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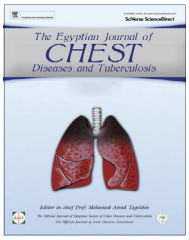

ORIGINAL ARTICLE

Is a composite reference standard (CRS) an alternative to culture in assessment and validation of a single tube nested in-house PCR for TB diagnosis?

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KEYWORDS

CRS;
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Abstract *Introduction:* Delay in diagnosis of paucibacillary extra pulmonary tuberculosis (TB) and of smear negative TB has hampered the efforts taken by Control Programs to curb its spread. Better efforts to control spread of TB require more accurate and rapid diagnostic test.

Aims: To facilitate early diagnosis of TB directly from clinical specimens, we have standardized and validated the use of a single tube in-house nested PCR in comparison against culture and composite reference standard (CRS).

Methods: Single tube nested PCR was performed using primers targeting Insertion Sequence (IS) 6110 of *Mycobacterium tuberculosis complex*. Microbiological techniques includes AFB smear microscopy, and cultivation on solid egg-based medium (Löwenstein–Jensen [LJ]) and on liquid culture medium using BACTEC MGIT 960 system, BD Microbiology Systems.

Results: The sensitivity and specificity of PCR against culture was observed to be 89.7% [95% CI: 84.1–93.5] and 73.1% [95% CI: 67.4–78.1] respectively and that against CRS criteria was 80.2% [95% CI: 75.1–84.5] and 97.1% [95% CI: 92.9–98.9] respectively. PCR showed 100% [111/111, 95% CI: 97–100] sensitivity for smear positive specimens. For smear negative specimens sensitivity and specificity of PCR against culture was observed to be 78.4% [69/88, 95% CI: 68.4–86.5] and 77.3%

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[204/264, 95% CI: 71.7–82.2] respectively and that against CRS was 68.1% [124/182, 95% CI: 60.8–74.8] and 97.1% [165/170, 95% CI: 93.3–99] respectively.

Conclusion: CRS criteria were observed to be better than culture for assessing the diagnostic accuracy of PCR test.

Introduction

Tuberculosis is globally still a leading cause of mortality. India contributes the highest number of new cases, accounting for 21% of the global burden [1]. Early diagnosis and identification of TB are essential in instituting effective and timely therapy. Intense efforts are being taken globally to establish reliable test for rapid and accurate diagnosis of TB which would further help in initiating early anti-tubercular therapy. The conventional methods for diagnosing TB includes: (1) acid fast bacilli (AFB) smear that has low sensitivity and specificity; and (2) culture that is time consuming. Because of these limitations, nucleic acid amplification (NAA) tests have emerged as promising alternatives and polymerase chain reaction (PCR) is the most widely used test.

Many studies have reported the use of PCR, using both commercial kits and in-house assays, for rapid detection of *Mycobacterium tuberculosis* (MTB) from various clinical specimens. Meta-analysis studies have reported sensitivity and specificity of commercial assays to be 36–100% and 54–100% and of in-house PCR assays to be 9–100% and 5–100% respectively [2,3]. The high cost of commercial tests has restricted its use in developing countries.

Hence in the present study we aim to standardize a single-tube nested PCR using Insertion Sequence (IS) 6110 and evaluate its utility for the diagnosis of pulmonary and extrapulmonary TB and also to evaluate its utility against gold standard culture technique and composite reference standard (CRS).

Materials and methods

Setting

The study was carried in the College of American Pathologist (CAP) accredited mycobacteriology laboratory at a tertiary care centre from August 2004 to April 2008.

Patients

The specimens included in the study were 164 biopsies/tissues, 94 pus/abscess/aspirates, 52 body fluids, 27 sputum, 13 bronchoalveolar lavage (BAL), 60 cerebrospinal fluid (CSF) and 53 urine samples (Fig. 1). Detailed clinical history (past and family history, treatment history, radiological scans, and histology/cytology) of each patient was taken by an authorized trained laboratory personnel by telephonic conversation for correlation of PCR results.

Inclusion/exclusion criteria

The patients were included if they could provide a detailed clinical history, radiological scans, histology/cytology, etc. and adequate amount of specimens (2 ml for CSF, 5 ml for any kind of body fluid, $1 \times 1\ cm^2$ for tissues) were available for both culture and PCR tests.

Specimen processing

The specimens were equally divided into 2 parts and assigned to the molecular technologist in the molecular diagnostic laboratory for the PCR test and to the technologist in the mycobacteriology laboratory for AFB microscopy (Ziehl–Neelsen [ZN] staining) and culture by both solid medium (egg-based Löwenstein–Jensen [LJ] [4] and liquid medium (BACTEC MGIT [mycobacteria growth indicator tube] 960 culture; BD Microbiology Systems). Positive cultures were confirmed for *Mycobacterium tuberculosis* (MTB) species by p-nitro benzoic acid assay (PNBA) [4,5].

Molecular testing

All specimens were processed as per the Clinical and Laboratory Standards Institute guidelines, in the increasing order of their smear status i.e., clinical specimens with smear negative status were processed first, followed by AFB scanty, 1+, 2+, and 3+. Also specimens from sterile sites were processed first followed by specimens obtained from non-sterile sites. The specimens were stored in −20 °C and processed for molecular testing on weekly basis.

(i) DNA extraction:

Decontamination using N-acetyl-L-cysteine (NALC)–sodium hydroxide (NaOH) method was performed on pulmonary specimens and extrapulmonary specimens like urine, pus, abscess, and aspirates. Sterile body fluids, CSF and biopsies were not subjected for decontamination procedure and were used directly for extraction protocol. Lysozyme buffer (300 µl; 300 mg lysozyme, 0.1% Trition X-100, 10 mM Tris, 1 mM EDTA, pH 8.0) was added to the decontaminated pellet and incubated at 37 °C in a water bath overnight. DNA was extracted from the lysate using QIAamp DNA mini kit (Qiagen) as per the manufacturer's instructions.

