 EPTB cases, 101 were pus, 16 were CSF, 105 were pleural fluid and 17 were ascitic fluid. Overall, 14.64% (35/239) cases were found positive for TB by GeneXpert MTB/RIF while 20% (7/35) cases were found to be RIF resistant. Among 35, 23.7% (24/101) pus cases were positive while 10.4% (11/105) pleural fluid cases were found positive for TB nucleic acid. None of CSF and Ascitic fluid sample were found positive for TB. Among these 35 GeneXpert cases 16 cases, were confirmed positive by fluorescent microscopy. Considering GeneXpert as a standard, fluorescent microscopy was found to be 45.7% (95% CI, 28.83% to 63.35%) sensitive and 100% specific (98.21% to 100.00%).

Conclusion: Present study showed 14.64% (35/239) cases positive for TB bacilli in EPTB while 20% (7/35) were found to be resistant for RIF. Lower sensitivity of microscopy may be intended to paucity of bacilli in these extra pulmonary sites.

Keywords: *Mycobacterium tuberculosis*, Sensitivity, Specificity

INTRODUCTION

Predominantly, *Mycobacterium tuberculosis* (MTB) affects the lungs and causes Pulmonary Tuberculosis (PTB). PTB constitutes 85% of majority of estimated number of incident TB cases [1] EPTB may be due to spread of bacterium to the different sites of the body such as gastrointestinal, pleural cavity, nervous system, joints etc., [2].

Tuberculosis (TB) kills more people in India and Southeast Asia than any other infectious disease. India alone constitute for approximately 23% of the global TB burden [3]. The percentage of EPTB cases varies from 15-20% to more than 50% among HIV co-infected cases in developing countries like India [4]. EPTB is a diagnostic and therapeutic challenge which usually occurs in opportunistic infection like AIDS and immuno-compromised conditions as Diabetes Mellitus and malnutrition [5]. According to a report in 2017, estimated incidence of TB in India was approximately 28,00,000 which constitutes one-fourth of world's TB cases. Global TB Report 2018 states that in 2017, 10 million people had illness with TB, and 1.6 million lost their lives. WHO states that there was resistance to rifampicin in 558,000 new cases, rifampicin is the most effective first-line drug, out of these 82% had MDR-TB [6]. TB is a leading cause of death of HIV-infected individuals. In 2017, 1 million children had illness with TB and 230,000 children died of TB. State wise TB case notification 2017, in Rajasthan 16% cases were EPTB out of all TB reported cases [7]. The estimated incidence of TB in India was 2.1 million cases in 2013, out of that 16 % constituted new EPTB cases, equating to 336,000 people with EPTB [5,8].

Ongoing research, diagnosis and treatment aim pulmonary TB as PTB is most occurring disease from this bacterium. However, EPTB have great impact on society in terms of disease burden and economy. Diagnostic delay can cause harm, however timely diagnosis and treatment may cure majority of the EPTB cases [5,9].

Due to paucibacillary nature, EPTB have low infectious potential. Further manifestation due to EPTB mimic other disease pathology which creates more diagnostic challenges leading to serious disease sequlae [10,11]. Compared to PTB, EPTB deserves an increasing focus for proper management of TB cases and TB Free India.

Microscopy with Ziehl-Nielsen (ZN) staining is the most common and often the only laboratory technique used to diagnose TB in most developing countries. Currently, the gold standard of diagnosing TB is culture. However, the use of this method is limited due to lack of trained staff, biosafety requirements and long turnaround time, further culture is considered to be less sensitive for EPTB [12-15]. Fluorescence microscopy yields a 10% higher sensitivity than Ziehl-Neelsen staining. Further, fluorescence microscopy requires less time than the Ziehl-Neelsen method. It requires only one or two sputum specimens for processing, hence fluorescence microscopy is less time consuming and has improved diagnostic method [16,17].

WHO approved Xpert MTB/RIF to test sputum samples from patients with active pulmonary TB and had been shown to have high accuracy for diagnosing TB in these patients [18]. Several investigators have tested the diagnostic test accuracy of Xpert MTB/RIF in non-respiratory specimens for the diagnosis of various forms of EPTB and have shown that accuracy varies considerably with specimen type and bacillary load [19].

