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Multiplex PCR targeting *IS6110* and *mpb64* Genes for Detecting Extra-Pulmonary Tuberculosis: A Cross-Sectional Study in a Tertiary Care Center, Kashmir, India

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ABSTRACT

Introduction: Extra-pulmonary tuberculosis (EPTB) is a significant cause of morbidity, and early diagnosis is critical for improving patient outcomes. Conventional diagnostic methods for EPTB often require improvement, highlighting the need for more rapid and sensitive diagnostic procedures. In this cross-sectional study, we aimed to evaluate the diagnostic usefulness of multiplex PCR (mPCR) using *IS6110* and *mpb64* as gene targets for detecting *Mycobacterium tuberculosis* in samples from suspected cases of EPTB. We compared the performance of mPCR with conventional methods, including Ziehl Neelsen (ZN) microscopy, culture in LJ media, and BacT/Alert system. Our study aimed to provide insight into the utility of mPCR and its different targets for diagnosing EPTB in our setting. **Methods**: We conducted a cross-sectional survey of 250 non-repeat clinical samples from extrapulmonary sites to detect *M. tuberculosis*. Both conventional diagnostic methods, including ZN microscopy, culture in LJ media, and BacT/Alert system, and molecular methods, including multiplex PCR (mPCR) using *IS6110* and *mpb64* as gene targets, were performed on the samples. Of the 250 samples, results for all the diagnostic methods were available for 116 samples, which were included in the final analysis. The study population comprised 83 patients with suspected EPTB and 33 controls. **Results**: Among the 83 samples in the EPTB group, conventional diagnostic methods, including ZN microscopy, LJ culture, and BacT/Alert system, showed low positivity rates of 6.02%, 8.43%, and 15.66%, respectively. In contrast, multiplex PCR (mPCR) using *IS6110* and *mpb64* as gene targets showed a significantly higher positivity rate of 79.51%. The *IS6110* gene was amplified in 79.51% of the samples, while *mpb64* was amplified in 49.39%. **Conclusion**: Our study demonstrates that multiplex PCR (mPCR) using *IS6110* and *mpb64* as gene targets is a more sensitive diagnostic method for extra-pulmonary tuberculosis (EPTB) than conventional methods. Both *IS6110* and *mpb64* showed high sensitivity of 100%, but *mpb64* was more specific when compared with the gold standard. Our findings suggest that mPCR, particularly with the inclusion of *mpb64* as the target gene, may be a valuable tool for the early and accurate diagnosis of EPTB.

INTRODUCTION

Tuberculosis (TB) is a major global health problem, classified as pulmonary TB (PTB), extrapulmonary TB (EPTB), or both. In 2019, EPTB cases accounted for 16% of the 7.5 million incident cases worldwide [1]. The diagnosis of EPTB is challenging due to the limitations of conventional diagnostic methods, including low sensitivity and long turnaround time [2]. To overcome these limitations, nucleic acid amplification tests (NAATs) targeting various mycobacterial genes have been explored [3, 4, 5].

In this study, we aimed to evaluate the diagnostic accuracy of multiplex PCR (mPCR) using *IS6110* and

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mpb64 as gene targets for detecting *M. tuberculosis* in samples from patients with suspected EPTB. We compared the performance of mPCR with conventional diagnostic methods, including Ziehl Neelsen (ZN) staining, culture in Lowenstein Jensen (LJ) media, and BacT/Alert system.

The study aimed to provide insight into the diagnostic usefulness of mPCR and its different targets for the accurate and rapid diagnosis of EPTB.

MATERIAL AND METHODS

Definitions. Extrapulmonary tuberculosis (EPTB) involves organs other than the lungs, including but not limited to the pleura, lymph nodes, abdomen, genitourinary tract, skin, joints, bones, and meninges. Bacteriologically confirmed cases were defined as those with the growth of *M. tuberculosis* on culture by any method. Clinically diagnosed cases were based on strong clinical or radiological evidence of tuberculosis. The growth of non-tuberculous mycobacteria (NTM) was considered culture negative for final analysis.

Study subjects. Between April 2016 and April 2017, clinical specimens were collected from patients at the Sher-i-Kashmir Institute of Medical Sciences in Srinagar, J&K, India, a tertiary care institute. Clinical and demographic details were recorded from the laboratory requisition forms.