(ii) PCR amplification:

PCR was performed using IS6110 primer sequences [6,7]. The 50 µl amplification mixture consisted of 1× Taq buffer, 4.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 µl Q-solution, 50 pmol of ISF, ISR primers, 10 pmol of INSF and INSR primers, 20 pmol of HBGF, HBGR primers and 1U Hotstar Taq polymerase. Table 1 shows the primer sequences and PCR cycling conditions. The PCR products were visualized on 3% agarose gel stained with ethidium bromide.

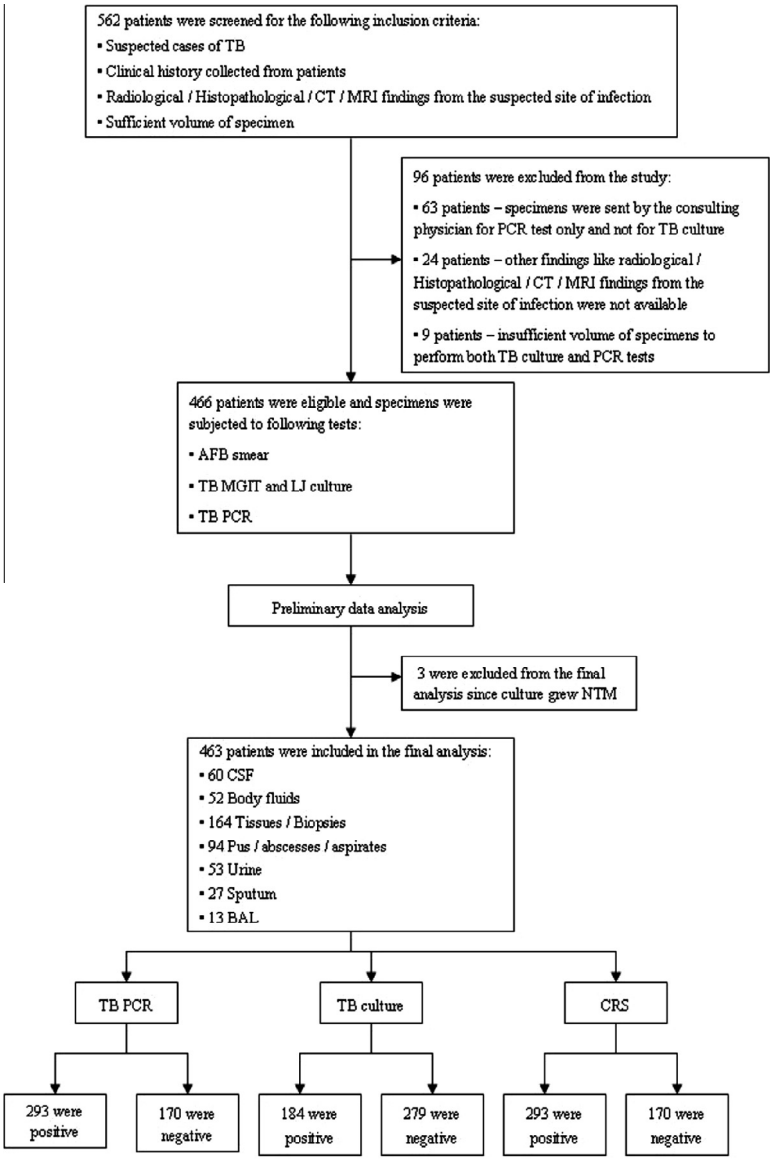


Figure 1 Flowchart of patients included and analyzed in this study.

Table 1 Primers sequences, corresponding product size and PCR conditions.

Gene	Primer sequences (5' – 3')	Product size (bp)	PCR cycling conditions
Human β-globin	HBGF: TGAACGTGGATGAAGTTGGTGGTG	291	94°C – 15' 94°C – 1' 66°C – 1' 72°C – 1' × 25
	HBGR: ACTTTCTTGCCATGAGCCTTCACCTT		94°C – 1' 70°C – 1' 72°C – 1' × 30
IS*6110*[a]	ISF: CGTGAGGGCATCGAGGTGGC	245	72°C – 10'
	ISR: GCGTAGGCGTCGGTGACAAA		4°C – Hold
	INSF: CTCGTCCAGCGCCGCTTCGG	123	
	INSR: CCTGCGAGCGTAGGCGTCGG		

[a] Published primer sequences [8,9].

PCR validation

(i) Determination of analytical sensitivity of PCR:

The analytical sensitivity of nested PCR was evaluated by preparing McFarland's dilutions of standard H37Rv strain ranging from 1 to 10^6 cfu/mL. To check for interferences from clinical specimens' sputum, pus and urine samples obtained from patients not having signs and symptoms of TB disease were spiked with serial dilutions of H37Rv strain.

(ii) Determination of analytical specificity of PCR

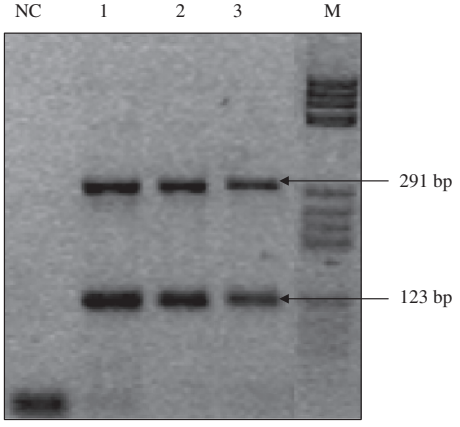
The analytical specificity of PCR was evaluated on healthy controls and non-TB diseased controls. Healthy controls comprised of 10 CSF samples from patients with non-infectious findings such as 4 patients had tumour, 5 patients had delayed milestones and one patient had a road traffic accident. Additionally sputum specimens were collected from 10 patients without any signs and symptoms of TB.

