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Comparative Evaluation of CFX96TM Real Time PCR with Conventional PCR for Rapid Diagnosis of *Mycobacterium tuberculosis* Complex in Clinical Isolates

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Abstract

Background: Globally, Tuberculosis (TB) still remains a major public health problem. In India, Pulmonary Tuberculosis (PTB) is the most common form of the disease; however, Extrapulmonary Tuberculosis (EPTB) comprises 10 to 20% of all TB cases. The diagnosis of EPTB cases is difficult because of paucibacillary nature and consequently is associated with low sensitivity of Zhiel-Neelson (ZN) smear and culture on Lowenstein-Jensen (LJ) media as gold standard. The present study comparatively evaluates the utility of real time quantitative PCR over nested PCR and other conventional techniques for the detection of M. tuberculosis Complex (Mtc) in clinical isolates of PTB and EPTB samples at a tertiary care centre, Shri Ram Murti Smarak Institute of Medical Sciences (SRMSIMS), Bareilly.

Methods and Findings: In total, 205 both pulmonary (24) and extrapulmonary (181) specimens were processed for ZN smear, culture on LJ media, nested PCR and real time quantitative PCR using IS*6110* as a gene target. Out of 205 samples, none of the sample was found to be smear-positive and only 28 (14%) samples were found to be culture positive. The nested PCR and real time quantitative PCR positivity was observed 100% in culture positive specimens. However in majority of culture negative specimens the sensitivity was 100% in both nested PCR and real time quantitative PCR assays for EPTB and PTB specimens. The specificity was better in case of real time quatitative PCR as compared to nested PCR, 45.8% and 20% in case of nested PCR for nonrespiratory and respiratory specimens respectively. The specificity was increased upto 86.1 and 100% with real time quantitative PCR for EPTB and PTB cases respectively.

Conclusions: The combined analysis of nested PCR, real time quantitative PCR and other lab investigations can be very useful in the rapid diagnosis of *M. tuberculosis* in paucibacillary extrapulmonary tuberculosis samples in Indian scenario.

Keywords: Pulmonary tuberculosis; Extrapulmonary tuberculosis; Nested PCR; Real time quantitative PCR; *Mycobacterium tuberculosis* complex

Introduction

Tuberculosis (TB) is a major global health problem. The World Health Organization (WHO) estimates 9.6 million incident cases annually, out of which it is estimated that 1.8 million cases are from India [1]. Early diagnosis and identification of TB is essential in introducing timely and efficient treatment. Diagnosis of TB is mainly based on clinical presentation, histopathology, and the demonstration of Acid-Fast Bacilli (AFB) in smears and the isolation of *Mycobacterium tuberculosis* (MTB) from culture. Culture for MTB usually takes four to six weeks to grow on solid media, delaying time to results and hence treatment. These diagnostic criteria have limitations that include atypical clinical presentations of disease, and poor sensitivity and specificity of AFB and microscopy, particularly with Extrapulmonary TB (EPTB) due to the paucibacillary nature of extrapulmonary specimens and because the signs and symptoms of disease can be non-specific [2,3]. EPTB is a significant health problem in both developing and developed countries [4]. EPTB constitutes about 15 to 20% of all cases of TB in immunocompetent patients and accounts for more than 50% of the cases in HIV-positive (immunocompromised) individuals [5].

Nucleic Acid Amplification Test (NAAT) offers new prospective for the diagnosis of EPTB in a few hours over culture with a higher sensitivity and specificity. There are different methods of amplification wherein the *Mycobacterium* is amplified by either the Polymerase Chain Reaction (PCR) technique; Transcription Mediated Amplification (TMA), or other forms of nucleic acid amplification methods. PCR technique can be based on conventional DNA amplification, nested-PCR, or real time PCR. One of the first amplification methods most widely used for the detection of *M. tuberculosis* in respiratory samples is the commercial PCR kit Cobas Amplicor MTB (Roche Diagnostics,

Indianapolis, IN), which is based on the amplification of a 584-bp region of the 16S rRNA gene common to all mycobacteria [6,7]. The main drawback has been reported using this kit, particularly with extrapulmonary samples, is due to the presence of taq inhibitors and contamination [8].

