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Original Research Article

Evaluation of *Mycobacterium tuberculosis* by conventional methods, GeneXpert and line probe assay in cases of extrapulmonary tuberculosis

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ABSTRACT

Background: Diagnosis of EPTB is often delayed due to its paucibacillary nature. Diagnostic modalities like conventional methods and molecular methods like GeneXpert are employed for *Mycobacteria* detection and their results are compared. Line Probe Assay is used for determination of resistance in first line and in second line anti-tubercular drugs.
Methods: In this study 302 extrapulmonary samples from suspected cases of all age groups were included. Samples were first homogenised and decontaminated and then subjected to various diagnostic modalities like microscopy, culture and GeneXpert for *Mycobacteria* detection. Culture and smear positive isolates were subjected to LPA for determination of drug resistance in first and second-line anti-tubercular drugs.
Results: Out of the 302 extrapulmonary samples, maximum samples were of lymph nodes (19.86%) followed by pus (17.88%). Male to female ratio was 1:3. GeneXpert detected 45.04% positive cases and 5.96% were rifampicin resistant. Positive samples detected by microscopy and culture were 21.19% and 24.17% respectively. When compared to culture, microscopy showed a sensitivity of 86.30% and specificity of 99.56%. GeneXpert reported 100% sensitivity and 72.48% specificity. LPA reported 9.45% isoniazid resistant cases, 4.05% rifampicin resistant cases and 5.40% both isoniazid and rifampicin resistant cases (MDR-TB). Out of the MDR-TB cases, 25% cases were resistant to fluoroquinolones indicating pre-XDR TB.
Conclusions: For *Mycobacterium tuberculosis* detection in extrapulmonary samples, multiple modalities should be employed so that the bacilli in these samples is not missed and the turn-around time is lowered which is a key to TB control strategy.

Keywords: Extrapulmonary tuberculosis, GeneXpert, LPA, MDR-TB, *Mycobacteria*, Rifampicin

INTRODUCTION

Tuberculosis is communicable disease which is a major cause of ill health and one of leading cause of death worldwide. In high TB burden countries worldwide, India accounts for 26% of the TB cases and 34% of global TB deaths. The death burden was highest among men 50% followed by women 40% and children 9.8%.[1] Infection with TB can result in two stages: asymptomatic latent tuberculosis infection (LTBI) or tuberculosis disease. Tuberculosis is primarily a disease affecting lungs known as pulmonary tuberculosis (PTB) but it can also have other manifestations such as pleura, lymph node, central nervous system, meninges, bones, gastrointestinal tract or

disseminated known as extrapulmonary tuberculosis (EPTB).[2,3]

The burden of EPTB is high, ranging from 15-20% of all TB cases.[4] EPTB is a paucibacillary disease, requires strong clinical suspicion to be diagnosed and special diagnostic procedures are required.[5,8] It possess a significant health problem in both developing and developed countries. The prevalence of disease in India accounts for 8.3 to 13.1%.[6,7]

The bacillary load is very less in extra pulmonary samples and because of difficulty in obtaining tissues from deep seated organs; diagnosis is delayed in most cases.[9] Culture of MTB is considered as 'gold standard' for definitive diagnosis of EPTB. Dependability on conventional methods often lead to considerable delays, compromising patient care and outcomes.

The World Health Organization (WHO) has recommended use of GeneXpert MTB/RIF assay for national tuberculosis programs in developing countries.[10] It is a rapid test based on nested real-time PCR assay, automated and user friendly.[11] This technique is not prone to cross-contamination, requires minimal biosafety facilities and has a high sensitivity in smear-negative pulmonary TB. It simultaneously detects rifampicin resistance and has the advantage of short turnaround time of 2 hours.[12,13]