Amongst 40 non-TB diseased controls 10 patients with culture-proven pyogenic meningitis, 10 patients with viral encephalitis and 30 patients with histopathology proven malignancy were included. Of the 10 patients with pyogenic meningitis 6 had *Streptococcus pneumoniae* grown on culture, 1 had *Haemophilus influenza* and 3 had detected the presence of *Cryptococcus neoformans*.

(iii) Cross reactivity:

Cross-reactivity was evaluated against commonly isolated *Non-Tuberculosis Mycobacteria* (NTM) strains such as *Mycobacterium fortuitum*, *Mycobacterium intracellulare*, *Mycobacterium abscessus*, *Mycobacterium avium*, *Mycobacterium chelonae*, *Mycobacterium kanasaii* and *Mycobacterium scrofulaceum*. Cross reactivity was also evaluated using standard bacterial and fungal ATCC strains such as *Staphylococcus aureus* (ATCC 25923), *Enterobacteriaceae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922), *Proteus vulgaris* (ATCC 8427), *Pseudomonas aeroginosa* (ATCC 27853), and *Candida albicans* (ATCC 10231).

(iv) Reproducibility:



NC – Negative Control

1 - 10^3 copies/ml.

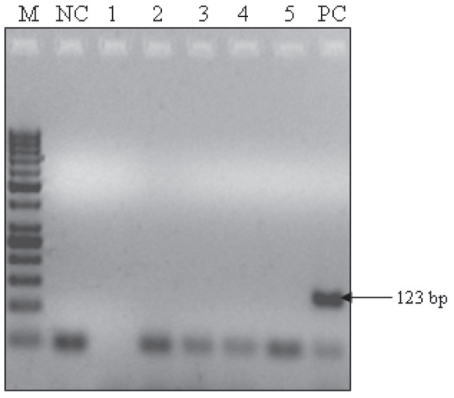
2 –.10^2 copies/ml.

3 – 10 copies/ml

M - pBR322/HaeIII ladder.

Figure 2 Determination of analytical sensitivity of PCR using spiked sputum specimens.

Sputum specimens from patients not suffering from TB disease were spiked with 10copies/ml of standard H37RV strain. Reproducibility was evaluated by performing extraction and amplification on spiked specimens for 10 times within batch and in between 10 different batches.



M - 50 bp ladder

NC - Negative control

1 - Healthy control (non-infectious CNS infections)

2 - Healthy control (healthy individual)

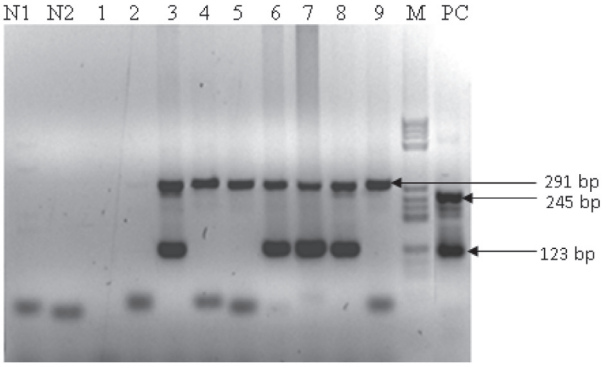
3 - Diseased control (pyogenic meningitis)

4 - Diseased control (viral encephalitis)

5 - Diseased control (malignancy)

PC - Positive control

Figure 3 Determination of analytical specificity of PCR in healthy and diseased controls.



N1	-	PCR Negative control
N2	-	Extraction negative control
1, 2	-	Samples showing inhibition by nested PCR
4, 5, 9	-	Samples negative by nested PCR
3, 6, 7, 8	-	Samples positive by nested PCR
M	-	pBR322/HaeIII ladder

Figure 4 Nested PCR in different clinical specimens.

Table 2 Positive results of smear, culture, PCR and CRS in different clinical specimens.

	Smear positive (%)	Culture positive (%)	PCR positive (%)	CRS positive (%)
CSF (60)	2 (3)	14 (23)	29 (48)	42 (70)
Body fluids (52)	3 (6)	16 (31)	19 (36)	27 (52)
Tissues (164)	44 (27)	60 (37)	77 (47)	91 (55)
Pus (94)	48 (51)	55 (58)	68 (72)	74 (79)
Urine (53)	6 (11)	20 (38)	24 (45)	36 (68)
Total extrapulmonary (423)	103 (22)	165 (39)	217 (51)	270 (64)
Sputum (27)	6 (22)	14 (52)	16 (59)	17 (63)
BAL (13)	2 (15)	5 (38)	7 (54)	6 (46)
Total pulmonary (40)	8 (20)	19 (47)	23 (57)	23 (57)
Total specimens (463)	111 (24)	184 (40)	240 (52)	293 (63)

Table 3 Comparison of smear with culture and CRS in different clinical specimens.

	Culture positive (184)	Culture negative (279)	CRS positive (293)	CRS negative (170)
Smear positive (111)	96	15	111	0
Smear negative (352)	88	264	182	170

Table 4 Comparison of culture with CRS in different clinical specimens.