There is paucity of data on the aspect of diagnostic comparison of GeneXpert MTB/RIF and fluorescent microscopy in EPTB. Hence we aim to evaluate the frequency of EPTB by GeneXpert MTB/RIF and fluorescent microscopy in tertiary care hospital in northern India and to estimate the efficacy of fluorescent microscopy in

comparison to GeneXpert MTB/RIF for the diagnosis of EPTB. We further aim to evaluate the post-test probability of developing EPTB by fluorescent microscopy and GeneXpert MTB/RIF respectively. Since data is limited from our region, present study will demonstrate the diagnostic performance and comparison between molecular versus fluorescent microscopy in EPTB cases.

MATERIALS AND METHODS

Patients

A retrospective analysis of EPTB cases between April 2018-January 2019. The authors have applied for Institutional Ethical clearance for the present study which is yet to be conducted. However editors judged the present study to be exhibiting lower than minimal risk, thus proceeded further for publication. In the absence of any previous data on EPTB from the region, we arbitrarily enrolled total 239 cases of EPTB who were presented to various OPD of New Hospital Medical College, Kota, Rajasthan, India (A tertiary care hospital). The Ethical Clearance No. for the present study is F3/Acad-2/2019/1706. 101 Pus, 105 Pleural fluid, 17 Ascitic fluid, 16 CSF were subjected for diagnosis of TB by GeneXpert MTB/RIF and fluorescent microscopy. Data related to these patients were obtained from Revised National TB Control Programme RNTCP register of DOT center.

Inclusion Criteria

Cases of all age who presented with signs and symptoms of presumptive EPTB (chronic lymphadenitis and body fluid accumulations like meningitis, pleural effusion, ascites).

Exclusion Criteria

Cases that were critically ill, patients who had active pulmonary and EPTB patients who were on anti-TB treatment.

Processing of Samples for Fluorescent Microscopy

Specimens were transported and stored at 2 to 8°C prior to processing (a maximum of 7 days). All the samples were processed for auramine staining according to protocol [20]. Auramine stained smear was studied under a fluorescent microscope. Samples were considered positive when there was presence of at least 2-3 Acid Fast Bacilli (AFB)/10 fields [21]. Grading of samples was further done according to RNTCP guideline.

Xpert MTB/RIF Assay

The Xpert MTB/RIF assay was performed according to the manufacturer's instructions (Cepheid, Sunnyvale, CA, USA). For Xpert MTB/RIF assay if sample was collected 1-5 ml, an equal volume of the sample was added to sample reagent and this mixture was directly added to the Xpert MTB/RIF cartridge. In case of sample volume less than 1 mL, sample was resuspended to final volume of 2 mL with Xpert MTB/RIF sample reagent. The mixture was further vortexed twice for 15 min at room temperature. 2 mL of the mixture was loaded into the test cartridge and then installed into the GeneXpert instrument [22, 23].

STATISTICAL ANALYSIS

Statistical analysis was performed with GraphPad Prism software version 5.0 (GraphPad Software, Inc., San Diego, USA). Sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were determined to evaluate the diagnostic efficacy. Mean and standard deviations were calculated by the numerical data using Column statistics. The degree of agreement was assessed by Kappa statistics [24]. The Kappa values ranging from 0.01-0.20 were considered as slight agreement, from 0.21-0.40 as fair agreement, from 0.41-0.60 as moderate agreement, from 0.61-0.80 as substantial agreement and from 0.81-0.99 as almost perfect agreement. Online tool

were used http://araw.mede.uic.edu/cgi-bin/testcalc.pl for construction of "Nomograph" [25].

RESULTS

A total of 239 patients with age range from 0 to 81 years from Hadoti region were recruited. Male: Female ratio of TB positive patients was 15:20 [Table/Fig-1].

Age and sex distribution	Number of patients
Age (years)	
0-1 year	02 (0.83%)
1-5 years	09 (3.7%)
5-18 years	26 (10.87%)
Above 18	202 (84.5%)
Sex	
Male: Female	129: 110

[Table/Fig-1]: Age and sex distribution.

Overall, 14.64% (35/239) cases were found positive for TB by GeneXpert MTB/RIF. Fluorescent microscopy was able to detect 6.69% (16/239) cases of TB. Among these GeneXpert MTB/RIF positive cases, 20% (7/35) cases were found to be RIF resistant. None of ascitic fluid or CSF sample was found positive for TB nucleic acid [Table/Fig-2].

