Original Article

DOI: 10.7860/JCDR/2019/37569.12485

Microbiology Section

Diagnostic Evaluation of Multiplex Real Time PCR, GeneXpert MTB/RIF Assay and Conventional Methods in Extrapulmonary Tuberculosis

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ABSTRACT

Introduction: Effective management of Extrapulmonary Tuberculosis (EPTB) requires simultaneous identification of *Mycobacterium tuberculosis* (*M.tuberculosis*) complex, Non-Tuberculous Mycobacteria (NTM) and Rifampicin (RIF) sensitivity pattern for early and effective evidence based Antitubercular Treatment (ATT).

Aim: To evaluate the diagnostic potential of multiplex Real-Time Polymerase Chain Reaction (mRT-PCR) versus GeneXpert and conventional microscopy and culture.

Materials and Methods: A total of 110 multivaried extrapulmonary specimens from an equal number of patients with strong clinical/radiological/histopatholgical evidence of EPTB were subjected to conventional microscopy, liquid culture MGIT 960 system, GeneXpert and mRT-PCR.

Results: Highest positivity of 88.8% (97/110) was shown by mRT-PCR followed by GeneXpert (52.72%, 58/110), liquid culture MGIT 960 system (44.54%, 49/110) and microscopy (4.54%, 5/110), ($p<0.01$, $\chi^2=156$). Comparing it with culture positive cases (n=49), the sensitivity, specificity, Positive and Negative Predictive Value (PPV and NPV) of GeneXpert was found to be 87.25%, 100%, 100% and 80.64% respectively while the same parameters were 100% each for mRT-PCR. mRT-PCR showed higher positivity over GeneXpert in various individual extrapulmonary sample category with significant difference seen in synovial fluid. The specific added advantage of mRT-PCR was seen in the detection of 17 NTM. GeneXpert advantage seen in detection of five cases of RIF resistance.

Conclusion: With individual feature of detection of NTM by mRT-PCR and RIF resistance by GeneXpert, this study may advocate the use of mRT-PCR adjunct to GeneXpert in the diagnostic armamentarium to identify more rapidly and effectively both *M.tuberculosis* and NTM along with RIF resistance information for early and specific ATT intervention of all EPTB cases including Multi Drug Resistant (MDR). However, further research may be required to enhance the sensitivity of both GeneXpert and mRT-PCR to exclude all possibilities of false negative EPTB cases.

Keywords: Molecular diagnosis, *Mycobacterium tuberculosis* complex, Nontuberculous mycobacteria, Nonpulmonary tuberculosis

INTRODUCTION

EPTB, a clinical infection caused either by *M.tuberculosis* complex or non tuberculous mycobacteria. It can affect any organ other than lungs like lymph node, pleura, abdomen, genitourinary tract, skin, joints, bones or meninges, etc. Out of annual global TB incidence of 9.6 million new cases, India alone is burdened with 2.2 million cases with 16% (3,36,000) cases exclusively attributed to EPTB [1]. This affected figure further rises to 40-50% in HIV positive cases [1]. India even has the highest burden of both TB and MDR-TB and second highest of HIV associated TB cases. TB kills more adults in India than any other infectious disease causing two deaths every five minutes [2]. A similar upward trend has also been noticed in NTM associated EPTB cases in India.

Therefore, effective therapeutic management of EPTB mandatorily requires the implementation of the specific diagnostic strategy, which over the last decade or so has transformed from the earlier single need of detection of *M. tuberculosis* to three pronged requirements of simultaneous, effective and rapid identification of (a) *M.tuberculosis* complex (b) NTM and (c) RIF drug resistance which is regarded as a marker of MDR-TB, directly from the clinical specimen to initiate early and specific ATT treatment for effective management of TB [3,4].