One of the most widely used molecular techniques for the detection of MTB is the commercial PCR kit GeNeiTM amplification reagent set for MTB (GeNei, Bangalore, India). This test is based on the principle of single-tube nested PCR method, which amplifies the 1191-bp repetitive insertion sequence IS*6110* that is usually present 6-20 times in the *M. tuberculosis* complex genome although as few as one copy has been observed [9]. IS*6110*-PCR is a more rapid, sensitive and specific method in comparison to others routinely used in clinical laboratories [10,11]. Commercially available PCR kits with specific procedures and controls may help to reduce interlaboratory variation. The major limitation using this kit is that we cannot quantitate the Mycobacteria in specimens. Even so, this is still generally considered the molecular reference technique [12].

Real time PCR has been proposed for detection and quantitation of *M. tuberculosis* due to faster results and lower risk of contamination [13]. MTB quantitation helps in the diagnosis and management of patients with TB and used to determine the response to the treatment and disease progression. This assay uses highly specific probes for accurate and sensitive detection of MTB complex. Compared with conventional PCR, quantitative real time PCR facilitates quantitation of nucleic acids, as well as automation and computerization of data with enhanced sensitivity and specificity in both pulmonary and extrapulmonary specimens [14]. Previous work has demonstrated that the real time PCR assay displays high percentages of sensitivity and specificity for the detection of MTB complex isolates, particularly in smear-positive respiratory specimens [15].

In the present study we comparatively evaluated the utility of CFX96TM real time quantitative PCR over nested PCR of the multi-copy insertion sequence IS*6110* along with smear and culture for the rapid detection of MTB complex in majority of paucibacillary extra pulmonary clinical isolates in Indian scenario.

Methods

Study specimens

The present study was conducted on the samples of suspected MTB patients attending the out and indoor patient departments (Medicine, TB and Chest Diseases, Obstetrics and Gynaecology, Paediatrics, Orthopedics and General Surgery) of Shri Ram Murti Smarak Institute of Medical Sciences (SRMS IMS), Bareilly, India from Nov 2014 to Nov 2015. The samples were processed at Central Research Laboratory, Department of Biochemistry at SRMS IMS, Bareilly. Ethical approval was not needed for the current study as all samples were received for the clinical diagnosis.

The clinical specimens used in this study were collected from patients of all age group and both sexes with suspected MTB infection on the basis of clinical criteria or to rule out these infections. After collection specimens were divided into two parts; one part of the specimen was kept at -20°C for PCR and real time PCR and another part of the sample was processed for staining and culture. Specimens contained both pulmonary and extrapulmonary specimens. Specimens from 17 unique anatomical sites were tested. In total, 205 specimens were tested. These comprised 13 ascitic fluid, 3 Bronchoalveolar Lavage (BAL) fluid, 3 blood specimen, 20 bronchial wash specimen, 8 Cerebrospinal Fluid (CSF), 17 endometrial tissue, 1 gastric aspirate, 46 menstrual blood, 1 paraventral swelling fluid, 1 pelvic drain fluid, 9 pericardial fluid, 56 pleural fluid, 3 pus, 1 splenic abscess aspirate, 1 sputum, 1 synovial fluid and 21 urine specimens (**Table 1**).

Table 1 Clinical specimens used for comparative evaluation of PCR and Real time PCR.

Specimen	Sample Type	No.
Pulmonary	BAL	3
	Bronchial wash	20
	Sputum	1
Extrapulmonary	Ascitic fluid	13
	Blood	3
	Cerebro Spinal Fluid	8
	Endometrial Tissue	17
	Gastric Aspirate	1
	Menstrual Blood	46
	Paraventral swelling fluid	1
	Pelvic drain fluid	1
	Pericardial fluid	9
	Pleural fluid	56
	Pus	3
	Splenic aspirate	1
	Synovial fluid	1
	Urine	21

Mycobacterial culture and smear

All the specimens were subjected to digestion and decontamination using N-acetyl L-Cysteine (NALC)-NaOH method and then centrifuged at 8,000 rpm for 15 minutes. The pellet was resuspended in 2 ml of sterile 0.067 M phosphate buffer [16]. All specimens used in this study were fully processed by digestion, decontamination and concentration. Processed specimen sediment was used to inoculate a Becton Dickinson Bactec MGIT tube and a Lowenstein-Jensen (LJ) slant (Remel, Lenexa, KS), which were incubated for 6 and 8 weeks, respectively. Smears of processed sediment were stained with

Ziehl Neelsen (ZN) stain and examined for Acid-Fast Bacilli (AFB) [17].