Ethical approval. The Institutional Ethics Committee approved the study and was exempted from individual informed consent as it was a purely laboratory-based study with anonymized data. Permission was granted under reference number SIMS 1 31/IEC-SKIMS/2016-146, dated April 18, 2016.

Eligibility criteria. Patients of all ages and genders with strong clinical or radiological evidence of tuberculosis and a requisition for AFB smear and mycobacterial cultures on LJ and BacT/Alert system were eligible for the study. Two hundred-fifty non-repeated clinical samples of new suspected tuberculosis cases were selected from extrapulmonary sites.

Exclusion criterion. Confirmed cases of pulmonary tuberculosis and patients currently receiving anti-tubercular treatment were excluded from the study. In addition, samples showing contamination in cultures or molecular methods were also excluded.

Inclusion criterion. The study included samples (n=116) with results available from all conventional (ZN staining and mycobacterial cultures on LJ and BacTAlert) and molecular methods.

Study groups. The samples (n=116) were divided into two groups. Group 1 consisted of the EPTB group (n=83), which was further subdivided into two subgroups: 1) confirmed EPTB cases (n=18) defined by either histopathological examination (HPE) positive (n=5) or culture positive (n=13); and 2) clinically suspected EPTB cases (n=65) defined by either a previous history positive for TB, tuberculin skin test positive (≥10 mm), positive AFB smear microscopy alone, or ambiguous chest X-ray abnormalities, ultrasonography (USG) findings, cytology, computerized tomography (CT) scan, and cystoscopy.

Table 1. Provisional diagnosis and specimen type for extrapulmonary tuberculosis (EPTB) cases (n=83)

Provisional diagnosis		Nature of specimen	No.	Total No. (%)
Tuberculous pleural effusion		Pleural fluid	4	5 (4.15)
		Pleural biopsy	1	
CNS TB	Tuberculous meningitis (TBM)	CSF	2	4 (3.32)
	Tubercular brain abscess	Pus	2	
Gastrointestinal TB (GITB)		Ascitic fluid	1	11 (9.13)
		Peritoneal surface biopsy	1	
		Biopsy from the terminal ileum	4	
		Biopsy from colon	3	
		Biopsy from rectum	2	
Tubercular lymphadenopathy		Aspirate	17	17 (14.11)
Cutaneous tuberculosis		Skin biopsy	5	5 (4.15)
Osteoarticular TB		Pus psoas abscess	1	1 (0.83)
Tubercular breast abscess		Pus	4	4 (3.32)
Genital TB	Infertility secondary to tubercular endometritis	Endometrial curettage	4	7 (5.81)
	Pelvic inflammatory disease	Pouch of Douglas fluid	1	
	Testicular swelling	Aspirate	1	
	Tubercular orchitis	Semen	1	
Urinary TB	TB of the urinary tract	Urine	27	29 (24.07)
	TB of the kidney	Percutaneous nephrostomy fluid	2	
Total				83 (100)

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The control group (n=33) comprised individuals with non-TB infections and various benign and malignant conditions. The samples included pleural fluid (n=1), CSF (n=2), pus (n=2), ascitic fluid (n=3), GI biopsies (n=5), endometrial curettings (n=13), and urine (n=7). Table 1 shows the distribution of the 83 EPTB specimens and their provisional diagnoses.

Sample processing. Each sample was subjected to conventional and molecular methods to detect *M. tuberculosis*. All extrapulmonary samples, except urine and semen, were collected by the treating physician under strict aseptic conditions and sent to the laboratory. Urine was collected as the first-morning sample on three separate days by the patient, while semen was collected in a sterile container by the patient. Sample processing was performed in a class II biosafety cabinet. Samples from "sterile" sites were concentrated by centrifugation, while others were decontaminated using Petroff's method with 4% NaOH. Biopsies were ground in 5 mL saline before processing with decontamination.

One to two milliliters of sediment obtained after concentration/decontamination were used to prepare smears for ZN staining and inoculate BacT/Alert MP (bioMérieux, France) and LJ bottles (HiMedia, India). The remaining sediment was preserved at -20° C for detection of *M. tuberculosis* by multiplex PCR (mPCR).