However, diagnosis of EPTB remains an uphill challenge and often more difficult than pulmonary TB amid the factor of paucibacillary non uniform lodgement of *M.tuberculosis* and NTM in a diseased tissue site making the task of obtaining an appropriate representative clinical specimen from deep seated organ more challenging [5-10]. The conventional methods of microscopy and culture have their own diagnostic limitations [8]. This often ends up with false negative reports resulting in undiagnosed EPTB cases leading to increased rates of mortality and morbidity in EPTB patients [11]. The low sensitivity of conventional test has instigated and prompted many researchers to develop more sensitive and rapid diagnostic tests. Many other laboratory tests like Interferon Gamma Release Assay (IGRA), histopathology, tubercular skin test and biochemical identification of culture isolates although have been employed for the diagnosis of EPTB, yet each reported there own limitations of low sensitivity and inability to differentiate between *M.tuberculosis* and NTM [7,10].

The last five to ten years have witnessed the development of the several new molecular methods playing a pivotal role in early detection of *M.tuberculosis,* NTM and drug resistance [8-12]. Various promising validated and standardised commercial tests includes line probe assays {Genotype *M.tuberculosis* Drug Resistance (MTBDR) plus (Hains Lifesciences Gmbh, Nehren, Germany), INNO-LIPA Rif. TB (+Innogenetics, Ghent, Belgium)} and Real Time PCR (GeneXpert *M.tuberculosis (MTB)*/RIF; a closed automated heminested real time PCR system of Cepheid, Sunnyvale, CA and various real time PCR commercial tests targeting various specific regions

of *M.tuberculosis* and NTM viz., 38kDa, 65kDa, 85 B, IS6110, MPB64, *rpo B*) [13-18]. Among these tests, GeneXpert MTB/RIF assay, an FDA approved technology has widely been promoted by Revised National Tuberculosis Control Program (RNTCP) and WHO in the diagnosis of both pulmonary TB and EPTB due to the added advantage of simultaneous detection of *M.tuberculosis* complex and RIF drug resistance directly from the clinical sample in less than three hours [1,2,5]. However, the inability of GeneXpert to detect NTM in clinical samples necessitates the requirement for any other suitable molecular test to cater the need of simultaneous detection of *M.tuberculosis* and NTM since treatment regimen is always different for both the infections. This may most ably be addressed by mRT-PCR test with its characteristic advantage of amplifying multiple genes to fulfill the need of ascertaining simultaneously the presence of both *M.tuberculosis* and NTM directly in the clinical sample.

Moreover, there is a paucity of data from India for evaluating the role of mRT-PCR versus GeneXpert and conventional test in the diagnosis of EPTB which has been confirmed by PUBMED search which retrieved only one study of Vadwai V et al., from India evaluating exclusively GeneXpert in the diagnosis of EPTB. Further, to best of authors' knowledge, no study has yet been published on mRT-PCR from the state of Chhattisgarh, a central region of India [5]. Accordingly, in this study, we have investigated the diagnostic efficacy of the mRT-PCR and its comparative evaluation with GeneXpert, conventional microscopy and liquid culture MGIT 960 system in the diagnosis of EPTB. In addition, rapid detection of RIF resistance was also determined and compared with phenotypic susceptibility testing.

MATERIALS AND METHODS

This prospective study was conducted in collaboration between the Molecular Diagnostic Laboratory of All India Institutes of Medical Sciences (AIIMS), Raipur, Chhattisgarh and Intermediate Regional Laboratory (IRL) under RNTCP of Government of India from January, 2017 to September, 2017. A total of 110 extrapulmonary clinical samples obtained from an equal number of EPTB cases with a strong clinical/radiological/histopathological evidence of TB referred from various clinical departments viz., Gynaecology, Orthopaedics, Paediatrics, Surgery, ENT, Medicine and Pulmonary Medicine of AIIMS, Raipur, Chhattisgarh were included in the study after obtaining written informed consent. Additionally, 10 samples (5 synovial tissue and 5 synovial fluid) from equal number of patients with joint damage of nontuberculous origin and 15 skin biopsy samples from the lesion of patients with cutaneous carcinoma were included as negative controls. The negative control group was clinically confirmed of not having any signs/symptoms or any past history of TB. All were found HIV negative.