		CRS positive	CRS negative
CSF	Culture positive	14	0
	Culture negative	28	18
Body fluids	Culture positive	16	0
	Culture negative	11	25
Tissues	Culture positive	60	0
	Culture negative	31	73
Pus	Culture positive	55	0
	Culture negative	19	20
Urine	Culture positive	20	0
	Culture negative	16	17
Total extrapulmonary	Culture positive	165	0
	Culture negative	105	153
Sputum	Culture positive	14	0
	Culture negative	3	10
BAL	Culture positive	5	0
	Culture negative	1	7
Total pulmonary	Culture positive	19	0
	Culture negative	4	17
Total specimens	Culture positive	184	0
	Culture negative	109	170

(v) Validation of PCR products by sequencing

The PCR products were validated by sending 10 representative samples for DNA sequencing (Bangalore Genei, Bangalore) and the specificity of the amplified sequence was confirmed by performing BLAST analysis.

Quality control measures

Measures to avoid false positive PCR results and to minimize cross contamination were followed as previously reported [8,9]. Every batch of PCR mixture included reagent controls for extraction reagents and PCR reagents, negative controls, and positive control. The incorporation of β globin gene PCR served as an internal control to monitor PCR inhibition in every sample.

Patient categorization

Patients were categorized into 4 categories: 'Confirmed TB', 'Probable TB', 'Possible TB' and 'Not TB' cases using a CRS algorithm as described in a study by Vadwai et al. [10]. Confirmed TB cases comprised of smear positive/culture positive and smear negative/culture positive patients. Probable TB cases comprised of culture negative patients showing clinical signs/symptoms of TB, radiological findings and/or histology/cytology are suggestive of TB. Possible TB cases include patients showing only clinical signs/symptoms of TB whereas all other tests including culture were negative. In these patients response to empirical anti-tuberculosis treatment (ATT) was determined after 3 months. Not TB cases comprised of culture and all other tests are negative with patient responding to non-TB treatment.

Table 5 Statistical analysis of culture results with CRS in different clinical specimens.

	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	PPV (95% CI)	NPV (95% CI)	Prevalence or pre test probability (95% CI)	Post test probability of positive result (95% CI)	Post test probability of negative result (95% CI)
CSF	33.3 (19.6–49.5)	100 (81.5–100)	12.81 (0.80–203.88)	0.68 (0.54–0.85)	100 73.24–100)	39.13 (25.46–39.13)	39.13 (25.46–39.13)	100 (65–100)	61 (56–66)
Body fluids	59.3 (38.8–77.6)	100 (86.3–100)	30.64 (1.93–485.31)	0.42 (0.27–0.65)	100 (75.93–100)	69.44 (51.73–83.08)	69.44 (51.73–83.08)	100 (68–100)	31 (23–41)
Tissues	65.9 (55.3–75.5)	100 (95.1–100)	97.326 (6.121–1547.6)	0.34 (0.26–0.466)	100 (92.5–100)	70.19 (60.31–78.56)	70.19 (60.31–78.56)	100 (88–100)	30 (24–36)
Pus	74.3 (62.8–83.8)	100 (83.2–100)	31.08 (2–482.25)	0.27 (0.18–0.39)	100 (91.87–100)	51.28 (35.03–67.29)	51.28 (35.03–67.29)	100 (88–100)	49 (40–59)
Urine	55.6 (46.2–79.2)	100 (80.5–100)	19.95 (1.28–311.47)	0.46 (0.32–0.66)	100 (79.95–100)	51.52 (33.85–68.83)	51.52 (33.85–68.83)	100 (73–100)	48 (40–58)
Total extrapulmonary	61.1 (55–67)	100 (97.6–100)	188.1 (11.8–2998.6)	0.39 (0.34–0.45)	100 (97.16–100)	59.3 (53.02–65.3)	59.3 (53.02–65.3)	100 (95–100)	41 (38–44)
Sputum	82.4 (56.6–96.2)	100 (69.2–100)	17.72 (1.17–268.38)	0.20 (0.08–0.53)	100 (73.24–100)	76.92 (45.98–93.84)	76.92 (45.98–93.84)	100 (67–100)	23 (12–47)
BAL	83.3 (35.9–99.6)	100 (59–100)	12.57 (0.83–189.23)	0.23 (0.05–0.95)	100 (46.29–100)	87.5 (46.68–99.34)	87.5 (46.68–99.34)	100 (42–100)	13 (4–45)
Total pulmonary	82.6 (61.2–95)	100 (80.5–100)	29.25 (1.89–452.9)	0.19 (0.08–0.44)	100 (79.08–100)	80.95 (57.42–93.71)	80.95 (57.42–93.71)	100 (72–100)	19 (10–38)
Total	62.8 (57–68.3)	100 (97.9–100)	214.62 (13.46–3422.2)	0.37 (0.32–0.43)	100 (97.45–100)	60.93 (54.91–66.64)	60.93 (54.91–66.64)	100 (96–100)	39 (36–43)

Statistical analysis

Diagnostic accuracy of PCR against culture and CRS criteria was performed using MetaDisc software to calculate sensitivity, specificity, predictive values, diagnostic odds ratios, and likelihood ratios. Wilson's binomial theorem was used to calculate 95% confidence intervals (CI) [11]. Post test probability for positive and negative results was calculated using Pre and post test probability calculator (http://www.mashpedia.com/Pre-_and_post-test_probability).

Results

PCR validation

Analytical sensitivity of PCR was found to be 10 cfu/ml (Fig. 2). No cross-reactivity was observed with the standard bacterial, fungal and NTM strains and also in 20 healthy and 40 diseased controls (Fig. 3). Reproducibility of nested PCR in-between and within the batches was observed. Sequencing and BLAST analysis demonstrated maximum homology of the amplified products with MTB complex strains published in the NCBI database. Different clinical specimens were then subjected to standardized and validated nested PCR (Fig. 4).