Another method employed by WHO for determination of drug resistance is line probe assay (LPA). Genotype MTBDRplus and Genotype MTBDRsl (Hain Life Science GmbH, Nehren, Germany) detect mutations in katG and rpoB genes for determination of drug resistance in isoniazid and rifampicin respectively. The WHO endorsed Genotype MTBDRsl 2.0 assay can also detect resistance conferring mutations of FLQ (gyrA and gyrB) and SLID (rrs and eis).[14]

These rapid molecular methods enable early detection and treatment of cases, which is essential in preventing MDR-TB from progression to XDR-TB.[15] With this background, various modalities for diagnosis of extrapulmonary tuberculosis like microscopy, culture and GeneXpert were compared. Resistance pattern between first and second-line anti-tubercular drugs was also seen using line probe assay.

METHODS

This was cross-sectional study. This study was conducted from August 2020 to December 2022. Total 302 different extrapulmonary samples were studied such as lymph node aspirates, pus, pleural fluid, CSF, Ascitic fluid, synovial fluid, etc.

Patients with extrapulmonary tuberculosis from different age groups were studied and samples received at the Intermediate Reference Lab in the Department of Microbiology, Government Medical College, Nagpur, India.

Inclusion criteria

Patients with extrapulmonary tuberculosis were included.

Exclusion criteria

Patients with pulmonary tuberculosis were excluded.

Suspected extrapulmonary tuberculosis specimens like lymph node aspirates, ascitic fluid, CSF, pleural fluid, pus, biopsy tissues, etc. were received in the laboratory and processed as per the standard decontamination procedures.[16,17] Samples were homogenised and decontaminated using N-acetyl-L-cysteine (NALC) and 4% NaOH. These samples were then subjected to various diagnostic modalities for the detection of *Mycobacteria*.

Extrapulmonary samples were processed by GeneXpert as per the instructions given in the manual.[18] The sample was loaded into the cartridge with the help of pipette along with the sample reagent and the cartridge was inserted in the machine to run the programme. Results were read after 2 hours. GeneXpert detects rifampicin resistance along with the detection of MTB.

Microscopy was done on all the suspected extrapulmonary tuberculosis samples by fluorescent microscopy using 0.1% Auramine and potassium permanganate and the smears were observed under 40X lens of fluorescent microscope. *Mycobacteria spp.* fluoresce yellow to orange.

Samples were inoculated on LJ media and was incubated at 35-37^0 C for 6-8 weeks. Cultures are examined weekly for growth until 8 weeks. The colonies are buff coloured and rough, having the appearance of breadcrumbs or cauliflower. They are not easily emulsified but give a granular suspension. The cultures are discarded and reported as negative if no growth seen after 8 weeks.

Genotype MTBDRsl (Hain Life Science GmbH, Nehren, Germany) and Genotype MTBDRsl 2.0 assay was performed on all culture positive isolates and was performed according to the manufacturer's instructions.[19] First line and second line anti-tubercular drugs are tested by reverse hybridization assay method. Results were interpretated as the post hybridization reaction leads to the development of coloured bands on nitrocellulose strip at site of probe binding and was observed by naked eye.

Statistical analysis

Categorical data were expressed in frequency and percentage. Categorical variables were compared by performing chi square test. For small amount Fischer's exact test was used wherever applicable. $p< 0.05$ was

considered statistically significant. Statistical software STATA version 14.0 was used for data analysis.

RESULTS

Total 302 Suspected cases of extrapulmonary tuberculosis were included in the study. Out of these 16 (53.31%) were males and 141 (46.68%) were females with a ratio of male to female was 1.14:1 as shown in Figure 1. Maximum cases belonged to the age group of 31-40 years 68 (22.51%). Maximum samples received for MTB detection were lymph node aspirates 60 (19.86%) followed by pus 54 (17.88%). When tested by fluorescent microscopy, 238 (78.80%) were negative and 64 (21.19%) were positive. When subjected to LJ culture, 229 (75.82%) samples were negative and 73 (24.17%) samples were positive. The number of lymph node aspirate samples were the highest (60) and out of these 21 were smear positive and 23 were culture positive. Total 54 pus samples were included and 17 out of these were culture and smear positive. Similarly, 50 pleural fluid samples were included and 5 samples were smear positive and 6 were culture positive as shown in Table 1.