Nucleic acid extraction

The deposits of the clinical samples obtained after processing with NALC-NaOH and other appropriate method described earlier (Kent & Kubica, 1985) were used for DNA extraction. DNA was extracted from the clinical specimens using the commercially available kit amplification reagent set for *M. tuberculosis* (GeneiTM, Bangalore, India) (Cat no. 610670300011730) and by QIAamp® DNA mini kit (Qiagen, Germany) (Cat no. 51304) with one initial additional step. The preliminary processed materials as described above were kept at 80°C for 10 min on dry heat block for inactivation of possible mycobacteria. The material was then further processed as per the guidelines of the manufacturer of the kit to obtain the DNA.

Amplification of the IS*6110* gene (123 bp) of *M. tuberculosis* by nested polymerase chain reaction (nPCR), a single tube nPCR was performed using the proprietary IS*6110* primer sequences (custom synthesized by Bangalore Genei, Bangalore, India) targeting *M. tuberculosis*. The DNA was amplified with forward (5'-CCTGTCCGGGACCACCCGCGGCAA-3') and reverse primer (5'GGATCCTGCGAGCGTAGGCGTCGG3') of the outer region of *M. tuberculosis* and the first product was amplified with the inner primer IS*6110*f (5'-CCT GCG AGC GTA GGC GTC GG-3') and IS*6110*r (5'-CTC GTC CAG CGC CGC TTC GG-3') in the second amplification per the manufacturer's instructions.

The PCR conditions for outer sense primers for the first round of amplification were as follows: initial denaturation at 22°C for 10 minutes, 94°C for 5 minutes, 20 cycles of 94°C for 30 seconds, 68°C for one minute, 72°C for one minute, and a final extension of 72°C for 7 minutes. The PCR conditions using the inner set of primers for the second amplification consisted of initial denaturation step at 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 68°C for 30 seconds, 72°C for 30 seconds and a final extension of 72°C for 7 minutes.

The detection of amplified PCR products was determined using agarose gel (2%) electrophoresis stained with ethidium bromide (0.5 µg/ml) and subsequently visualized on the 260 nm wavelength UV transilluminator of the gel documentation system (UVP, Upland, CA, USA). A result was considered positive for the target when a well-defined DNA band corresponding to the sample was observed along with the controls and molecular weight marker. Samples which had amplified products measuring 123 bp for IS*6110* were considered positive.

Real time PCR

Real time PCR amplification for MTB Complex was performed using the careTB PCR assay kit (Qiagen, Germany) (Cat no. 4831024), in accordance with the manufacturer's protocol in a CFX96TM real time system (BIO-RAD). This qualitative real time PCR test kit for *M. tuberculosis* uses in vitro nucleic acid amplification by PCR in combination with real time detection of fluorescent probes for the detection and quantification of MTB DNA (gene target IS*6110* fragment), with a sensitivity of ten bacteria in every milliliter of sample. The cycling conditions were 1 min at 95°C and 40 cycles of 5 s at 95°C, 30 s at 60°C (single acquisition of fluorescence signals). The CFX96TM analyzer determined the Cycle threshold value (Ct) for the DNA of *M. tuberculosis* in sample and checked whether the Ct values of the one high positive control, one critical positive control, 4 quantification standards (QS 1-4) and one negative control were within the normal ranges. The MTB (+) control contains approximately 20 copies/test of *M. tuberculosis* plasmid DNA sequence, for QS 1-4, Ct values were found to be 21, 25, 29 and 32 copies respectively, and (-) control the Ct values was above the limit for the assay or no Ct value was obtained.