Patients

A total of 562 patients were screened for the inclusion/exclusion criteria and 466 patients following the criteria were included in the study. In the preliminary data analysis 0.64% (3/466) patients were excluded since culture grew non-tuberculosis mycobacterial (NTM). Thus 463 patients were included in the final data analysis. The mean age of the patients was 33 (±21 years, standard deviation) years and the male:female sex ratio was 1.2:1. None of the patients included in the study were positive for human immunodeficiency virus (HIV).

Of the 463 patients 24% were smear positive, 40% were culture positive, 52% were PCR positive and 63% were positive by CRS criteria (Table 2). Of the total specimens 20.73% (96/463) were positive by both smear and culture, 19% (88/463) were smear negative/culture positive, 3.24% (15/463) were smear positive/culture negative and 57.02% (264/463) were negative by both smear and culture. Of the total specimens 50.76% (235/463) were positive by both PCR and CRS criteria and 12.52% (58/463) were positive by CRS criteria only.

AFB smear findings

AFB smear showed sensitivity and specificity of 52.2% (95% CI: 44.7–59.6) and 94.6% (95% CI: 91.3–97) respectively with culture (Table 3) and 37.9% (95% CI: 32.3–43.7) and 100% (95% CI: 97.9–100) respectively against CRS criteria.

Culture results

Of the total 463 specimens 39% (182/463) were positive by MGIT liquid culture and 25% (116/463) showed growth on conventional LJ media. Of the total 184 culture positive specimens, 37% (68/184) were positive only by MGIT, 1% (2/184)

Table 6 Comparison of PCR results with smear and culture in different clinical specimens.

Total = 463	Smear positive (111)				Smear negative (352)			
	Culture positive (96)		Culture negative (15)		Culture positive (88)		Culture negative (264)	
	PCR positive	PCR negative	PCR positive	PCR negative	PCR positive	PCR negative	PCR positive	PCR negative
CSF (60)	2	0	0	0	8	4	19	27
Body fluids (52)	3	0	0	0	9	4	7	29
Tissue (164)	37	0	7	0	20	3	13	84
Pus (94)	43	0	5	0	10	2	10	24
Urine (53)	4	0	2	0	11	5	7	24
Extra pul (423)	89	0	14	0	58	18	56	188
Sputum (27)	5	0	1	0	8	1	2	10
BAL (13)	2	0	0	0	3	0	2	6
Pul (40)	7	0	1	0	11	1	4	16
Total (463)	96	0	15	0	69	19	60	204

was positive only on LJ media and 62% (114/184) were positive both by MGIT and LJ media.

The comparison of culture results with CRS in different specimens is given in Table 4. Overall culture showed sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 62.8%, 100%, 100% and 60.93% respectively with CRS (Table 5). The positive likelihood ratio (PLR), negative likelihood ratio (NLR), post test probability of positive result and post test probability of negative result were observed to be 214.62%, 0.37%, 100% and 39% respectively.

Diagnostic accuracy of PCR with culture

Table 6 represents the comparison of PCR results with smear and culture in different clinical specimens. Amongst smear positive specimens 86.5% (96/111) were positive by both culture and PCR and additionally 13.5% were positive only by PCR. Amongst smear negative specimens 19.6% (69/352) were positive by both culture and PCR, additionally 17% (60/352) were positive only by PCR, 5.4% (19/352) were positive by culture only and 57.9% (204/352) were negative both by culture and PCR.

The diagnostic accuracy of PCR against culture in different clinical specimens is depicted in Table 7. Overall sensitivity, specificity, PPV and NPV were observed to be 89.7%, 73.1%, 68.75% and 91.48% respectively. The PLR, NLR, post test probability of positive result, and post test probability of negative result were observed to be 3.34%, 0.14%, 69% and 8.5% respectively. PCR showed 100% [111/111, 95% CI: 97–100] sensitivity for smear positive specimens. For smear negative specimens sensitivity and specificity were observed to be 78.4% [69/88, 95% CI: 68.4–86.5] and 77.3% [204/264, 95% CI: 71.7–82.2] respectively.

Diagnostic accuracy of PCR with CRS

Table 8 represents the comparison of PCR results with smear and CRS in different clinical specimens. All smear positive specimens were positive by both PCR and CRS. Amongst smear negative specimens 35.27% (124/352) were positive by both PCR and CRS, 1.4% (5/352) were positive only by PCR, 16.48% (58/352) were positive by CRS only and 46.87% (165/352) were negative both by culture and PCR.

The diagnostic accuracy of PCR against CRS in different clinical specimens is depicted in Table 9. Overall PCR showed sensitivity, specificity, PPV and NPV of 80.2%, 97.1%, 97.92% and 73.99% respectively. The PLR, NLR, post test probability of positive result and the post test probability were 27.27, 0.204, 98% and 26% respectively. For smear positive specimens PCR showed 100% sensitivity with CRS criteria also. For smear negative specimens sensitivity and specificity of PCR against CRS was 68.1% [124/182, 95% CI: 60.8–74.8] and 97.1% [165/170, 95% CI: 93.3–99] respectively.

PCR results in different categories of patients

Based on the CRS criteria 42.98% (n = 199/463) patients were 'Confirmed TB' cases, 20.3% (n = 94/463) patients were 'Probable TB' cases and 36.72% (n = 170/463) patients were 'Not TB' cases. None of the patients were classified as ''Possible TB'' cases. Nested PCR was positive in 89% of ''confirmed TB cases'' and in 59% of ''probable TB'' cases (Table 10). In 'not-TB' cases 2.94% (5/170) were positive by nested PCR.