Samples	Total numbers (n-239)	Number of TB positive cases by Gene Xpert MTB/RIF	Number of TB positive cases by Microscopy	Number of RIF resistant cases
Pus	101 (42.2%)	24 (23.7%)	09/101 (8.9%) 9/24 (37.5%)*	4/101 (3.9%) 4/24 (16.6%)
Pleural fluid	105 (43.9%)	11 (10.4%)	07/105 (6.6%) 7/11 (63.6%)*	3/105 (2.8%) 3/11 (27.1%)
Ascitic fluid	17 (7.1%)	0	0	0
CSF	16 (6.6%)	0	0	0
Total	239	35 (14.64%)	16/239 (6.69%) 16/35 (45.7%)*	7/35 (20%)

[Table/Fig-2]: Frequency of Tuberculosis.
CSF: Cerebrospinal fluid; Percentages has been showed in parenthesis; *Percentage of positivity in comparison to positive cases by GeneXpert MTB/RIF

Sixteen cases were found to be true positive by fluorescent microscopy in concordance with GeneXpert MTB/RIF while false negative cases were 19. Microscopy was 45.71% sensitive, 100% specific and PPV and NPV was 100%, 91.48% respectively. 14.6% Disease prevalence was calculated by this method [Table/Fig-3].

| Method	Sensitivity	Specificity	PPV	NPV	Disease prevalence
Microscopy vs GeneXpert (n-279)	45.71% (95% CI, 28.83% to 63.35%)	100% (95% CI, 98.21% to 100%)	100.00%	91.48% (95% CI, 88.79% to 93.57%)	14.6% (95% CI, 10.42% to 19.77%)
Microscopy vs GeneXpert for Pus (n-101)	37.5% (95% CI, 18.80% to 59.41%)	100% (95% CI, 95.3% to 100%)	100.00%	83.7% (95% CI, 79.16% to 87.18%)	23.76% (95% CI, 15.81% to 33.26%)
Microscopy vs GeneXpert For pleural fluid (n-105)	63.64% (95% CI, 30.79% to 89.7%)	100% (95% CI, 96.5% to 100%)	100.00%	95.92% (95% CI, 91.41% to 98.09%)	10.48% (95% CI, 5.3% to 17.9%)

[Table/Fig-3]: Diagnostic accuracy of Microscopy vs GeneXpert MTB/RIF.

A 'moderate agreement' (Kappa coefficient r-0.590, SE of Kappa-0.082) between fluorescent microscopy and GeneXpert MTB/RIF was observed. Kappa coefficient was further calculated for pus and pleural fluid, it was observed that pus had 'moderate agreement' while pleural fluid had 'good agreement' with GeneXpert MTB/RIF [Table/Fig-4]. For the prediction of post-test probability, A

nomograph was created which can determine diagnostic test characteristics (sensitivity, specificity, likelihood ratios) and/or determine the post-test probability of developing infection. It was observed that spectrum of post test probability of developing disease by fluroscent microscopy is narrow [Table/Fig-5] in comparison to GeneXpert MTB/RIF assay. 'Nomograph' constructed for pus and pleural fluid revealed that post-test probability of developing disease symptoms was much higher in pleural fluid in comparison to pus [Table/Fig-6].

Method	Kappa coefficient (r)	SE of kappa	95% CI	Strength of agreement
Microscopy vs GeneXpert	0.590	0.082	0.428 to 0.751	'moderate'
Microscopy vs GeneXpert for pus (n-101)	0.478	0.106	0.270 to 0.686	'moderate'
Microscopy vs GeneXpert for pleural fluid (n-105)	0.758	0.115	0.532 to 0.984	'good'