Quality control

Reagents were aliquoted and each aliquot was used only once. Sterile microfuge tubes and PCR tubes were used for the PCR assay. Reagent preparation, DNA extraction, DNA amplification and detection were performed in separate rooms to avoid cross-contamination of amplicons. A positive control was included in each test and distilled water was included as a negative test control. Uracil-N-Glycosylase (UNG) was used in the amplification process to avoid post PCR DNA contamination.

Statistical Analysis

Data were analysed using SPSS 15.0 (Statistical Package for the Social Sciences, Chicago, IL, USA) for Windows. Performance of PCR-based NAAT was reported in terms of sensitivity and specificity at 95% confidence interval along with their p-values.

Results

This study established the significance of nested PCR and real time PCR over other conventional gold standard techniques for the early diagnosis of MTB complex in clinical samples. A total of 205 clinically suspected TB samples from 17 different pulmonary (24) and extrapulmonary (181) parts of the body were collected and processed for diagnosis of MTB complex (**Table 1**). Out of 205 samples, none of the sample was found to be AFB smear-positive after ZN staining and 14% (28/205) were found to be culture positive and 86% (177/205) were found to be culture negative on LJ media (as gold standard). Furthermore, maximum 100% samples were positive in case of splenic aspirate (1/1), 33% in case of BAL (1/3), 25% in case of pleural fluid (14/56), 23% in case of ascitic fluid (3/13), 15% in case of bronchial wash (3/20), 10% in case of urine (2/21), 9% in case of menstrual blood (4/46). No samples were found positive for blood, CSF, endometrial tissue, gastric aspirate, paraventral swelling fluid, pelvic drain fluid, pericardial fluid, pus, synovial fluid and sputum. For

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comparative analysis all the specimens were subjected to PCR and real time PCR analysis (**Table 2**).

Table 2 Culture positivity (gold standard) in both pulmonary and extrapulmonary suspected TB samples.

Samples		**Positive N (%)**
Type	No.	
Ascitic fluid	13	3 (23)
Blood	3	0 (0)
Cerebro Spinal Fluid	8	0 (0)
Endometrial Tissue	17	0 (0)
Gastric Aspirate	1	0 (0)
Menstrual Blood	46	4 (9)
Paraventral swelling fluid	1	0 (0)
Pelvic drain fluid	1	0 (0)
Pericardial fluid	9	0 (0)
Pleural fluid	56	14 (25)
Pus	3	0 (0)
Splenic aspirate	1	1 (100)
Synovial fluid	1	0 (0)
Urine	21	2 (10)
Total EPTB cases	181	24 (13)
BAL	3	1 (33)
Bronchial wash	20	3 (15)
Sputum	1	0 (0)
Total PTB cases	24	4 (17)

PCR Results

This study showed that the PCR positivity of IS*6110* gene target was found to be 100% in culture positive paucibacillary TB cases. Further the specificity was determined >95% for both tests in true negative healthy controls or non-TB subjects. The sensitivity and specificity of PCR with Positive Predictive Value (PPV) and Negative Predictive Value (NPV) in culture negative various clinical TB cases are given in **Table 3**. Our data revealed sensitivity and specificity of PCR for paucibacillary extrapulmonary cases were 100% (95% CI: 82.8-100%) with p-value 0.45 and 45.8% (95% CI: 37.9-54%) with P value 0.21 respectively. The PPV was 22% (95% CI: 14.9-31.2%) with P value 0.04 and NPV was 100% (95% CI: 93.7-100%) with P value 0.07. The sensitivity and specificity for pulmonary cases were found to be 100% (95% CI: 39.6-100%) with P value 0.87 and 20% (95% CI: 6.7-44.3%) with P value 0.72 respectively. The PPV was 20% (95% CI: 6.7-44.3%) with P value 0.16 and NPV was 100% (95% CI: 39.6-100%) with P value 0.03 (**Table 3**). Though blood, CSF, gastric aspirate, paraventral swelling fluid, pelvic drain fluid and pus were completely negative in culture but gave positive result in PCR. Synovial fluid and sputum did not give any positive result in both methods (culture and PCR) but it's not conclusive in comparative analysis because sample numbers were too low for comparison.