Inhibition rate

The inhibitory rate was observed to be 3.4% (16/463) and the contamination rate of MGIT culture was 1.9% (9/463) and that of LJ was 3.02% (14/463). The inhibition rates for different specimen matrices were observed to be 3.7% (1/27), 3.33% (2/60), 5.76% (3/52), 1.83% (3/164), 2.13% (2/94) and 9.43% (5/53) for Sputum, CSF, body fluids, tissues/biopsies, pus/abscess, and urine specimens respectively.

Discussion

Diagnosis of TB relies on the detection of AFB by microscopy and culture which lack sensitivity and rapidity respectively. The major dilemma lies in the diagnosis of extrapulmonary TB due to atypical clinical presentations. Several meta-analysis

Table 7 Statistical analysis of PCR results with smear and culture in different clinical specimens.

	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	PPV (95% CI)	NPV (95% CI)	Prevalence or pre test probability (95% CI)	Post test probability of positive result (95% CI)	Post test probability of negative result (95% CI)
CSF	71.4 (41.9–91.6)	58.7 (43.2–73)	1.73 (1.07–2.79)	0.49 (0.20–1.15)	34 (19–54)	87 (69–96)	23 (14–36)	34 (25–46)	13 (6–26)
Body fluids	75 (47.6–92.7)	80.6 (64–91.8)	3.86 (1.87–7.94)	0.31 (0.13–0.74)	63.16 (38.63–82.77)	87.88 (70.86–96.04)	31 (19–45)	63 (45–78)	12 (5–25)
Tissues	95 (86.1–99)	80.8 (71.9–87.8)	4.94 (3.32–7.36)	0.06 (0.02–0.19)	74.03 (62.57–83.05)	96.55 (89.55–99.11)	36.6 (29–44)	74 (66–81)	3 (1–10)
Pus	96.4 (87.5–99.6)	61.5 (44.6–76.6)	2.5 (1.68–3.74)	0.06 (0.01–0.24)	77.94 (65.94–86.74)	92.31 (73.4–98.66)	58 (48–68)	78 (70–84)	8 (1–25)
Urine	75 (50.9–91.3)	72.7 (54.5–86.7)	2.75 (1.49–5.07)	0.34 (0.16–0.75)	62.5 (40.76–80.45)	82.76 (63.51–93.47)	38 (25–52)	62 (47–75)	17 (9–32)
Total extra pulmonary	89.1 (83.3–93.4)	72.9 (67–78.2)	3.28 (2.67–4.04)	0.15 (0.10–0.23)	67.74 (61.02–73.82)	91.26 (86.33–94.59)	39 (34–44)	68 (63–72)	9 (6–13)
Sputum	92.9 (66.1–99.8)	76.9 (46.2–95)	4.02 (1.48–10.97)	0.09 (0.01–0.63)	81.25 (53.69–95.03)	90.91 (57.12–99.52)	52 (32–71)	81 (61–92)	9 (1–40)
BAL	100 (47.8–100)	75 (34.9–96.8)	3.3 (1.12–9.72)	0.11 (0.01–1.69)	71.43 (30.26–94.89)	71.43	38 (15–68)	71 (41–86)	0 (0–51)
Total pulmonary	94.7 (74–99.9)	76.2 (52.8–91.8)	3.98 (1.84–8.61)	0.07 (0.01–0.47)	78.26 (55.79–91.71)	94.12 (69.24–99.69)	47 (32–64)	78 (62–89)	6 (1–30)
Total	89.7 (84.1–93.5)	73.1 (67.4–78.1)	3.34 (2.73–4.07)	0.14 (0.09–0.22)	68.75 (62.41–74.47)	91.48 (86.82–94.66)	40 (35–44)	69 (64–73)	8.5 (6–13)

Table 8 Comparison of PCR results with smear and CRS in different clinical specimens.

Total = 463	Smear positive (111)				Smear negative (352)			
	CRS positive (111)		CRS negative (0)		CRS positive (182)		CRS negative (170)	
	PCR positive	PCR negative	PCR positive	PCR negative	PCR positive	PCR negative	PCR positive	PCR negative
CSF (60)	2	0	0	0	26	14	1	17
Body fluids (52)	3	0	0	0	15	9	1	24
Tissue (164)	44	0	0	0	33	14	0	73
Pus (94)	48	0	0	0	20	6	0	20
Urine (53)	6	0	0	0	17	13	1	16
Extra pul (423)	103	0	0	0	111	56	3	150
Sputum (27)	6	0	0	0	10	1	0	10
BAL (13)	2	0	0	0	3	1	2	5
Pul (40)	8	0	0	0	13	2	2	15
Total (463)	111	0	0	0	124	58	5	165

Table 9 Statistical analysis of PCR results with smear and CRS in different clinical specimens.