[Table/Fig-4]: Diagnostic agreement between Microscopy vs GeneXpert MTB/RIF. The Kappa values ranging from 0.01 to 0.20 were considered as slight agreement, from 0.21 to 0.40 as fair agreement, from 0.41 to 0.60 as moderate agreement, from 0.61 to 0.80 as substantial agreement and from 0.81 to 0.99 as almost perfect agreement
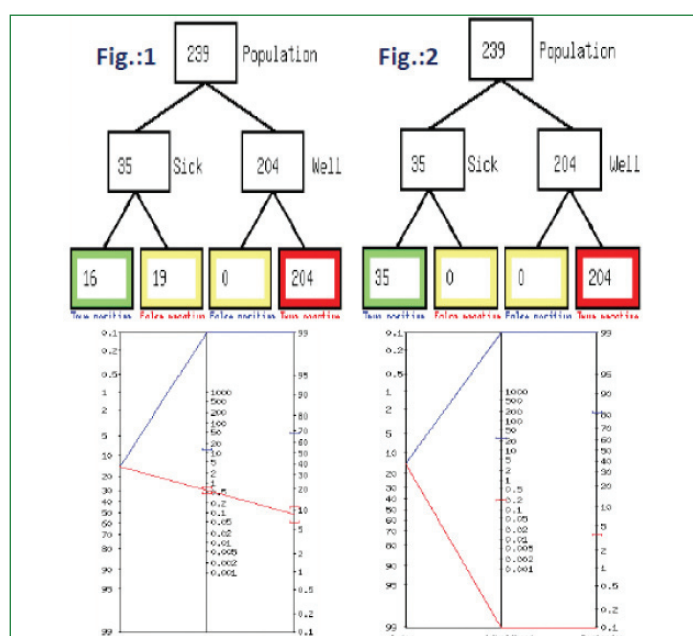
[Table/Fig-5]: 'Nomograph' constructed for fluorescent microscopy in comparison to GeneXpert MTB/RIF assay.
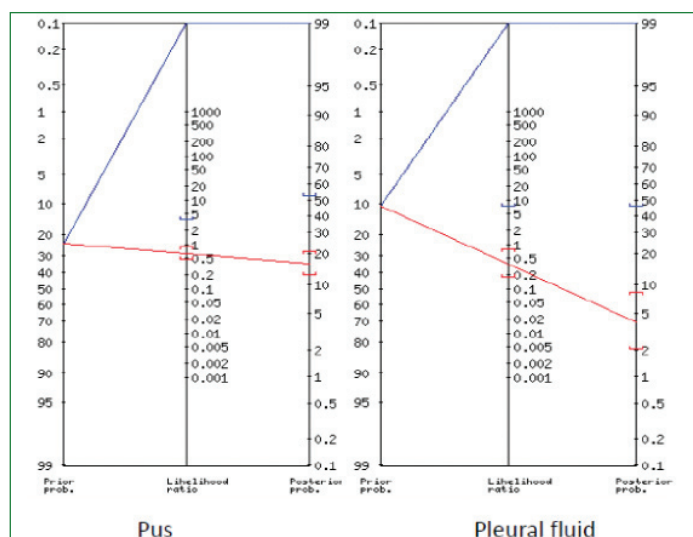
[Table/Fig-6]: 'Nomograph' constructed for pus and pleural fluid revealed that post-test probability of developing disease symptom is higher in pleural fluid in comparison to pus.

DISCUSSION

The public health emphasis on infectious PTB is central to the health of the Indian people [5]. Nevertheless, EPTB remains extremely common and is probably under recognised and untreated or overtreated [9]. There are no specific tools or technique to diagnose EPTB even culture have low sensitivity [14,15], hence various methods are adopted for sample processing for accurate diagnosis by Xpert MTB/RIF as well as microscopy.

In present study, our prevalence data of EPTB was in concordance to the other previous national and international data [5,7]. Fluorescent microscopy in this study demonstrated 45.7% sensitivity in comparison to Xpert MTB/RIF assay however it showed 100% specificity, showing none of the false positive case was detected by fluorescent microscopy. Sensitivity was increased up to 63.6% in pleural fluid samples in comparison to Xpert MTB/RIF. This finding may be due to high occurrence of pleural fluid cavity TB in this region as the high incidences of pleural cavity TB have also been found in Indian studies [26]. However, Lymphatics and lymph node TB is the most common form of EPTB [26]. This suggest, fluorescent microscopy cannot be overlooked as a diagnostic tool for EPTB in resource limited settings where Xpert MTB/RIF is not available further sensitivity and specificity of microscopy may be enhanced by experienced observers. This is to state that present study had concordance with a recent study conducted in Pakistan in terms of sensitivity and specificity [27]. Khan AS et al., demonstrated that fluorescent microscopy has 40% of sensitivity and 100% of specificity in comparison to Xpert MTB/RIF. Performance of fluorescent microscopy and ZN stain has been tabulated and it showed sensitivity and specificity of microscopy between 22-41% [Table/Fig-7] [27-31]. As suggested by others, we also observed that careful processing of clinical specimens may enhance rate of detection in EPTB cases [16, 32, 33].

Present study demonstrated comparable results of fluorescent microscopy versus Xpert MTB/RIF. Results demonstrated Xpert MTB/RIF is more sensitive than fluorescent microscopy however fluorescent microscopy showed diagnostic 'moderate agreement' with Xpert MTB/RIF. To further understand the diagnostic performance, we constructed a mathematical model called 'Nomograph' which revealed that post-test probability of developing EPTB symptoms were higher when EPTB samples were diagnosed by Xpert MTB/RIF in comparison to fluorescent microscopy. These analyses were further done at pus and pleural fluid sample level and it was found that pleural fluid has greater chance of developing disease symptoms in comparison to pus. Fluorescent microscopy of pleural fluid showed 'good diagnostic agreement' with XpertMTB/RIF in comparison to pus.