***M. tuberculosis* identification**. Positive cultures obtained on either LJ or BacT/Alert MP media were subjected to the SD BIOLINE TB Ag MPT64 Rapid test by Standard Diagnostics, Inc. (Republic of Korea) for confirmation as *M. tuberculosis*. No attempt was made to speciate the isolates further.

DNA extraction. The sediments of samples preserved at -20 °C were subjected to DNA extraction using the Quick-gDNA ™ MiniPrep kit (ZymoResearch, Cat No. D3024) and the spin column method, according to the manufacturer's instructions. The extracted DNA was stored at -20 °C until the test was performed.

Multiplex PCR

PCR protocol. Multiplex PCR (mPCR) was performed using primers previously described for amplifying a 123-bp product of the *IS6110* gene (F: 5' CCT GCG AGC GTA GGC GTC GG 3', R: 5' CTC GTC CAG CGC CGC TTC GG 3') and a 240-bp product of the *mpb64* gene (F: 5' TCC GCT GCC AGT CGT CTT CC 3', R: 5' GTC CTC GCG AGT CTA GGC CA 3') [7, 8]. Each reaction mixture consisted of 39.5 µL distilled water, 5 µL PCR buffer (10x), 1 µL dNTPs (10mM), 0.5 µL each of primers P1, P2, P3, and P4 (10 µM), and 0.5 µL Taq polymerase (5 U/µL). Two µL of DNA template was added to 48 µL of the reaction mixture to make a final volume of 50 µL. Amplification was performed on a thermocycler (Applied Biosystems) with an initial denaturation of DNA at 94 °C for 1 min, followed by 40 cycles of 1 min at 94 °C, 1.5 min at 65 °C, and 1.5 min at 72 °C, with a final extension period of 10 min at 72 °C. Amplified products (10 µL) were visualized by electrophoresis in 2% agarose gels stained with ethidium bromide. A 100-bp DNA ladder was used as a molecular weight standard.

A 123-bp band indicated amplification of the *IS6110* gene, while a 240-bp band indicated amplification of the *mpb64* gene. After DNA extraction, all clinical samples were subjected to mPCR. The presence of either band indicated a positive PCR reaction, while the absence of both bands was considered negative (Fig. 1).

Limit of Detection (LOD) of PCR. To validate the sensitivity of our targets, we performed mPCR using known positive and negative samples. We prepared doubling dilutions (10^4, 10^2, 10, 5, 2.5, 1.25, and 0.75 CFU/mL) of 0.5 McFarland (1.5×10^8 CFU/mL) of H37Rv spiked samples, as described previously [6]. After DNA extraction, mPCR was performed on all dilutions and repeated twice. On gel electrophoresis, 123-bp bands of *IS6110* were observed in all dilutions, while 240-bp bands of *mpb64* were observed in all dilutions except 0.75 CFU/mL. Thus, the detection limit was >1 CFU/mL for *mpb64*-PCR and <1 CFU/mL for *IS6110*-PCR.

Quality control. For each PCR batch, we included a positive control (DNA from H37Rv) and a negative control (sterile distilled water). The reaction was considered valid when no band was present in the negative control, and both bands were in a positive control. We used a three-room setup for all PCR experiments to prevent amplicon contamination.

Statistical analysis. Categorical variables were presented as frequency and percentage. We evaluated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the PCR results against the BacT/Alert culture method, which was used as the gold standard, using bivariate two-by-two tables (binary classification method). We used the Online MedCalc software for statistical analysis.

RESULTS

Of the 83 subjects in the EPTB group, the age range was 4-85 years, with a mean of 39.46 years and a median of 37 years. Most patients (N=56) were in the age group of 21-60 years, with a maximum number in the 21-40 age group (N=32). Of the 83 patients, 35 (42.2%) were male, and 48 (57.8%) were female, resulting in an overall male-to-female ratio of 0.72. Females outnumbered males in the 21-40 age group, while males predominated in the >60 age group. The male-to-female ratio was 1 in both the <20 years and 41-60 age groups.