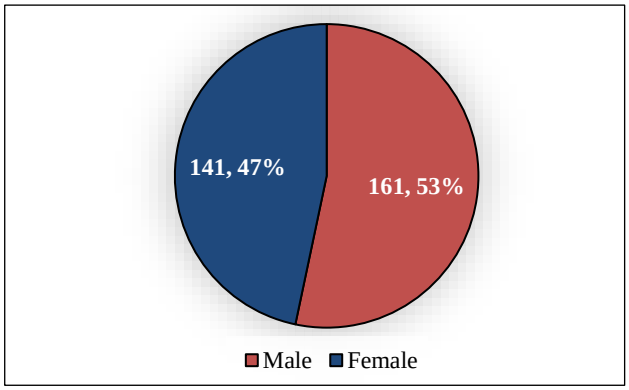

Figure 1: Gender-wise distribution of extrapulmonary samples (n=302).

Table 1: Distribution of positive samples by different methods (n=302).

Sample	Total samples	Microscopy	Culture	GeneXpert
Lymph node aspirate	60	21	23	41
Pus	54	17	17	35
Pleural fluid	32	0	1	12
Cerebrospinal fluid	50	5	6	9
Bronchial wash	21	8	10	13
Ascitic fluid	15	1	1	2
Gastric aspirate	13	1	2	5
Endometrial tissue	11	1	1	2
Fine needle aspirate	8	3	3	4
Tissue	6	3	4	4
Biopsy	5	1	2	3
Breast abscess	4	0	0	1
Synovial fluid	4	0	0	2
Others	19	3	3	3
Total	302	64	73	136

Total 302 samples were subjected to (CBNAAT) GeneXpert, out of which 166 (55.96%) were negative for *Mycobacterium tuberculosis*. A 136 (45.04%) samples detected *Mycobacterium tuberculosis.* An 18 (5.96%) out of the 136 samples showed resistance to rifampicin. Distribution of extrapulmonary samples by GeneXpert were shown in Figure 2.

Out of the 136 positive samples maximum were of lymph node aspirates (41/60=68.33%) followed by pus (35/54= 64.81%), bronchial wash (13/21= 61.90%). Rifampicin resistance were detected in 18 of the positive MTB samples. Maximum rifampicin resistant samples belonged to pus 16.66% (9/54), followed by bronchial washings 14.28% (3/21) pleural fluid 2% (1/50) and lymph node aspirates 6.66% (4/60).

As shown in Table 2, GeneXpert MTB/RIF also provided with semi-quantitative assay of positive samples for MTB. A total of 136 samples that tested positive for MTB, maximum samples 56 belonged to cycles with very low threshold (>28 cycles) followed by low threshold cycles (23-28 cycles) 33, medium (16-22 cycles) 25 and high threshold cycles (<16 cycles) 22. This shows that Extrapulmonary tuberculosis is paucibacillary in nature.

When the extrapulmonary specimens were compared by fluorescent microscopy and LJ culture, the sensitivity of microscopy was 86.30% and a high specificity of 99.56%. Results of GeneXpert and LJ culture were compared, sensitivity of GeneXpert was found to be high (100%) and a specificity of 72.48%. Specificity of LJ culture was high. On comparing GeneXpert and

fluorescent staining methods, microscopy had a lower sensitivity (27.51%). Sensitivity of GeneXpert was high (100%) but a specificity of 70%. Microscopy showed a high specificity.