Real Time PCR results

Simultaneously, with conventional PCR, all the samples (205) were subjected to real time PCR analysis. All the culture and PCR positive samples were also positive for real time PCR. Statistical data for performance are shown in **Table 4**. Out of 205 (181 extrapulmonary and 24 pulmonary) paucibacillary TB cases, the sensitivity and specificity of real time PCR for extrapulmonary cases were found to be 100% (95% CI: 95.7–100%) with P value 0.87 and 86.1% (95% CI: 75.4–92.8%) with P value 0.04 respectively. The PPV was 91.6% (95% CI: 84.7–95.7%) with P value 0.02 and NPV was 100% (95% CI: 92.7–100%) with P value 0.44. The sensitivity and specificity for pulmonary cases were found to be 100% (95% CI: 79.9–100%) with P value 0.37 and 100% (95% CI: 39.6–100%) with P value 0.90 respectively. The PPV was 100% (95% CI: 79.9–100%) with P value 0.47 and NPV was 100% (95% CI: 39.6–100%) with P value 0.98 (**Table 4**).

Table 3 PCR positivity in culture negative suspected TB specimens.

Samples		**Culture**	**PCR positive N (%)**	**Sensitivity (%) (95%CI)**	**Specificity (%) (95%CI)**	**PPV (95%CI)**	**NPV (95%CI)**
Type	**No.**						
Ascitic fluid	13	Pos (n=3)	3 (100)	100	20	27.3	100
		Neg (n=10)	8 (80)	(31.0-100)	(3.1-56.7)	(7.4-61.0)	(20.0-100)
		Total no. (%)	11 (85)	p-value-0.23	p-value- 0.07	p-value0.12	p-value-0.32
Blood	3	Pos (n=0)		-	33.4	0	100
		Neg (n=3)	2 (67)		(1.3-87.3)	(0-81.2)	(5.1-100)
		Total no. (%)	2 (67)		P value-0.43	P value-0.57	P value-0.44

Cerebro Spinal Fluid	8	Pos (n=0)		-	25.3	0	100
		Neg (n=8)	6 (75)		(4.2-64.1)	(0-48.3)	(20-100)
		Total no. (%)	6 (75)		P value-0.43	P value-0.43	P value-0.43
Endometrial Tissue	17	Pos (n=0)		-	71.1	0	100
		Neg (n=17)	5 (29)		(44.2-88.3)	(0-54.3)	(70.2-100)
		Total no. (%)	5 (29)		P value-0.12	P value-0.23	P value-0.11
Gastric Aspirate	1	Pos (n=0)		-	0	0	-
		Neg (n=1)	1 (100)		(0-94.5)	(0-94.5)	
		Total no. (%)	1 (100)		P value-0. 74	P value-0.43	
Menstrual Blood	46	Pos (n=4)	4 (100)	100	53.9	17.7	100
		Neg (n=42)	20 (48)	(40.1-100)	(37.7-68.6)	(5.8-38.1)	(82.3-100)
		Total no. (%)	24 (52)	P value-0.43	P value-0.31	P value-0.02	P value-0.01
Paraventral swelling fluid	1	Pos (n=0)		-	0	0	-
		Neg (n=1)	1 (100)		(0-94.5)	(0-94.5)	
		Total no. (%)	1 (100)		P value-0.32	P value-0.43	
Pelvic drain fluid	1	Pos (n=0)		-	0	0	-
		Neg (n=1)	1 (100)		(0-94.5)	(0-94.5)	
		Total no. (%)	1 (100)		P value-0.15	P value-0.25	
Pericardial fluid	9	Pos (n=0)		-	77.8	0	100
		Neg (n=9)	2 (22)		(40.1-96.1)	(0-80.2)	(56.1-100)
		Total no. (%)	2 (22)		P value-0.75	P value-0.27	P value-0.33
Pleural fluid	56	Pos (n=14)	14 (100)	100	47.6	38.8	100
		Neg (n=42)	22 (52)	(73.2-100)	(32.2-63.3)	(23.6-56.4)	(79.9-100)
		Total no. (%)	36 (64)	P value-0.54	P value-0.25	P value-0.70	P value-0.22
Pus	3	Pos (n=0)		-	33.4	0	100
		Neg (n=3)	2 (67)		(1.3-87.3)	(0-81.2)	(5.1-100)
		Total no. (%)	2 (67)		P value- 0.04	P value-0.15	P value-0.07
Splenic aspirate	1	Pos (n=1)	1 (100)	-	0	0	-
		Neg (n=0)			(0-94.5)	(0-94.5)	
		Total no. (%)	1 (100)		P value- 0.23	P value-0.37	
Synovial fluid	1	Pos (n=0)		0	-	-	0
		Neg (n=1)	0	(0-94.5)			(0-94.5)
		Total no. (%)	0	P value-0.45			P value-0.44
Urine	21	Pos (n=2)	2 (100)	100	21.1	11.2	100
		Neg (n=19)	15 (79)	(19.8-100)	(7.0-46.1)	(2.1-37.7)	(39.6-100)
		Total no. (%)	17 (81)	P value-0.07	P value-0.23	P value-0.32	P value-0.05
Total EPTB cases	181	Pos (n=24)	24 (100)	100	45.8	22	100
		Neg (n=157)	85 (54)	(82.8-100)	(37.9-54.0)	(14.9-31.2)	(93.7-100)
		Total no. (%)	109 (60)	P value-0.45	P value-0.21	P value-0.04	P value-0.07