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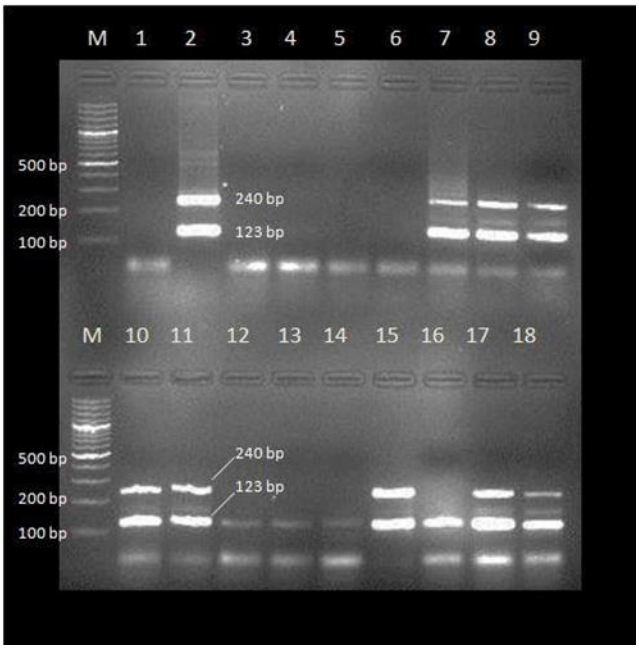

Fig. 1. Detection of *M. tuberculosis* using mPCR targeting *IS6110* and *MPB64*. Electrophoresis separation of the amplicons into a 2% agarose gel is shown across Lanes 1-18. Lane M: 100-bp molecular weight marker. Lane 1: negative control, and Lanes 3, 4, 5, and 6 show no bands in patient samples. Lane 2: positive control, and Lanes 7-11, 15, 17, and 18 showing 123-bp and 240-bp bands indicating the presence of IS6110 and MPB64. Lanes 12, 13, and 14 show 123-bp bands only in patient samples.

Table 2. *M. tuberculosis* (MTB) detection rates for different diagnostic modalities by type of cases

Type of cases	The MTB detection rate with various diagnostic modalities					
	Conventional methods			Molecular methods		
	ZN staining (No./%)	BacT/Alert culture (No./%)	LJ culture (No./%)	mPCR (No./%)	*IS*6110 PCR (No./%)	*mpb*64 PCR (No./%)
Confirmed EPTB cases n=18 (15.51%)	3 (16.66)	13 (72.22)	7 (38.88)	17 (94.44)	17 (94.44)	15 (83.33)
Suspected EPTB cases n=65 (56.03%)	2 (3.07)	0 (0)	0 (0)	49 (75.38)	49 (75.38)	26 (40)
Non -TB cases/ Controls n=33 (28.40%)	4 (12.12)	0 (0)	0 (0)	3 (9.09)	3 (9.09)	0 (0)
Total n=116	9 (7.75)	13 (11.20)	7 (6.03)	69 (59.48)	69 (59.48)	41 (35.34)

In the control group, majority of patients were 21-60 years old, with an overall male-to-female ratio of 0.65. Among the various diagnostic modalities used in the study, mPCR/*IS6110* PCR showed the highest positivity rate (59.48%), followed by *mpb64* PCR (35.34%), BacT/Alert culture (11.20%), ZN staining (7.75%), and LJ culture (6.03%). Among controls, four patients had false-positive results on ZN microscopy, later identified as non-tubercular mycobacteria (NTM) on culture. Additionally, three samples were false-positive on mPCR/*IS6110* PCR, despite being negative on other diagnostic modalities (See Table 2 for details).

In EPTB cases (n=83), the ZN smear was positive in only 5 cases (6.02%), two of which were gastrointestinal TB and three were urinary TB. All of the smears showed paucibacillary TB. BacT/Alert culture showed the highest MTB positivity rates in cases of CNS TB (50%), followed by tubercular lymphadenopathy (29.41%), genital TB (28.57%), tubercular pleural effusion (20%), and urinary TB (10.34%). LJ culture was less efficient than BacT/Alert culture in detecting all forms of EPTB. mPCR showed 100% positivity in CNS, gastrointestinal, lymph node, and cutaneous TB and more than 50% positivity in other forms of EPTB. *IS6110* was a better PCR target for detecting EPTB than *mpb64* (Table 3).