Inclusion Criteria: All new cases of EPTB accompanied with appropriate clinical/radiological/histopathological information indicative of TB infection and response to ATT therapy on follow-up were included in the study.

Exclusion Criteria: Smear and molecular test (GeneXpert and mRT-PCR) negative EPTB patients not responding to ATT treatment after four weeks of treatment were excluded from the study. Any patients who had received TB treatment within the last two years were also excluded.

Break up of Clinical Samples: A total of 110 extrapulmonary samples included synovial fluid (33), endometrial tissue biopsy (20), lymph node biopsy (16), menstrual blood (08), pleural fluid (08), pus (07), ovarian cyst (05), right ulnar biopsy (04), hip joint aspiration (02), Urine (02), peritoneal fluid (02), foot aspiration (01), CSF (01) and pericardial fluid (01).

Criteria for Extrapulmonary Diagnosis: Since culture considered a suboptimal reference standard for evaluation of nucleic acid amplification based methods, the response to ATT was taken as the gold standard in the study. All the 110 patients had responded to ATT treatment.

The ethical permission was obtained from AIIMS, Raipur, Chhattisgarh with registration number AIIMSRPR/IEC/2016/036.

Processing of the Clinical Specimens

All the fluid samples were divided into two portions with first portion of 1-2 mL used for GeneXpert and rest of the 2 to 5 mL sample was used for microscopy, culture and mRT-PCR. Biopsy samples were first homogenized in a mortar and pestle before dividing it in the similar two portion described above.

Microscopy and culture: Both direct and concentrated smears were prepared, fixed, and stained by Ziehl Neelsen Staining before examining under oil immersion lens for the presence or absence of the Acid Fast Bacilli (AFB). All non-sterile clinical specimens were processed by the standard conventional N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) method as per standard protocol [19]. The deposit obtained was neutralized with phosphate buffer saline (pH 7.2) and divided into three portions. First portion was used for the preparation of concentrated smear, whereas second portion was used for inoculation into the Liquid Mycobacterial Growth Indicator Tube (MGIT) medium (Beckton Dickenson, Sparks, MD, USA) with subsequent incubation in the MGIT 960 system according to manufacturer's instructions [20]. The third portion was used for DNA extraction.

DNA extraction: DNA was extracted by QIAmp DNA mini kit (QIAGEN Company, Germany) with slight modification to the DNA procedure by keeping vortexed suspension of 200 µL of the NALC-NaOH leftover pellet in 200 µL ATL buffer at 85°C for 20 minutes for mycobacterial inactivation with rest of the procedure followed as per the manufacturer recommendation. Briefly it involves addition of 20 µL of proteinase K to suspension with brief vortexing and further incubation at 56°C for one hour. A 200 µL buffer AL was then added and followed with brief vortexing and further incubation at 70°C for 10 minutes and thereafter washing and elution to obtain genomic DNA.

mRT-real time PCR: Commercially available Genefinder™ TB and NTM mRT-PCR kit (Infopia Co., Ltd., Korea) was used in the present study. The mRT-PCR amplify the specific region of most abundantly present Insertion Sequence (IS) 6110 and secretary protein MPB64 gene of *M.tuberculosis* and Internal Transcribed Spacer (ITS) gene specific for 42 pathogenic NTM. External transcribed spacer region of *Paulownia tomentosa* was used as internal control. Probes specific for *M.tuberculosis* and NTM were labelled with the fluorophore FAM and HEX. Black Hole Quencher (BHQ) was used as quenching dye. Probes for internal controls was tagged with Cy5 reporter and BHQ quenching fluorophore to check PCR inhibition. To check and confirm the absence of contamination during each of the mRT-PCR tests, the various controls viz., positive (plasmid for IS6110, MPB 64 and ITS), negative (ultrapure water), no template and internal control were also run along with the samples. mRT-PCR was done as per manufacturer instruction with 10 µL of TB and NTM reaction mixture, 5 µL of probe and 5 µL of respective DNA of the sample or control mixed strictly under aseptic condition inside of the biosafety cabinet type 2 A and run on CFX 96 Real Time PCR system, BioRad Laboratories, Pvt., Ltd., USA with thermal cycling profile of 1 cycle each of 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C/15 seconds and 60°C/1 minute.

