Original Article

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Role of Real Time Polymerase Chain Reaction Targeting *mpb64* Gene in the Diagnosis of Genitourinary Tuberculosis: A Cross-sectional Study

Microbiology Section

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ABSTRACT

Introduction: Genitourinary Tuberculosis (GUTB) remains an important cause of infertility in India. The clinical manifestations of GUTB are non specific, resulting in delayed diagnosis and initiation of Anti Tubercular Treatment (ATT). This delay can lead to various complications such as kidney dysfunction, ureteral strictures, and a shrunken bladder.

Aim: To evaluate the diagnostic importance of Real Time Polymerase Chain Reaction (RT-PCR) targeting the *mpb64* gene for the rapid and accurate diagnosis of GUTB.

Materials and Methods: A cross-sectional hospital-based observational study was conducted in the Department of Microbiology at Subharti Medical College and associated Chhatrapati Shivaji Subharti Hospital in Meerut, Western Uttar Pradesh, India. The study was conducted over a three-year period, from January 2019 to December 2021. A total of 200 genitourinary samples were collected from female patients with clinical suspicion of Tuberculosis (TB) and subjected to direct microscopy, mycobacterial culture by BacT/Alert 3D (Biomerieux, France), and RT-PCR targeting the *mpb64* gene (Qiagen). Demographic details were recorded in a predesigned proforma. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 26.0 (IBM Corp., Armonk, NY, USA). The p-value was calculated using the Chi-square test.

Results: RT-PCR showed a higher positivity rate of 56 (28.9%) compared to 47 (24.2%) by culture. Acid Fast Bacilli (AFB) microscopy was the least sensitive, detecting only 7 (3.6%) cases. When comparing the results of RT-PCR with conventional methods, RT-PCR had a sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) of 91.9%, 99.4%, 85.1%, and 93.8%, respectively.

Conclusion: RT-PCR targeting the *mpb64* gene is a specific and effective additional test that aids in the early and accurate diagnosis of Extrapulmonary Tuberculosis (EPTB), including GUTB, compared to conventional methods. Early diagnosis facilitates timely initiation of ATT, leading to better clinical outcomes.

Keywords: Extrapulmonary tuberculosis, Molecular diagnosis of tuberculosis, *Mycobacterium tuberculosis*

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a leading and significant cause of morbidity and mortality worldwide [1]. TB accounts for approximately 90% of total cases and deaths in the developing world, with 75% of the cases occurring in the economically productive age group [2]. Early diagnosis of TB is crucial for the timely initiation of ATT [3]. Multidrug Resistant (MDR) or Extensively Drug Resistant (XDR) TB is a matter of concern. The diagnosis of EPTB poses a major challenge due to its pauci-bacillary nature. GUTB is a serious form of EPTB commonly observed in India, accounting for 20-73% of all EPTB cases in the general population, but it is less frequently reported in children [4]. Concurrent GUTB has been observed to develop in 2-20% of patients with pulmonary TB [5]. Clinical manifestations of GUTB are non specific, making diagnosis difficult and leading to delayed initiation of ATT, which can result in various genitourinary complications such as kidney dysfunction, ureteral strictures, and a shrunken bladder [6].

Although the demonstration of AFB by microscopic examination and culture on Lowenstein Jensen (LJ) medium remains the cornerstone of TB diagnosis, these conventional methods are time-consuming and have low sensitivity, especially in clinical samples with a low bacterial load [7]. The availability of a quick and effective method to diagnose TB using RT-PCR has significantly reduced false positivity, as amplification and detection occur in the same reaction tube. Thus, RT-PCR can also be used to detect TB in specimens where culture yields negative results due to low bacterial load [8].

Most of the RT-PCR studies conducted on MTB diagnosis have targeted the IS6110 gene. However, authors hypothesised that targeting the *mpb64* gene could also serve as an important tool in diagnosing EPTB. Since only a few studies have been conducted on the importance of RT-PCR targeting the *mpb64* gene, present study further attempted to evaluate its role in the diagnosis of GUTB and contribute to the existing literature [2,4,8].

Considering that some strains of *Mycobacterium tuberculosis* circulating in the Asian and Indian population lack the insertion sequence 6110 (IS6110) [9-11], present study used RT-PCR targeting the *mpb64* gene of *Mycobacterium tuberculosis*. Additionally, aimed to compare the diagnostic efficacy of culture and microscopy with RT-PCR. The study aimed to evaluate the role of RT-PCR targeting the *mpb64* gene in the diagnosis of GUTB, while the objective was to compare culture and microscopy for diagnosing GUTB.