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BAL	3	Pos (n=1)	1 (100)	100	0(0-80.2)	33.3	-
		Neg (n=2)	2 (100)	(5.4-100)	P value-0.89	(1.8-87.5)	
		Total no. (%)	3 (100)	P value-0.06		P value- 0.27	
Bronchial wash	20	Pos (n=3)	3 (100)	100	17.6	17.6	100
		Neg (n=17)	14 (82)	(31.0-100)	(4.7-44.2)	(4.7-44.2)	(31.0-100)
		Total no. (%)	17 (85)	P value-0.05	P value-0.48	P value-0.04	P value-0.32
Sputum	1	Pos (n=0)		0	-	-	0
		Neg (n=1)	0	(0-94.5)			(0-94.5)
		Total no. (%)	0	P value-0.36			P value-0.57
Total PTB cases	24	Pos (n=4)	4 (100)	100	20	20	100
		Neg (n=20)	16 (80)	(39.6-100)	(6.7-44.3)	(6.7-44.3)	(39.6-100)
		Total no. (%)	20 (83)	P value- 0.87	P value-0.72	P value-0.16	P value-0.03

Table 4 Real time PCR positivity in PCR negative suspected TB specimens.

Samples		PCR	Real time PCR positive N (%)	Sensitivity (%) (95%CI)	Specificity (%) (95%CI)	PPV (95%CI)	NPV (95%CI)
Ascitic fluid	13	Pos (n=11)	11 (100)	100	100	100	100
		Neg (n=2)	0	(67.9-100)	(19.8-100)	(67.9-100)	(19.8-100)
		Total no. (%)	11 (85)	P value- 0.76	P value- 0.42	P value- 0.33	P value- 0.07
Blood	3	Pos (n=2)	2 (100)	0	0	0	0
		Neg (n=1)	0	(0-94.5)	(0-80.2)	(0-80.2)	(0-94.5)
		Total no. (%)	2 (67)	P value- 0.87	P value- 0.92	P value- 0.56	P value- 0.09
Cerebro Spinal Fluid	8	Pos (n=6)	6 (100)	100	100	100	100
		Neg (n=2)	0	(51.7-100)	(19.8-100)	(51.7-100)	(19.8-100)
		Total no. (%)	6 (75)	P value- 0.32	P value- 0.17	P value- 0.26	P value- 0.04
Endometrial Tissue	17	Pos (n=5)	5 (100)	100	91.7	83.3	100
		Neg (n=12)	1 (8)	(46.3-100)	(59.7-99.6)	(36.4-99.1)	(67.8-100)
		Total no. (%)	6 (35)	P value- 0.15	P value- 0.18	P value- 0.20	P value- 0.27
Gastric Aspirate	1	Pos (n=1)	1 (100)	100	-	100	-
		Neg (n=0)		(4.0-100)		(5.4-100)	
		Total no. (%)	1 (100)	P value- 0.31		P value- 0.33	
Menstrual Blood	46	Pos (n=24)	24 (100)	100	90.9	92.3	100
		Neg (n=22)	2 (9)	(82.8-100)	(69.3-98.4)	(73.4-98.6)	(79.9-100)
		Total no. (%)	26 (57)	P value- 0.40	P value- 0.43	P value- 0.41	P value- 0.47
Paraventral swelling fluid	1	Pos (n=1)	1 (100)	100	-	100	-
		Neg (n=0)		(4.0-100)		(5.4-100)	
		Total no. (%)	1 (100)	P value- 0.76		P value- 0.99	
Pelvic drain fluid	1	Pos (n=1)	1 (100)	100	-	100	-
		Neg (n=0)		(4.0-100)		(5.4-100)	