GeneXpert MTB/RIF assay: The MTB/RIF assay was performed as per the instruction of the manufacturer. Briefly, sample reagent was added in 3:1 ratio to the clinical specimen by taking 1 mL of resuspended sample and 2 mL Xpert sample reagent. The closed specimen container was manually agitated/shaken several times and incubated for 15 minutes at room temperature and then this suspension was transferred to the test cartridge. The inoculated test cartridges were inserted into the GeneXpert instrument. Cycle

Threshold (CTs) of 5 *rpo B* gene probes automatically reported the presence or absence of *M.tuberculosis* [21]. RIF resistance was reported automatically by calculation of a change in C_T (ΔCT) between the highest and the lowest signal of the five probes. ΔC_T greater or equal to 3.5 indicated RIF drug resistance.

Patient Groups

Comparative evaluation of GeneXpert MTB/RIF and mRT-PCR test was done by comparing their diagnostic utility among three different classes of patients namely:

(A) Smear and culture positive TB;

(B) Smear negative and culture positive TB;

(C) Smear and culture negative TB.

STATISTICAL ANALYSIS

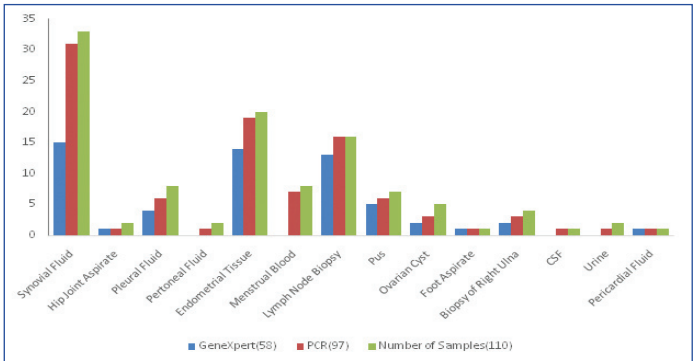
Statistical analysis was performed with the SPSS for Windows (version 16.0) software package. Sensitivity and specificity were compared with chi-square test (χ^2) along with 95% Confidence Interval (CI).

RESULTS

One hundred ten (110) extrapulmonary specimens obtained from an equal number of patients (mean age 31.35 yrs; 61 males and 49 females) were processed by conventional methods (microscopy and culture), GeneXpert and mRT-PCR test. ZN stained smear microscopy and culture showed positivity in 05 (4.5%, CI=1.4-10.2%) and 49 (44.54%, CI=35-54.33%) clinical samples respectively. GeneXpert overall showed positivity of 52.72% (58/110, CI=42.9-63.3%) whereas mRT-PCR detected *M.tuberculosis* and NTM in 88.18% (97/110, CI=80.6-93.5%) with a statistically significant difference over the other tests ($p<0.01$, $\chi^2=156$) [Table/Fig-1]. Even in multi-varied sample category, mRT-PCR showed statistically significant sensitivity over GeneXpert in synovial fluid ($p<0.01$, $\chi^2=16.4$, CI=79.77-99.26%) [Table/Fig-2,3]. In various individual specimen categories too, mRT-PCR showed either higher or equivalent sensitivity in comparison with GeneXpert [Table/Fig-3].

Test	Positive	Negative	Positivity (%)	CI
Microscopy	5	105	4.5	1.4-10.2%
Culture	49	61	44.54	35-54.33%
GeneXpert	58	52	52.72	42.9-63.3%
mRT-PCR	97	13	88.18	80.6-93.5%

[Table/Fig-1]: Positivity rate of smear microscopy, liquid MGIT 960 TB culture, GeneXpert MTB/RIF and Multiplex RT-PCR.