MATERIALS AND METHODS

A cross-sectional, hospital-based observational study was conducted in the Department of Microbiology at Subharti Medical College and associated Chhatrapati Shivaji Subharti Hospital in Meerut, Western Uttar Pradesh, India, over a period of three years from January 2019 to December 2021. The study included a total of 200 genitourinary samples collected from female patients suspected of having GUTB. Approval from the Institutional Ethics Committee (IEC) was obtained before the study commenced, with reference number SMC/IEC/2018/81A. Informed consent was obtained from patients after explaining the study's purpose before collecting clinical specimens.

Inclusion criteria: The study included genitourinary samples received from female patients suspected of having GUTB.

Exclusion criteria: Genitourinary samples received from male patients were excluded from the study.

Sample size calculation: The sample size was determined using the following formula:

$$n=Z^2 * p * (1-p)/d^2$$

Where:

n=sample size.

Z=level of confidence (1.96 at a 5% error).

p=expected prevalence (19%).

d=precision/expected error (5%).

n=1.96 * 1.96 * 0.19 * (1-0.19)/0.05 * 0.05.

Study Procedure

The sample collection, transportation, and processing involved the following samples: Endometrial biopsy (125), endometrial curettings (30), menstrual blood (20), placenta (5), and urine (20). These samples were collected in clean sterile universal containers following aseptic precautions and transported to the testing laboratory within two hours in a cold chain (4°C).

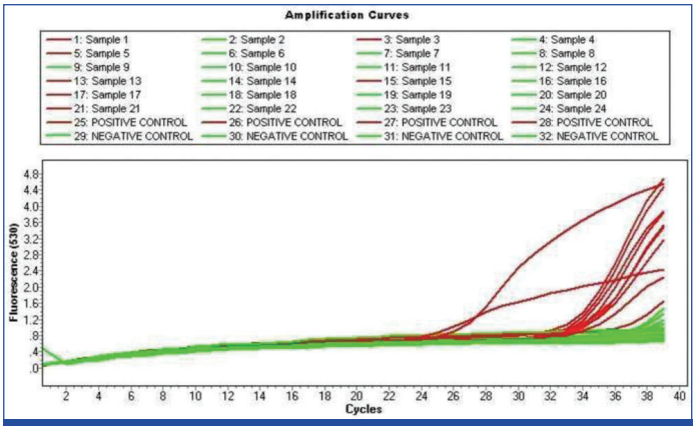
Briefly, tissue specimens were grinded with the help of a tissue grinder in a 2 mL screw-capped tube, and silica beads were added. Decontamination of all the clinical specimens was performed using the modified Petroff's method, which involved N-acetyl-L-cysteine and NaOH (NALC/NaOH) [12-14]. Subsequently, all specimens were centrifuged in 15 mL sterile centrifuge tubes at 5,000 revolutions per minute for 20 minutes, and the resulting pellet was used for both DNA extraction and AFB smear preparation. Additionally, all decontaminated specimens underwent culture using the BacT/Alert 3D (bioMerieux, France) automated blood culture system, following the standard protocol [15].

DNA extraction: DNA was extracted using the Quick-DNA™ Miniprep Kit (Catalogue Numbers: D3024, D302) and innovative Zymo-Spin™ Technology, following the manufacturer's instructions. The extracted DNA aliquot was stored at -80°C until further testing [16].

RT-PCR: The extracted DNA was then subjected to RT-PCR to detect the *mpb64* gene of the *Mycobacterium tuberculosis* Complex (MTC). The extracted DNA was amplified using QuantiFast® SYBR® Green PCR (Qiagen) and primers (Sigma Aldrich) specific to the *mpb64* gene of MTC. The amplification was performed using the 2× QuantiTect SYBR Green PCR Master Mix. The forward primer sequence was 5´-TCC GCT GCC AGT CGT CTT CC, and the reverse primer sequence was 5´-GTC CTC GCG AGT CTA GGC CA [17]. The amplification process involved an initial hold at 95°C for 15 minutes, followed by cycling with denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and primer extension at 72°C for 30 seconds. This cycle was repeated for 45 rounds, and a final extension was performed at 72°C for seven minutes.