		Total no. (%)	1 (100)	P value- 0.66		P value- 0.55	
Pericardial fluid	9	Pos (n=2)	2 (100)	100	71.4	50	100
		Neg (n=7)	2 (29)	(19.7-100)	(30.2-94.8)	(9.1-90.8)	(46.3-100)
		Total no. (%)	4 (44)	P value- 0.43	P value- 0.23	P value- 0.73	P value- 0.07
Pleural fluid	56	Pos (n=36)	36 (100)	100	80	90	100
		Neg (n=20)	4 (20)	(87.9-100)	(55.7-93.3)	(75.4-96.7)	(75.9-100)
		Total no. (%)	40 (71)	P value- 0.13	P value- 0.15	P value- 0.23	P value- 0.42
Pus	3	Pos (n=2)	2 (100)	100	100	100	100
		Neg (n=1)		(19.8-100)	(5.5-100)	(19.8-100)	(5.5-100)
		Total no. (%)	2 (67)	P value- 0.05	P value- 0.04	P value- 0.74	P value- 0.72
Splenic aspirate	1	Pos (n=1)	1 (100)		0	0	
		Neg (n=0)		-	(0-94.5)	(0-94.5)	-
		Total no. (%)	1 (100)		P value- 0.43	P value- 0.99	
Synovial fluid	1	Pos (n=0)		0			0
		Neg (n=1)	0	(0-94.5)	-	-	(0-94.5)
		Total no. (%)	0	P value- 0.21			P value- 0.88
Urine	21	Pos (n=17)	17 (100)	100	75	94.4	5.5
		Neg (n=4)	1 (25)	(77.1-100)	(21.9-98.6)	(70.6-99.7)	(0.3-29.4)
		Total no. (%)	18 (86)	P value- 0.45	P value- 0.02	P value- 0.04	P value- 0.04
Total EPTB cases	181	Pos (n=109)	109 (100)	100	86.1	91.6	100
		Neg (n=72)	10 (14)	(95.7-100)	(75.4-92.8)	(84.7-95.7)	(92.7-100)
		Total no. (%)	119 (66)	P value- 0.87	P value- 0.04	P value- 0.02	P value- 0.44
BAL	3	Pos (n=3)	3 (100)		0	0	
		Neg (n=0)		-	(0-69.0)	(0-69.0)	-
		Total no. (%)	3 (100)		P value- 0.52	P value- 0.99	
Bronchial wash	20	Pos (n=17)	17 (100)	100	0	85	
		Neg (n=3)		(77.1-100)	(0-69.0)	(61.1-96.0)	-
		Total no. (%)	17 (85)	P value- 0.68	P value- 0.97	P value- 0.56	
Sputum	1	Pos (n=0)			0	0	
		Neg (n=1)	0	-	(0-94.5)	(0-94.5)	-
		Total no. (%)	0		P value- 0.86	P value- 0.77	
Total PTB cases	24	Pos (n=20)	20 (100)	100	100	100	100
		Neg (n=4)		(79.9-100)	(39.6-100)	(79.9-100)	(39.6-100)
		Total no. (%)	20 (83)	P value- 0.37	P value- 0.90	P value- 0.47	P value- 0.98

Discussion

In India, TB remains a major global public health problem. Conventional methods including smear and culture, used in the diagnosis of EPTB, have poor sensitivity due to the paucibacillary load in the samples. The uses of less sensitive conventional methods have contributed to the difficulties in managing patients with EPTB [18]. Problems can arise when clinical specimens contain very few *Mycobacterium* species and their slow growth rate limit detection by the conventional method such as acid-fast staining and bacterial culture. The early diagnosis of TB helps in early treatment and thus preventing the possible transmission of the infection. In recent years, rapid diagnostic tests based on molecular techniques with high sensitivity have been developed [18,19]. With the development of novel and rapid molecular techniques, this

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delay in the accurate detection of pathogen in AFB smear-negative paucibacillary specimens, is minimized.