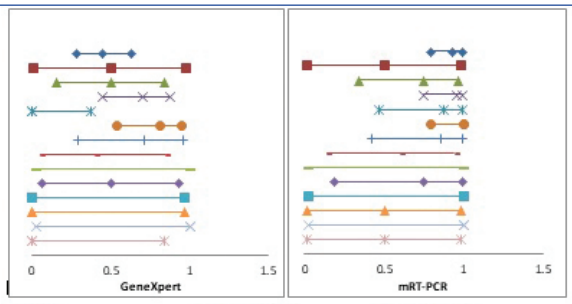
[Table/Fig-2]: Detection rate of GeneXpert versus mRT-PCR in individual EPTB samples.

In 25 negative control samples, all the four tests showed accurate result with 100% specificity.

In culture positive cases (n=49), the sensitivity, specificity, PPV, NPV and CI for GeneXpert was found to be 87.25%, 100%, 100%, 80.64% and 0.87 (75.23-95.37%) respectively while the same parameters for mRT-PCR were calculated 100% each with CI of 1.00 (92.75-100%) [Table/Fig-4,5].

Since all five smear positive samples were found positive by culture also, GeneXpert versus mRT-PCR were compared in the three patient categories comprising the first of smear and culture positive TB cases (05), the second of smear negative but culture positive cases (44) and the third category of both smear and culture negative cases (61) [Table/Fig-6].

In the first category of smear and culture positive cases (05), both GeneXpert and mRT-PCR showed 100% positivity and CI of 55-100%. In smear negative and culture positive cases (44), GeneXpert notched 86.36% positivity (CI=76-95%) by detecting



Samples	GeneXpert(95% CI)	mRT-PCR(95% CI)
Synovial Fluid	0.45(0.28-0.63)	0.93(0.79-0.99)
Hip Joint	0.50(0.01-0.98)	0.50(0.01-0.98)
Pleural Fluid	0.50(0.15-0.84)	0.75(0.34-0.96)
Endometrial tissue	0.70(0.45-0.85)	0.95(0.75-0.99)
Menstrual Blood	0.00(0.00-0.37)	0.87(0.47-0.99)
Lymphnode Biopsy	0.81(0.54-0.95)	1.00(0.79-1.00)
Pus	0.71(0.29-0.96)	0.85(0.42-0.99)
Overian Cyst	0.40(0.05-0.85)	0.60(0.14-0.94)
Foot Aspirate	1.00(0.02-1.00)	1.00(0.02-1.00)
Right Ulna Biopsy	0.50(0.06-0.93)	0.75(0.19-0.99)
CSF	0.00(0.00-0.97)	1.00(0.02-1.00)
Urine	0.00(0.00-0.97)	0.5(0.01-0.98)
Pericardial Fluid	1.00(0.02-1.00)	1.00(0.02-1.00)
Peritoneal Fluid	0.00(0.00-0.84)	0.5(0.01-0.98)

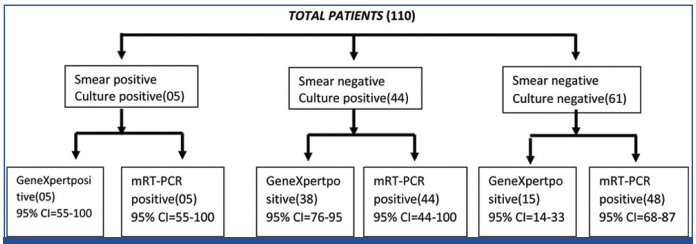
[Table/Fig-3]: Forest plot showing sensitivity of mRT-PCR versus GeneXpert in various types of extrapulmonary specimens.