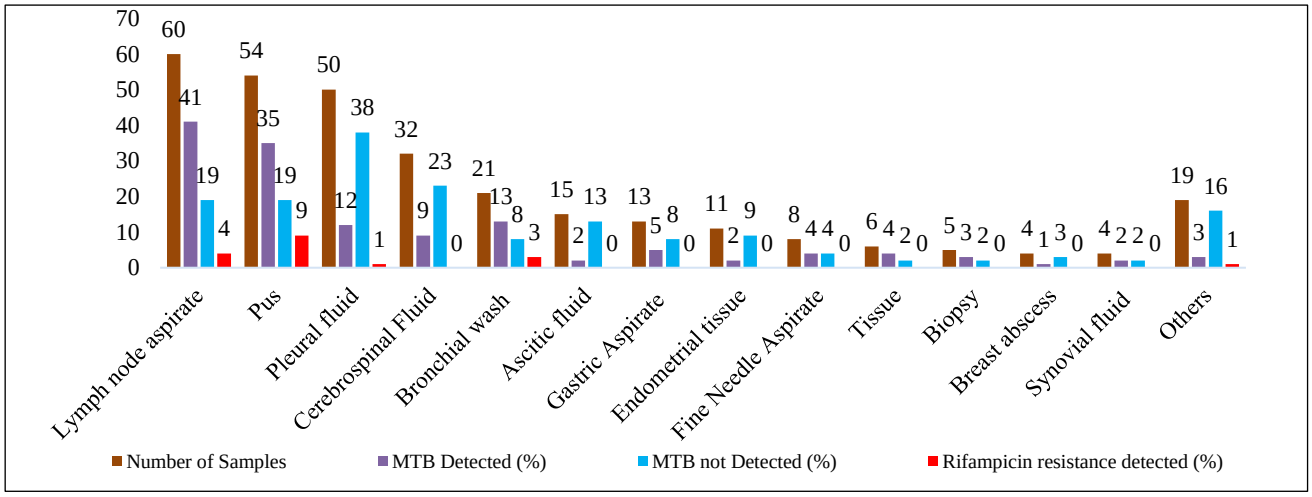

Figure 2: Distribution of extrapulmonary samples by GeneXpert MTB/RIF assay (n=302).

Table 2: Semi-quantitative results by GeneXpert MTB/RIF assay (n=136).

High cycles <16 cycles	Medium 16-22 cycles	Low 23-28 cycles	Very low > 28 cycles	Total positive cases
22	25	33	56	136

Culture and microscopy positive isolates (74) were subjected to LPA and reported 9.45% (7/74) isolates were resistance to isoniazid (HR-TB), 4.05% (3/74) isolates were resistant to rifampicin only and 5.40% (4/74) were resistant to both isoniazid and rifampicin indicating RR-TB and MDR-TB respectively. There were 25% (1/4) of the MDR-TB isolates also showed resistance to fluoroquinolones suggesting pre-XDR-TB.

DISCUSSION

Total 302 extrapulmonary samples were included in the study from all the age groups. Most of the cases belonged to the age group of 31-40 years with a male to female ratio of 1.14:1. In a study by Mukherjee et al, the mean age of the EPTB patients was 36.49 years. Diriba et al had an age group of 32.3 years whereas Sharma et al had a mean age of 35.29 years. All of them had a male predominance.[20-22]

In this study, maximum samples received were of lymph node aspirates (60=19.86%), followed by pus (54=17.88%), pleural fluid (50=16.55%) and cerebrospinal fluid (32=10.59%) as shown in Table 1.