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Table 3. Detection rates for different diagnostic modalities in extrapulmonary tuberculosis (EPTB) cases by provisional diagnosis

Provisional diagnosis	The positivity rate of various diagnostic modalities in EPTB					
	Conventional methods			Molecular methods		
	ZN staining (No. / %)	BacT/Alert culture (No. / %)	L-J culture (No. / %)	mPCR (No. / %)	*IS*6110 PCR (No. / %)	*mpb*64 PCR (No. / %)
Tubercular breast abscess (n=4)	0 (0)	0 (0)	0 (0)	2 (50)	2 (50)	2 (50)
CNS TB (n=4)	0 (0)	2 (50)	2 (50)	4 (100)	4 (100)	4 (100)
Cutaneous TB (n=5)	0 (0)	0 (0)	0 (0)	3 (60)	3 (60)	2 (40)
Genital TB (n=7)	0 (0)	2 (28.57)	1 (14.28)	6 (85.71)	6 (85.71)	4 (57.14)
GITB (n=11)	2 (18.18)	0 (0)	0 (0)	11 (100)	11 (100)	8 (72.72)
Tubercular lymph adenopathy (n=17)	0 (0)	5 (29.41)	3 (17.64)	17 (100)	17 (100)	10 (58.82)
Osteo articular TB (n=1)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)
Tubercular pleural effusion (n=5)	0 (0)	1 (20)	1 (20)	3 (60)	3 (60)	1 (20)
Urinary TB (n=29)	3 (10.34)	3 (10.34)	0 (0)	19 (65.51)	19 (65.51)	9 (31.03)
Total (n= 83)	5 (6.02)	13 (15.66)	7 (8.43)	66 (79.51)	66 (79.51)	41 (49.39)

Table 4. Comparison of Multiplex PCR (mPCR), *IS6110* PCR, and *mpb64* PCR with culture for *M. tuberculosis* (MTB) detection in extrapulmonary tuberculosis (EPTB) group (n=83)

BacT/Alert Culture	mPCR/ *IS*6110 PCR			*mpb*64 PCR		
	Positive	Negative	Total	Positive	Negative	Total
Positive	13	0	13	13	0	13
Negative	54	16	70	28	42	70
Total	67	16	83	41	42	83
Sensitivity	100 % (95% CI,75.29;100)			100% (95%CI,75.29;100)		
PPV*	24.48%(95% CI, 22.20;26.91)			38.46% (95% CI, 31.93;45.44)		
NPV*	100%			100%		
Accuracy*	38.29%(95% CI, 27.82;49.61)			68% (95% CI, 56.85;77.82)		
% of agreement	34.93%			66.26%		
Cohen's k	0.084 (Slight agreement)			0.319 (Fair Agreement)		

*Disease prevalence of 20%

We compared mPCR, *IS6110* PCR, and *mpb64* PCR with BacT/Alert culture in the EPTB and control groups to calculate the sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), and accuracy of these tests. The sensitivity of *IS6110* PCR and *mpb64* PCR was 100%, while the specificity of *mpb64* PCR was also 100%, compared to 90.91% for *IS6110* PCR. *mpb64* PCR also showed better accuracy and percentage of agreement with the gold standard (See Tables 4 and 5 for detailed results).

Table 5. Comparison of Multiplex PCR (mPCR), *IS6110* PCR, and *mpb64* PCR with culture for *M. tuberculosis* (MTB) detection in the control group (n=33)

BacT/Alert Culture	mPCR/*IS*6110 PCR			*mpb*64 PCR		
	Positive	Negative	Total	Positive	Negative	Total
Positive	0	0	0	0	0	0
Negative	3	30	33	0	33	33
Total	3	30	33	0	33	33
Specificity	90.91% (75.67% to 98.08%)			100% (89.42% to 100.00%)		

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DISCUSSION

EPTB presents a significant health challenge in developed and developing countries [9]. Early and accurate diagnosis is crucial in establishing effective and timely therapy, as EPTB is associated with high morbidity and mortality rates [10]. However, diagnosing EPTB remains a challenge due to various factors, including the varied clinical presentation, paucibacillary nature of specimens, inadequate sample volumes, need for invasive procedures to obtain specimens for diagnosis, uneven distribution of microorganisms in samples, and the lack of a universal sample processing technique [5, 10, 11, 12]. In this study, we investigated the detection of EPTB using various conventional and molecular methods. The positivity of all diagnostic modalities was significantly higher in confirmed EPTB cases than in suspected EPTB cases (See Table 2 for more details).