GeneXpert	True positive	False positive
	43(a)	0(b)
	False negative	True negative
	06(c)	25(d)
Sensitivity	a/a+c×100=87.25%	
Specificity	d/d+b×100=100%	
Positive predictive value	a/a+b×100=100%	
Negative predictive value	d/d+c×100=80.64%	
95% CI	0.87 (75.23-95.37%)	

[Table/Fig-4]: Comparison of GeneXpert in 49 culture positive cases.

mRT-PCR	True positive	False positive
	49(a)	0(b)
	False negative	True negative
	0(c)	25(d)
Sensitivity	a/a+c×100=100%	
Specificity	d/d+b×100=100%	
Positive predictive value	a/a+b×100=100%	
Negative predictive value	d/d+c×100=100%	
95% CI	1.00 (92.75-100%)	

[Table/Fig-5]: Comparison of mRT-PCR in 49 culture positive cases.



[Table/Fig-6]: Study flow chart showing results of conventional microscopy, culture and molecular GeneXpert and mRT-PCR.

38 cases while mRT-PCR showed 100% positivity (CI=44-100%). However, GeneXpert noticeably found to show very low positivity of 24.59% (CI=14-33%) in smear and culture negative extrapulmonary specimens whereas mRT-PCR detected 48 such cases with a positivity of 78.68% (CI=68-87%), and thus the difference of positivity was statistically significant ($p<0.01$, $\chi^2=35.74$) [Table/Fig-6].

The individual advantage of the GeneXpert in the detection of five RIF drug resistance showing complete corroboration with conventional 1% proportion based drug sensitivity testing was noticed. mRT-PCR showed the advantage in the exclusive detection of 17 cases of NTM.

The specificity for all the above tests was found to be 100%. No significant difference was observed in the detection of *M.tuberculosis* related to age and sex.

DISCUSSION

Effective laboratory diagnostic strategy of EPTB requires to identify both *M.tuberculosis* complex and NTM and RIF sensitivity pattern to enable initiation of specific ATT for early containment of the disease as per the guidelines of RNTCP/WHO recommending different treatment strategy for the *M.tuberculosis* and NTM [2].

Our results clearly indicated the inefficiency of conventional tests (microscopy and culture) in the diagnosis of EPTB and thus should not be considered as sole laboratory diagnostic modalities for EPTB. Even various earlier documented studies also pointed out the low sensitivity of microscopy and culture in the detection of *M.tuberculosis* in extrapulmonary specimens [8,9,18].

GeneXpert assay on the other hand although showed good sensitivity in two categories, namely smear and culture positive and smear negative and culture positive TB, but failed to detect *M.tuberculosis* in smear and culture negative cases with only 24.59% positivity raising a serious concern of providing diagnosis in such cases. The low positivity of 47.7% and 65.5% by GeneXpert were also reported by Kim MJ et al., and Zeka AN et al., respectively in smear negative extrapulmonary specimens [8,9]. Armand S et al., also found low sensitivity of 37% by GeneXpert in smear negative extrapulmonary specimen in comparison to 69% sensitivity obtained with IS6110 Taqman RT-PCR [22]. Zeka AN et al., too found the sensitivity of the GeneXpert statistically higher for the pulmonary specimen in comparison to the extrapulmonary specimen ($p<0.01$) [9]. However, there were relatively very few studies investigating the positivity of the GeneXpert in extrapulmonary samples especially from India which had been recorded at 16-20% EPTB cases every year. A study by Vadwai V et al., also reported 64% sensitivity of GeneXpert in smear negative cases of EPTB [5]. This raised a concern of utmost requirement of tests sensitive enough to detect both *M.tuberculosis* and NTM in clinical samples.

GeneXpert low sensitivity in EPTB cases could be due to various factors i.e., inability to detect NTM, paucibacillary mycobacterial lodgement, presence of any inhibitory substance, manufacturer non recommendation of its use in blood and urine and importantly its detection requirement of capturing intact bacilli from the sample within the cartridge. However, this low sensitivity may be compensated and overlooked with the added advantage of the GeneXpert in direct identification of RIF resistance in the clinical samples within three hours as evident in the present study where in five cases of rifampicin drug resistance was detected by GeneXpert. This particular feature strongly augments GeneXpert diagnostic utility in EPTB.