Validation and standardisation of RT-PCR: The RT-PCR validation was conducted using known positive and negative specimens, as well as specimens spiked with the American Type Culture Collection (ATCC) standard strain H37Rv. The *mpb64* gene was detected in all three repeat runs and all dilutions. The analytical sensitivity of the RT-PCR was found to be as low as 10 bacteria/mL of the specimen. The cycling and melting curves of the RT-PCR are shown in [Table/Fig-1].

The diagnostic importance of RT-PCR in the diagnosis of EPTB was evaluated using appropriate statistical parameters, as mentioned in the statistical analysis, considering *Mycobacterium Tuberculosis* culture as the gold standard for the diagnosis of EPTB.



[Table/Fig-1]: Representative image showing amplification curves of positive samples.

STATISTICAL ANALYSIS

The statistical analysis was performed using SPSS software version 26.0 (IBM Corp., Armonk, NY, USA), and the interpretation was based on a p-value <0.05, indicating statistical significance. The analysis aimed to calculate the sensitivity, specificity, PPV, NPV, and p-value of RT-PCR in diagnosing EPTB, considering MTB culture as the gold standard. The p-value was calculated using the Chi-square test.

RESULTS

The overall age of the patients who participated in the study ranged from 20 to 50 years, with a mean age of 27.35±3.93 years. The majority of the patients (130 or 67.01%) were under 30 years old, indicating that genital TB is more common in relatively younger females. A total of 194 samples were included in the study, as six samples failed during DNA extraction and were excluded. Among the tested samples, 56 (28.9%) were positive by RT-PCR, compared to 47 (24.2%) by culture using BacT/Alert 3D. The lowest positivity rate was observed by microscopy, with only 7 (3.6%) positive results [Table/Fig-2].

	Culture		AFB Microscopy	
RT-PCR	Positive	Negative	Positive	Negative
Positive 56 (28.9%)	45	11	7	49
Negative 138 (71.1%)	2	136	0	138
Total (n=194)	47 (24.2%)	147 (75.8%)	7 (3.6%)	187 (96.4%)

[Table/Fig-2]: Comparison of results of RT-PCR with conventional methods (n=194).

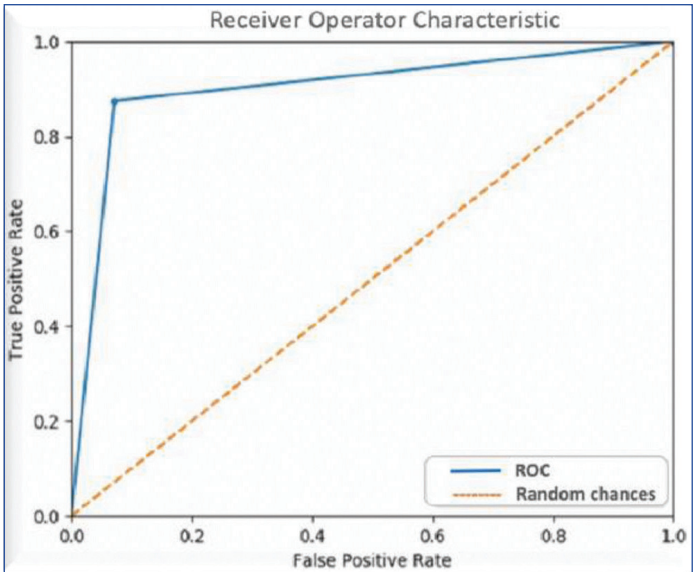
However, 138 (71.1%) samples tested negative by RT-PCR, despite being obtained from clinically suspected cases of GUTB. The average time for detection by culture was 23.6 days. The amplification curves of positive specimens are shown in [Table/Fig-1]. When comparing the results of RT-PCR with conventional methods, RT-PCR showed a sensitivity of 91.9%, specificity of 99.4%, Positive Predictive Value (PPV) of 85.1%, and Negative Predictive Value (NPV) of 93.8% [Table/Fig-3]. The Receiver Operator Characteristic (ROC) curve is shown in [Table/Fig-4].