It is well established that EPTB cases are paucibacillary and numbers of bacteria are very low in all positive case [19]. In present study, as expected, most of the EPTB cases were very low in bacterial load by Xpert MTB/RIF as well as by fluorescent microscopy [Table/Fig-8]. This is worthwhile to discuss here that there are several reasons why the Xpert MTB/RIF may perform differently with EPTB samples. Essentially, Xpert MTB/RIF has a specimen treatment step specifically designed to liquefy sputum but this may not be suitable for pre-test processing for EPTB samples [34]. Although this test has a limit of detection of 131 colony forming units per mL, it has been shown to underperform in paucibacillary disease; as many forms of EPTB require invasive sampling methods, the size and quality of the specimens may affect the sensitivity of the test [19]. In 2016, a new version of Xpert MTB/RIF, Xpert MTB/RIF Ultra, has been introduced with a lower limit of detection for paucibacillary EPTB cases [5].

Study reference	Area	Methods	Samples	Sensitivity of microscopy	Specificity of microscopy
Bagdia M et al., [28]	Nagpur, India	NAAT, cytology, LED fluorescent, ZN Stain, culture	Extra pulmonary	Not specified	98%
Khan AS et al, [27]	Pakistan	LED fluorescence microscopy vs GeneXpert	Extra pulmonary	40%	100%
Agrawal M et al., [29]	New Delhi, India	ZN stain, culture, GeneXpert (Xpert(®) MTB/RIF assay)	Pulmonary	22.2%	100 %
Kanwal FK et al., [30]	Pakistan	GeneXpert (Xpert(®) MTB/RIF vs ZN stain	Extra pulmonary	39.53%	100%
Barnard DA et al., [31]	South Africa	ZN stain, culture, GeneXpert (Xpert(®) MTB/RIF assay)	Pulmonary	41%	98.6%
Present study	Kota, Rajasthan, India	Fluorescence microscopy vs GeneXpert	Extra pulmonary	45.7%	100%

[Table/Fig-7]: Various diagnostic studies in pulmonary/extra pulmonary tuberculosis [27-31].

Bacterial density	
Bacterial load	(n-35)
By GeneXpert	
Very low	13 (37.1%)
Low	10 (28.5%)
Medium	08 (22.8%)
High	04 (11.4%)
Grading by Microscopy	(n-16)
Scanty	4 (25%)
+1	7 (43.7%)
+2	3 (18.7%)
+3	2 (12.5%)

[Table/Fig-8]: Bacterial density by GeneXpert and Fluorescent microscopy.

There are lacunae on data on sensitivity and specificity in EPTB cases worldwide. Collectively, present study demonstrated overall prevalence of EPTB cases in tertiary care hospital in northern India. Study demonstrated diagnostic importance of fluorescent microscopy in comparison to Xpert MTB/RIF, early detection of EPTB may contribute to a better management of disease.

LIMITATION

In terms of fluorescent microscopy, a positive staining reaction provides presumptive evidence of the presence of mycobacteria while it cannot differentiate between MTB and atypical mycobacteria. A negative staining reaction does not indicate that the specimen will be culturally negative. Therefore, cultural methods must be employed since it is the gold standard method. Another limitation of the study was that sample size calculation could not be done due to the absence of EPTB prevalence in the region.

Gene Xpert MTB/RIF had its own limitation in terms as it has only 81.3% sensitivity in EPTB samples and further mutations of *rpoB* gene in approximately13% of Indian population may lead to misdiagnosis by this technique as well as it doesn't differentiate between live and dead bacteria [12,13,34].

FUTURE RECOMMENDATIONS

Data analysis of larger sample size of EPTB will be more helpful for clearer picture and to establish these findings. Considering a prospective study will be more useful in terms of identification of risk factors for EPTB like age, sex, smoking, alcohol use and to some extent genetic factors.

CONCLUSION

GeneXpert MTB/RIF is highly sensitive and specific method for detection of EPTB hence it should be method of choice for diagnosing of EPTB however fluorescent microscopy may be helpful in resource limited settings where GeneXpert MTB/RIF are yet to be established. Lower sensitivity of fluorescent microscopy may be intended to paucity of bacilli in these extra-pulmonary sites while sensitivity of fluorescent microscopy may be enhanced by experienced observers. The rapid and accurate detection of TB and drug resistance by GeneXpert MTB/RIF could help to start early and appropriate treatment in EPTB.

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