Cerebrospinal fluid (CSF) samples had the highest culture positivity among the extrapulmonary samples, followed by lymph node aspirates. ZN smear positivity was only detected in urinary samples and suspected gastrointestinal tuberculosis (GITB) cases (For more details, refer to Table 3).

In our study, mPCR showed the highest positivity rate of 100% in the central nervous system (CNS) and osteoarticular tuberculosis for both targets. In comparison, *IS6110* showed 100% positivity in lymph nodes and gastrointestinal tuberculosis (GITB). *IS6110* was also superior to *mpb64* in detecting confirmed and suspected EPTB cases. However, none of the samples in our study that were negative for *IS6110* tested positive for *mpb64* by PCR. This finding contrasts with the results of Raj *et al*. (2016), who found *mpb64* positive in 16 samples where *IS6110* was absent [5].

In the control group, four positive samples for ZN smear were identified as non-tubercular mycobacteria (NTM) on culture. At the same time, none tested positive in the molecular tests, demonstrating the specificity of our PCR assays for *M. tuberculosis*. Three false positives were observed with *IS6110* PCR (specificity=90.91%) but none with *mpb64* PCR (specificity=100%). These false positives could be due to cross-contamination during PCR or very low bacterial loads that other modalities, such as microscopy and culture, could not detect. Additionally, the higher copy number of *IS6110* in each mycobacterial cell may lead to the generation of more amplicons and increase the likelihood of laboratory contamination.

Our study found higher rates of EPTB among females compared to males. These findings have been validated by various studies conducted in Southern and Southeastern Asia and the Middle East [13, 14, 15, 16], although many studies have reported a male preponderance [17, 18].

Our study found a lower AFB smear positivity rate of 6.02% compared to the 11.67% reported by other studies [19]. ZN staining has several drawbacks, including its poor sensitivity (25-75% compared to culture), low predictive value, the requirement for high bacterial counts ($>10^4$ organisms/ml) to yield positive results, inability to differentiate between *M. tuberculosis* and non-tuberculous mycobacteria (NTM), and inability to distinguish between viable and dead organisms [4, 12, 20]. In contrast to studies by Pingle *et al*. (2014), which reported a low positivity of 0%, and Fazal-ur-Rehman *et al*. (2013), which reported a high positivity of 26%, our study found a 10.34% smear positivity rate in genitourinary TB. This higher rate could be due to *Mycobacterium smegmatis*, which may be present as a commensal in the genitourinary tract. To avoid false positives, decolorization with acid-alcohol should be performed for all genitourinary samples [19].

In our study, culture on Lowenstein-Jensen (LJ) medium showed a positivity rate of 8.43%, which is higher than the rate reported by Angeby *et al*. (2003) [22]. Variable positivity rates ranging from 12% to 80% have been observed on LJ media in different body fluids from suspected EPTB cases due to the uneven distribution of acid-fast bacilli in these specimens. Culture on LJ medium requires 6-8 weeks and at least 10-100 bacilli/ml to yield positive results [4]. Using ingredients from different sources to prepare LJ medium in other studies could also be a reason for variable LJ positivity rates. Automated cultures such as BacT/Alert have shortened diagnosis time from 4-6 weeks to 2 weeks, but cultures need to be incubated for six weeks before being reported as negative. However, they lack sensitivity and are not cost-effective [4, 23]. Culturing also requires a Biosafety level (BSL-3) laboratory facility [24]. In our study, the BacT/Alert positivity rate was 15.66%, which is higher than the rate reported by Angeby *et al*. (2003), with a positivity rate of 3.45% [22] and lower than that reported by Ghadage *et al*. (2016) with a positivity rate of 30.30% [25].