Table 3 shows extrapulmonary samples distribution by various studies. In this study, various extrapulmonary samples that were subjected to fluorescent microscopy and LJ culture, 21.19% (64) were detected positive by fluorescent microscopy and about 24.17% (73) were positive by LJ culture. Among these lymph aspirates were the highest (21/60) by fluorescent microscopy and by LJ culture (23/60) followed by pus samples (17/54) by fluorescent microscopy as well as by LJ culture. Khan et al included a total of 737 extrapulmonary tuberculosis samples and out of these 52 (7%) were positive by fluorescent microscopy and 130 (18%) were positive by culture. Pleural fluid samples contributed maximally to positive samples, around 20% (52) by culture followed by CSF samples (12) and pus samples (12). Pleural fluid samples contributed maximally to positive samples, around 8% (22) by fluorescent microscopy followed by CSF (5) 8% and ascitic fluid 6% (8).[23]

Total 302 samples were tested by CBNAAT, of these 136 (45.04%) samples detected MTB. Out of the 136 samples that detected MTB, 18 (5.96%) showed resistance to rifampicin. Maximum positive samples belonged to lymph node aspirates 68.33% followed by pus samples 64.81% and bronchial washings 61.90%. Ascitic fluid and endometrial tissue however detected a lesser percentage of MTB, 13.33% and 18.18% respectively. Lawn et al found maximum positive samples in lymph node aspirates (35%) followed by gastric aspirates (23%) and pus (21%).[24] Fuladi et al also detected maximum positivity rate in lymph node aspirates 62.74%, followed by ascitic fluid 41.66% and pleural fluid 31.57%.[25]

Table 3: Distribution of extrapulmonary tuberculosis samples in different studies.

Study	Total samples	PUS N (%)	Lymph node N (%)	Gastric aspirate N (%)	Pleural fluid N (%)	Ascitic fluid N (%)	CSF N (%)
Kumari et al	510	304 (59.60)	-	91 (17.84)	62 (12.15)	4	18
Metaferia et al	353	-	15 (4.3)	-	109 (30.9)	-	184 (52.1)
Mukherjee et al	502	51	114	-	284	26	4
Sharma et al	623	184 (29.5)	63 (10.1)	-	44 (7)	18 (3)	222 (35.7)
Present study	302	54 (17.88)	60 (19.86)	13 (4.30)	50 (16.55)	15 (4.96)	32 (10.59)

Among the 136 positive cases that were detected by GeneXpert, 18 (5.96%) cases were rifampicin resistant. This is very similar to Zahoor et al which detected rifampicin resistance in 3 samples (5.88%).[26] Habous et al found out that 5 (11.6%) samples were RIF resistance with GeneXpert MTB/RIF assay. Of these, 2 (4.65%) were pus, 2 (4.65%) were body fluid, and 1 (2.32%) were urine samples.[27] This difference in rifampicin resistance can be due to demographical variation of the populations included in the various studies.[28]

GeneXpert Ct values were taken as the mean of PCR cycles obtained from the five probes (A-E) of the GeneXpert machines. Ct values inversely correlate with bacterial load i.e. lower Ct values represent a higher starting concentration of DNA template whereas higher Ct values represent a lower concentration of DNA template. The mean Ct values are also categorized by the GeneXpert system semi-quantitatively in relation to sample positivity as very low (>28 cycles), low (23-28 cycles), medium (16-22 cycles) and high (<16 cycles).[29]

The semi-quantitative results by GeneXpert were described. In this study, 136 samples that were tested positive by GeneXpert, 56 (41.17%) samples had 'very low' threshold (>28 cycles) which were maximum. This was followed by samples with 'low' threshold cycles (23-28 cycles) which were 33 (24.26%), 25 (18.38%) samples were detected with 'medium' threshold cycles (16-22cycles) and 22 (16.17%) samples detected with 'high' threshold cycles (>16 cycles).

Fuladi et al detected "very low" or "low" in the 70.5% of the samples that reported MTB positive.[25] Tortoli et al found out the semi quantitative results of" very low", or "low" cycle thresholds in the large majority (78.4%) of the samples that scored positive.[30] Similarly, Kumari et al detected maximum positive EPTB cases in "very low" and "low" threshold cycles (58.53% and 26.82% respectively).[28] This indicates the paucibacillary nature of extrapulmonary tuberculosis.