	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	PPV (95% CI)	NPV (95% CI)	Prevalence or pre test probability (95% CI)	Post test probability of positive result (95% CI)	Post test probability of negative result (95% CI)
CSF	66.7 (50.5–80.4)	94.4 (72.7–99.9)	12.0 (1.76–81.58)	0.35 (0.23–0.55)	96 (80–99)	55 (36–72)	70 (57–81)	97 (81–99)	45 (35–56)
Body fluids	66.7 (46–83.5)	96 (79.6–99.9)	16.67 (2.40–115.84)	0.35 (0.20–0.59)	94.7 (71.89–99.72)	72.73 (54.21–86.06)	52 (38–66)	95 (72–99)	27 (18–39)
Tissues	84.6 (75.5–91.3)	100 (95.1–100)	124.67 (7.86–1977.4)	0.16 (0.10–0.25)	100 (94.08–100)	83.91 (74.13–90.61)	55.5 (47–63)	100 (91–100)	16 (11–24)
Pus	91.9 (83.2–97)	100 (83.2–100)	38.36 (2.48–593.79)	0.09 (0.04–0.19)	100 (79.95–100)	100 (93.34–100)	79 (69–86)	100 (90–100)	23 (13–41)
Urine	63.9 (46.2–79.2)	94.1 (71.3–99.9)	10.86 (1.60–73.88)	0.38 (0.24–0.60)	95.83 (76.88–99.78)	55.17 (35.98–73.05)	68 (53–80)	96 (77–99)	45 (34–56)
Total extrapulmonary	79.3 (73.9–83.9)	98 (94.4–99.6)	40.42 (13.16–124.15)	0.21 (0.17–0.27)	98.62 (95.68–99.64)	72.82 (66.11–78.65)	64 (59–68)	99 (96–100)	27 (23–32)
Sputum	94.1 (71.3–99.9)	100 (69.2–100)	20.17 (1.34–303.60)	0.09 (0.02–0.41)	100 (65.55–100)	90.91 (57.12–99.52)	63 (42–80)	100 (69–100)	9 (3–41)
BAL	83.3 (35.9–99.6)	71.4 (29–96.3)	2.92 (0.86–9.926)	0.23 (0.04–1.48)	71.43 (30.26–94.89)	83.33 (36.48–99.12)	46 (20–74)	71 (42–89)	16 (3–56)
Total pulmonary	91.3 (72–98.9)	88.2 (63.6–98.5)	7.76 (2.1–28.7)	0.10 (0.03–0.37)	91.3 (70.49–98.48)	88.24 (62.25–97.94)	57 (41–73)	91 (74–98)	12 (4–33)
Total	80.2 (75.1–84.5)	97.1 (92.9–98.9)	27.27 (11.48–64.79)	0.204 (0.16–0.26)	97.92 (94.93–99.23)	73.99 (67.63–79.51)	63 (59–68)	98 (95–99)	26 (22–31)

studies have reported the diagnostic accuracy of in-house and commercial NAAT for the diagnosis of pulmonary and extrapulmonary TB and have concluded that the accuracy could not be determined for in-house PCR assays due to heterogeneity across studies [12]. Hence in this study we have attempted to determine the diagnostic accuracy of in-house PCR for the diagnosis of TB focusing mainly on extrapulmonary TB.

Meta-analysis studies have reported that the use of IS6110 target sequence and nested PCR produced higher diagnostic odds ratio compared to those using other targets and regular PCR protocols respectively [3,12]. Accordingly in this a study single tube nested PCR was meticulously standardized by performing modifications in the extraction protocol which included overnight incubation with lysozyme, increasing lysis time up to 2 h and concentrating DNA by using 75 µl AE buffer. To the best of our knowledge the combination of outer and inner primers used in this study to standardize a single tube nested PCR has not been reported, though these primer sets have been used separately.

In the present study sensitivity of smear against culture and CRS was observed to be 52.2% and 37.9% respectively, of PCR was 89.7% and 80.2% respectively and of culture was 62.8% against CRS. These were greater than those reported in the previous studies [13,14]. Of the CRS positive patients 37.2% (109/293, Table 4) were culture negative which attributed to the low sensitivity of culture. Low sensitivity of culture could be explicated to the fact that 68% (51/75) of culture negative, and PCR positive/CRS positive patients were receiving anti-tuberculosis treatment (ATT) for varying periods of time ranging from 2 to 8 days. Other possible explanations include paucibacillary nature of extrapulmonary specimens and loss of viable bacilli during NALC–NaOH decontamination procedure.

Diagnostic accuracy of PCR

The sensitivity of PCR was observed to be 89.7% and 80.2% respectively with culture and CRS criteria which were greater than 83% [14] and 74.2% [13] sensitivity reported in the previous studies. Specificity of PCR was observed to be 58.7% and 94.4% respectively with culture and CRS criteria. Specificity against CRS criteria was comparable with that of 94.59% [14] and 95% [13] reported in the previous studies. The specificity against culture was lower because 59% of patients with 'Probable TB' were positive by PCR and negative by culture. Hence CRS criteria were observed to be better than culture in determining the diagnostic accuracy of PCR test. In smear positive specimens PCR showed sensitivity of 100% both with culture and CRS criteria which was similar to that reported [14]. In smear negative specimens PCR showed a sensitivity of 78.4% and 68.1% respectively with culture and CRS criteria which was greater than that reported.

In the smear negative specimens 58 were CRS positive/PCR negative (Table 8) of which 19 were culture positive and 39 were culture negative. In the 39 CRS positive patients the negative results by smear, culture, and PCR indicate either absence or extremely low mycobacterial load. Of the 19 culture positive patients, 16 showed PCR inhibition. Though the inhibition effect was neutralized after repetition of PCR in all 16 specimens, these samples did not show the presence of MTB specific PCR product. Thus the absence of MTB specific

Table 10 PCR positivity in different categories of patients.

	Confirmed TB cases	Probable TB cases	Not TB cases
CSF	10/14 (71%)	18/28 (64%)	1/18
Body fluids	12/16 (75%)	6/11 (55%)	1/25
Tissues	64/67 (96%)	13/24 (54%)	0/73
Pus	55/60 (92%)	10/14 (71%)	0/20
Urine	17/22 (77%)	6/14 (43%)	1/17
Total extrapulmonary	158/179 (88%)	53/91 (58%)	3/153
Sputum	14/15 (93%)	2/2 (100%)	0/10
BAL	5/5 (100%)	0/1 (0%)	2/7
Total pulmonary	19/20 (95%)	2/3 (67%)	2/17
Total	177/199 (89%)	55/94 (59%)	5/170

product in all these specimens could be attributed to the extremely paucibacillary nature of these specimens.