The WHO has approved NAATs such as PCR, real-time PCR, and LAMP for the diagnosis of both pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB) due to their ability to yield results within a few hours and detect low numbers of mycobacteria (10 bacilli/ml), providing higher accuracy than AFB smear microscopy and faster results than culture [4]. Using two or more gene targets for amplification has increased the diagnostic yield of *M. tuberculosis* infection [2]. However, a significant limitation of NAATs is their inability to differentiate between viable and non-viable organisms, which can result in long-term positive tests in patients on anti-TB medications. False positivity can also occur due to cross-contamination during initial handling or amplicon/carryover contamination [26]. Lower positivity rates can be due to PCR inhibitors, more commonly associated with extrapulmonary specimens than pulmonary specimens [12]. Other factors contributing to lower positivity rates include poor lysis of mycobacteria during the extraction procedure due to the complexity of the cell wall and the non-uniform distribution of the microorganisms in extrapulmonary

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samples. Some clinical strains of *M. tuberculosis* have either a single or no copy of *IS6110*, which can lead to false-negative results and lower positivity rates with *IS6110* PCR in some studies [3, 4].

In our study, the overall positivity rates by mPCR/*IS6110*PCR and *mpb64*PCR were 79.51% and 49.39%, respectively. Similar studies conducted by Negi *et al*. (2007) reported a positivity rate of 77%, while Maurya *et al*. (2011), Raveendran *et al*. (2016), and Makeshkumar *et al*. (2014) reported lower positivity rates of 62.88%, 44.6%, and 26.96%, respectively, using *IS6110*-PCR in EPTB samples [17, 6, 26]. Raveendran *et al*. (2016) reported a low *mpb64* positivity rate of 18.9% [6]. In their study, only one sample out of 148 (0.68%) was positive for *mpb64* and negative for *IS6110*; however, in our study, none of the negative samples for *IS6110* tested positive for *mpb64*.

Our study found that mPCR/*IS6110*PCR and *mpb64*PCR had 100% sensitivity and negative predictive value (NPV) in the EPTB group but a low positive predictive value (PPV) (Table 4). Despite the high positivity rates of our molecular tests compared to culture, our study's accuracy and percentage agreement with the gold standard was low (Tables 2, 3, and 4). This discrepancy could be due to using a suboptimal gold standard for validating PCR results [3, 5]. The absence of a proper gold standard remains a primary hurdle for evaluating new diagnostics in individuals with EPTB [3]. Many studies have used composite gold standards to overcome this shortcoming for comparison.

The Xpert MTB/RIF assay is a cartridge-based NAAT recommended by the WHO for diagnosing EPTB that is revolutionizing TB control by facilitating rapid diagnosis of TB and drug resistance. This user-friendly system yields results within 2 hours, is less prone to cross-contamination, and requires minimal biosafety facilities [28]. However, the cost per test, like other commercial NAATs, is high compared to in-house PCR, which can be a limitation of this system in resource-poor settings like India, which carries nearly one-third of the global TB burden [29].

Our study found that multiplex PCR using *IS6110*PCR and *mpb64*PCR as targets was the most sensitive method for diagnosing EPTB. Although PCR is a rapid, reasonably sensitive, and specific method for diagnosing EPTB cases, it should be interpreted alongside clinical, radiographic, and other laboratory findings. PCR cannot replace the need for smear microscopy for acid-fast bacilli, culture for mycobacteria, and growth-based drug susceptibility testing.

In-house mPCR provides a sensitive, specific, and rapid method for detecting paucibacillary samples. It is an alternative diagnostic assay for early diagnosis and treatment of EPTB in resource-poor settings where the Xpert MTB/RIF assay is unavailable.

Future studies should be conducted on larger samples using a composite gold standard for validating diagnostic methods.

Although our study provides valuable insights, some limitations should be considered when interpreting our findings. Our study was laboratory-based, and clinical definitions were based on the laboratory requisition form. The HIV status of most patients was unknown, and the number of samples from different sites was relatively low and needed to be more uniform. While 250 samples were tested, only 116 with results available for all tests were included in the study, which could be considered a limitation due to the small sample size. Additionally, using culture as a gold standard for evaluating our molecular methods may not be ideal, which is another limitation of our study.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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