In smear negative cases of EPTB where in GeneXpert exhibited low sensitivity, mRT-PCR appeared to be a good tool with its ability to target multiple specific genes corresponding to *M.tuberculosis* and NTM. mRT-PCR showed the clear advantage of diagnostic sensitivity with statistically significant difference in positivity over GeneXpert and conventional tests in the overall analysis of 110 extrapulmonary samples ($p<0.01$). In various individual sample categories like peritoneal, pericardial and pleural fluid, biopsy (endometrial, lymph node), pus and CSF, mRT-PCR showed higher positivity over other tests used. Significant difference was observed between mRT-PCR and others in EPTB diagnosis in synovial fluid ($p<0.01$). However, due to a certain limitation of the present study with the low sample number in individual representative sample categories, the authors believe in the support of more such studies involving large number of specimen in various individual specimen categories. Marouane C et al., also showed low sensitivity of the GeneXpert in pleural fluid samples (25%) and urine samples (47.8%) [23]. Vadwai V et al., also found low sensitivity (29%) of the GeneXpert in CSF and moderate sensitivity of 63 to 73% for tissues, lymph nodes and pleural fluid [5]. Suzana S et al., found 67 and 69% sensitivity by GeneXpert in CSF and fluid sample category respectively [24].

Although GeneXpert is not recommended in blood and urine samples, various published studies and evaluation of all diagnostic modalities used in the study necessitates to evaluate its diagnostic efficacy in such samples also [9,23,25].

In other two categories of smear and culture positive and smear negative culture positive samples, no significant difference was found in positivity of mRT-PCR and GeneXpert ($p>0.01$).

Nevertheless, the specific individual advantage of mRT-PCR in detection of both *M.tuberculosis* and NTM and GeneXpert in detecting *M.tuberculosis* and RIF resistance augment diagnostic importance of both the tests for increasing detection rate in EPTB. Early detection of RIF resistance by GeneXpert helps in early screening of MDR-TB. It would thus increase the cure rate and reduce the transmission rate as well as associated mortality. On the other hand, identification of NTM by mRT-PCR provides the opportunity to also initiate the specific treatment for atypical mycobacteria. Thus, specific identification and treatment would help in bringing down the TB associated mortality and morbidity.

The average turnaround time for culture to come positive was 20.35 days (range 3 to 36 days) in the liquid MGIT medium. The turnaround time for GeneXpert and mRT-PCR were less than three hours.

LIMITATION

The limitation of this study includes the fact that some of the individual sample categories like pus, CSF, urine, fluids (Pericardial, Peritoneal and Pleural) and aspirates were represented by few number of samples, due to which the authors of the present study advocates for more such comparative studies on large pool of samples from multivaried extrapulmonary sites for better comparison between various diagnostic modalities in EPTB.

CONCLUSION

With its promising advantage of identifying NTM which was otherwise found undetected in GeneXpert, incorporation of mRT-PCR may be used as an adjunct to GeneXpert to increase the diagnostic sensitivity for early evidenced based management of EPTB.

Authors' Contribution

All authors were involved in conceptualizing and designing the study. SSN, AB, AJB, NMN were involved in initial designing of the study. SSN, PS and SC were involved in samples processing and analysis. SSN, PD, UG, PS were involved in statistical analysis. All authors were involved in critical analysis of the manuscript. All authors read and approved the submitted manuscript.

Funding: It was supported by intramural grant AIIMS.RPR/2017-18/71 provided by AIIMS, Raipur, Chhattisgarh, India.

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FINANCIAL OR OTHER COMPETING INTERESTS: As declared above.

Date of Submission: **Jun 17, 2018**
Date of Peer Review: **Aug 04, 2018**
Date of Acceptance: **Oct 22, 2018**
Date of Publishing: **Jan 01, 2019**