In order to identify TB in suspected patients, PCR was performed by amplification of the target nucleic acid regions that uniquely identify the MTB complex. The only repetitive target which is so far available is an IS*6110* insertion sequence, which belongs to the IS3 family and is found in almost all members of the MTB complex [9,10]. IS*6110* specific for the MTB complex generally occurs in 10 to 15 copies per cell, which are present in a wide variety of chromosomal sites, making it an ideal target for amplification [9,18,19].

Current study showed the different PCR positive rates in various categories of body fluids. Various sensitivity of PCR using IS*6110* has been reported in extrapulmonary samples i.e. 74.1% in tissue [20], 83% in pleural fluid, pleural tissue, and lymph node [21], 69.1 % and 87.5% in lymph node [22,23], 75% in clinical samples [24], 40% in EPTB [25]. But in our findings by IS*6110* gene target, sensitivity was increased in ascitic fluid (100%), pleural fluid (100%), menstrual blood (100%) and urine (100%). On the other hand, sensitivity was found to be less in few clinical samples i.e. blood, CSF, endometrial tissue, paraventral swelling fluid, pelvic drain fluid, pericardial fluid, pus, synovial fluid and sputum. We cannot conclude that the sensitivity is poor in these fluids because the sample size is less for analysis. Our data revealed the significant importance of *M. tuberculosis* PCR in the diagnosis of paucibacillary smear negative cases in Indian scenario.

Present study showed that real time PCR appears to be equally effective in both respiratory and nonrespiratory samples. Results for sensitivity were found to be similar in both PCR and real time PCR methods (ranging from 0-100% indifferent clinical specimens; overall sensitivity, 100%). Sensitivity for EPTB and PTB was 100% for both PCR and real time PCR assays. Findings for specificity were better in case of real time PCR as compared to PCR, 45.8% ($P = 0.21$) and 20% ($P = 0.72$) in case of PCR for nonrespiratory and respiratory specimens respectively. The specificity was significantly higher 86.1% ($P = 0.04$) and 100% ($P = 0.90$) with real time PCR for EPTB and PTB cases. PPV was found to be 20% ($P = 0.16$) and 22% ($P = 0.04$) for PTB and EPTB respectively but the NPV was found to be 100% ($P = 0.05$) for both the PTB and EPTB specimens in PCR assay. In case of real time PCR assay the PPV was found to be 91.6% ($P = 0.02$) and 100% ($P = 0.47$) for EPTB and PTB respectively whereas the NPV was found to be 100% ($P = 0.71$) for both the specimens as in PCR assay. In our study the sensitivity and PPV were comparatively better than, reported for the Cobas Amplicor MTB test in nonrespiratory samples while specificity and NPV were similar to those recorded for other molecular techniques used in nonrespiratory samples [26]. In general terms, the CFX96TM real time PCR assay performed better than the conventional PCR assay for EPTB in terms of sensitivity and specificity.

Molecular techniques obviously, are considerably more expensive than traditional culture methods. Since these can provide results in a matter of hours, whereas the reference culture method takes days, so these techniques represent a major contribution to the detection of *M. tuberculosis*. Real time PCR techniques with enhanced sensitivity and specificity facilitate quantitation of nucleic acids in a much-reduced response time and also provides visualization of amplification curves. The major limitation of these techniques is that we cannot distinguish between DNA of viable and non-viable microorganisms. Hence for this reason, although these assays give quantitative analysis, these should not be used for monitoring patient progress or treatment efficacy.

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Competing and Conflicting Interests

The authors declare no conflicts of interest.

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