Total patients (n=194)	Sensitivity	Specificity	Positive Predictive Value (PPV)	Negative Predictive Value (NPV)	p-value
	91.9%	99.4%	85.1%	93.8%	0.0000088

[Table/Fig-3]: Sensitivity and specificity of RT-PCR.
1. The sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and p-value of RT-PCR was calculated considering MTB culture as gold standard for the diagnosis of EPTB.
2. Chi-square statistics was used to calculate the p-value

DISCUSSION

The TB is a major public health issue, particularly in the developing world, including India, and is one of the important causes of morbidity and mortality worldwide. EPTB, though common, is often difficult



[Table/Fig-4]: Receiver Operator Characteristic (ROC) curve.

to diagnose. About 5-45% of all TB cases have extrapulmonary manifestations, and out of the total EPTB cases, 30-40% involve the urogenital tract. It has been observed that about 2-20% of females can develop GUTB after a latency of 5-40 years of PTB [18]. The available routine diagnostic tools do not have sufficient sensitivity to aid in the early diagnosis of EPTB. Conventional methods like AFB smear microscopy have poor sensitivity and specificity, and culture is often time-consuming, resulting in retrospective diagnosis [19].

The clinical utility of early detection of *Mycobacterium tuberculosis* targeting the *mpb64* gene by RT-PCR, including its accuracy in diagnosing GUTB, was evaluated in the present study. Although female genital TB is a chronic disease, the symptoms are often mild. In most cases of GUTB, the fallopian tubes are affected, leading to infertility in females. Women frequently present with atypical symptoms that mimic other gynaecological conditions [20]. A recent study conducted by Chaudhari KV et al., reported *mpb64* RT-PCR as a highly specific test for the diagnosis of *Mycobacterium tuberculosis*. According to them, the sensitivity can be increased by combining IS6110 RT-PCR with *mpb64* RT-PCR for the diagnosis of EPTB [21].

In the present study, RT-PCR was found to be the most sensitive method among the three used to detect MTC. The sensitivity, specificity, PPV, and NPV of RT-PCR were 91.9%, 99.4%, 85.1%, and 93.8%, respectively [Table/Fig-3]. Conventional PCR can also aid in the rapid detection of MTC, but without strict quality control, it may introduce false positives and negatives [22]. On the other hand, various studies have shown that RT-PCR is less prone to contamination and has reported sensitivities of 51.9%-90.3% and specificities of 81.8%-100% for diagnosing TB, depending on the target gene used [23,24]. Therefore, RT-PCR can serve as an important diagnostic test for cases of EPTB where culture and AFB microscopy yield negative results.

In the present study, two samples that were culture positive tested negative by RT-PCR. This discrepancy may be due to the misidentification of Non Tubercular Mycobacteria (NTM) as MTC, as culture and conventional biochemical identification are susceptible to subjective errors. Similar findings were reported by Raveendran R and Wattal C, where out of six samples that were microbiologically positive but PCR negative, two samples were identified as NTM upon further testing [25]. However, in the present study, the authors found 11 samples that were positive by RT-PCR but negative by culture. This could be attributed to the presence of non viable mycobacteria or a low bacillary load resulting from prior treatment with ATT before sample collection.

The present study highlights that RT-PCR provides a significantly higher diagnostic yield, good sensitivity, and specificity in the diagnosis of GUTB compared to microscopy and culture. It may serve as a powerful tool for the early detection of GUTB and the timely initiation of ATT, leading to a significant reduction in morbidity, mortality, and financial burden on the community. Mycobacterial culture, despite being the gold standard method for TB diagnosis, rarely aids in the initiation of ATT due to its time-consuming nature and low sensitivity [26]. Smear microscopy for AFB, although more economical and less time-consuming, suffers from low sensitivity, which is a major drawback. The present study highlights the role of RT-PCR targeting the *mpb64* gene, which can contribute to the early diagnosis of EPTB, enabling prompt initiation of ATT and subsequently reducing morbidity and mortality. Further studies comparing the diagnostic utility of various target genes can aid in selecting the most effective target gene for the early and accurate diagnosis of EPTB.

Limitation(s)

The present study had a few limitations due to a lack of resources:

i) Diagnostic utility of various other target genes, such as IS6110, IS1081, hsp65, mtp40, using multiplex PCR, could not be assessed.

ii) Further testing to rule out the possibility of NTM in RT-PCR positive and culture-negative samples was not performed.

iii) This study was confined only to a limited geographical area with a relatively small sample size. Therefore, the findings cannot be generalised to the entire population.

CONCLUSION(S)

RT-PCR targeting the *mpb64* gene is a specific and effective additional test that helps in the early and accurate diagnosis of EPTB, including GUTB, compared to conventional methods. However, to establish its role as the sole diagnostic test for EPTB, more studies from different geographical regions with a larger sample size are needed. Furthermore, sensitivity can be improved by using multiplex PCR with genus and *Mycobacterium tuberculosis*-specific primers for the accurate detection of *Mycobacterium* spp., thereby minimising false positives and false negatives.

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