Diagnostic accuracy of PCR in pulmonary specimens

Previous studies using IS*6110* PCR have reported higher sensitivity in pulmonary specimens (92–99%) than extrapulmonary specimens (39–91%) [15]. Wide variability in sensitivity and specificity ranging from 9.4% to 100% and 5.6% to 100% respectively for the diagnosis of pulmonary TB using in-house PCR test was reported [3]. Likewise in the present study the sensitivity and specificity of PCR in pulmonary specimens was observed to be 94.7% and 76.2% respectively against culture and 91.3% and 88.2% respectively against CRS criteria.

Sensitivity of in-house PCR for smear positive pulmonary specimens has been reported to be 96% [16] and for smear negative pulmonary specimens wide variability was reported in sensitivity (9–100%) and specificity (25–100%) [17]. In present study 100% sensitivity was observed for smear positive pulmonary specimens, and 87% and 92% sensitivity was observed respectively against culture and CRS criteria for smear negative pulmonary specimens. The specificity was observed to be 88% and 80% respectively against culture and CRS.

Diagnostic accuracy of PCR in extra pulmonary specimens

Previous studies using IS*6110* PCR have reported sensitivity ranging from 39% to 91% for extrapulmonary specimens [15]. In the present study sensitivity of PCR in extra pulmonary specimens was observed to be 89.1% and 79.3% respectively with culture and CRS criteria. Wide variability (0–100%) between sensitivities of in-house PCR tests has been reported for the diagnosis of TBM [18]. In the present study sensitivity of PCR from CSF specimens was observed to be 71.4% with culture and 66.7% with CRS. Similarly wide variability in sensitivity (20–100%) and specificity (53–100%) has been reported between in-house PCR assays for the diagnosis of TB pleuritis [12]. Present study showed sensitivity and specificity of 75% and 80.6% respectively with culture and 66.7% and 96% respectively with CRS criteria for detection of MTB from body fluids.

High sensitivity and specificity for tissues/biopsies specimens against both culture and CRS were observed in the present study. Sensitivity of 74.07–87.5% has been reported for various tissues specimens [19–23]. In the present study a very high sensitivity of >90% was observed for aspirates/pus/abscess specimens against both culture and CRS criteria which is greater than the reported sensitivity of 69.2% [22].

PCR showed sensitivity of 75% and 63.9% against culture and CRS criteria respectively for urine specimens which are comparable to that reported [22].

Different groups

With CRS criteria, nested PCR assay showed good overall sensitivity of 89% (177/199) in confirmed-TB cases (Table 10). Nested PCR showed sensitivity of 59% (55/94) in probable-TB cases who had strong clinical indications of TB (had positive radiological tests and/or positive histology/cytology reports, while some of the patients were already on antitubercular treatment at the point of enrolment in the study).

Inhibition rate and false positive rate

In the present study the overall inhibition rate of nested PCR was observed to be 3.4% which was lower than the reported rate of 4.8% [19] and 12.5% [24].

The false positive rate of nested PCR was observed to be very low 2.9% (5/170; 95% CI: 1–7) than that reported in the earlier study [25]. Several precautions were followed to minimize cross contamination: (1) Nested PCR was standardized in a single tube to prevent cross contamination; (2) reagent control was included in every batch to monitor cross contamination; (3) big batches of specimen processing were avoided; and (4) specimens were processed according to CLSI guidelines in increasing order of smear status.

Likelihood ratios and Post test probability of PCR

The PLR and NLR in total specimens when compared with culture were 3.34 and 0.14 respectively. With CRS criteria enhancement in likelihood ratios was observed. The high PLR (27.27) and very low NLR (0.204) indicate good probability for 'ruling in' and 'ruling out' disease respectively. If PCR test was positive, the likelihood that this patient has TB increases from 63% (pre-test probability) to 98%, a probability that is sufficiently high to justify initiation of anti-tuberculosis treatment (ATT). The post test probability was higher with CRS criteria (98%) than culture (69%) in 'ruling in' TB disease. Likewise if the PCR result was negative, the probability of TB disease was reduced to 26%. Different clinical specimens showed high post test probability for positive result which would definitely help in initiation of early ATT particularly in paucibacillary extrapulmonary TB cases. However in extrapulmonary specimens the post test probability for negative result was not sufficiently low to rule out TB with cer-

tainty. This could be attributed to the paucibacillary nature of specimens obtain from the extra pulmonary sites. Overall the likelihood ratios and the post test probability were observed to be greater with CRS criteria than those with culture. This highlights the usefulness of CRS criteria for determining the diagnostic accuracy of highly sensitive PCR test.

Strength and limitation of the study

Strength of the study includes: (1) large number of extra pulmonary specimens were analyzed for smear, culture and PCR tests; (2) evaluation of PCR against CRS criteria showed better diagnostic accuracy of the test; and (3) post test probability gave better understanding of clinical utility of the PCR test as a diagnostic tool especially for extra pulmonary TB. The lack of incorporation of uracil N-glycosylase (UNG) could be considered as a limitation of this PCR assay. However we have attempted to address this issue of carry-over contamination by standardizing the nested PCR in a single tube, thereby precluding transfer of amplified products for the nested reaction.

We conclude that CRS criteria were better than culture for assessing the diagnostic accuracy of PCR test and that PCR showed good clinical utility for 'ruling in' TB with certainty in extra pulmonary specimens.

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