In this study, we reported sensitivity of fluorescent microscopy 86.30% and specificity of 99.56% when compared to LJ culture. Positive predictive value was 98.43% and negative predictive value was 95.79%. Similarly, Bhalla et al reported sensitivity of 83.1% and specificity of 82.4%, positive predictive value 72.6% negative predictive value 89.7%.[31]

When GeneXpert was compared to LJ culture, sensitivity of GeneXpert was 100% for culture positive specimens and 27.5% in culture negative specimens. Specificity was 72.48%. Vadwai et al in comparison to culture isolates of EPTB cases reported specificity of 79% of GeneXpert which was very similar to this study. However, they reported a lower sensitivity of 83%. This is possibly because of higher proportion of treatment-experienced cases in their study contributed to lower culture positivity rates.[32] The variable performance of GeneXpert in different EPTB specimens could explain the difference in sensitivities and specificities reported from different studies due to heterogeneous sample populations.[30,33,34]

The sensitivity of GeneXpert as compared to fluorescent microscopy was 100% in smear positive cases and 30.52% in smear negative cases. The specificity reported was 70% in this study. This study was in concordance with a study done by Zeke et al which reported sensitivity of GeneXpert of 100% in smear positive cases and 63% in smear negative cases. However, they reported a higher specificity of 100%.[35] Niveditha et al reported sensitivity of 75% and specificity of 4% of GeneXpert in extrapulmonary specimens which was lower than our study.[36]

The present study showed 9.45% cases of HR-TB and 4.05% cases of RR-TB. MDR-TB cases were 5.40%. 25% cases of MDR-TB showed resistance to fluoroquinolones indicating pre-XDR TB. Similar to this study, Desikan P et al from central India, demonstrated 7.6% mono-isoniazid resistant, 18% MDR isolates and 11.6% mono-rifampicin resistant isolates.[39] Mchaki et al reported a high accuracy of 95.2% in LPA compared to GeneXpert's 84.9%. The ability of the tests to distinguish rifampicin-sensitive and rifampicin-resistant strains to be 87.9% for GeneXpert and 92.0% for LPA. The results indicate the superiority of LPA over GeneXpert regarding detection of rifampicin monoresistance. However, logistical issues such as longer turnaround time and requirement of trained laboratory personnel may limit the use of LPA, especially in low-income countries, where the burden of TB- and rifampicin-resistant TB is highest in the world.[40]

One of the most important and obvious reason for the use of the GeneXpert MTB/RIF is significantly reduced turnaround time for detection. Not only is the TAT reduced to 2 to 3 h, this test can also detect rifampicin resistance simultaneously.[37] However, after its wide use and analyses of several hundred thousand samples, reports have started emanating that it can give false-negative and false-positive RIF resistance results.[34,37,38] Further testing of EPTB by LPA is required to determine the extent of drug resistance and to know the prevalence of MDR and XDR-TB cases.

This study has some limitations. HIV status of the patients were not known as HIV infection is known risk factor in EPTB cases. As LPA detects hetero-resistance and requires a higher bacillary load, use of liquid media for culture and DST would have been improved isolation rate and individual drug susceptibility would have been known for first and second-line drugs.

CONCLUSION

Extrapulmonary tuberculosis cases are likely to be missed out due to its paucibacillary nature and limitations of conventional methods to detect higher bacterial load. Therefore, GeneXpertis essential for the diagnosis of EPTB as well as for determination of rifampicin resistance in the clinical settings where rapid results are required to initiate anti-tubercular treatment. Rifampicin resistance by GeneXpert should be further tested by LPA for determination of resistance to other drugs and to know the MDR, pre-XDR and XDR-TB prevalence in the community. In resource constrained laboratories, detection of *Mycobacterium tuberculosis* in extrapulmonary samples, multiple modalities should be employed so that *Mycobacterium tuberculosis* in these samples is not missed and also lowers the turn-around time which is a key to TB control strategy.

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