Sensitivity of PCR *IS6110* in relation to culture and staining in Pott's disease

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ABSTRACT

Background: Rapid diagnosis is essential to decrease the morbidity and mortality of Pott's disease. The bacteriological methods are time-consuming or insensitive. Polymerase chain reaction (PCR) provides a rapid diagnostic tool and hope for early diagnosis of this disease. The aim of this study was to compare and assess of a rapid and effective method among diagnostic battery (Ziehl–Neelsen (ZN) microscopy, BACTEC culture and PCR) of Pott's disease. **Materials and Methods:** Sixty-five specimens from clinico-radiological suspected cases of Pott's disease were included in this study. They were processed for ZN microscopy, BACTEC culture, and PCR *IS6110*. The tests tool's efficiency, positive agreement K^c (Kappa coefficient), and significance level (*P* value) were calculated for correlation between PCR and performed tests. **Results:** The PCR sensitivity reached to 96% and 46.3% among positive and negative specimens on ZN microscopy. Further, 94% and 36.4% sensitivity were found among positive and negative specimens by BACTEC culture. The total 38 (58.5%) specimens were detected either ZN microscopy or by BACTEC culture. Thus, the overall sensitivity and specificity of PCR were 95% and 74.1%. The kappa coefficient and *P* value, calculated for PCR against BACTEC culture and combined results of performed bacteriological tests were (K^c=0.60, (*P*<0.001)) and (K^c=0.70, (*P*<0.001)), respectively. Above statistical relations showed a fair agreement with significant differences. **Conclusion:** The PCR *IS6110* may be useful in rapid detection of clinico-radiological suspected cases of Pott's disease and those that are negative with bacteriological methods.

Key words: *M. tuberculosis,* polymerase chain reaction *IS6110*, pott's disease and kappa coefficient

INTRODUCTION

Pott's disease accounts for half the cases of skeletal tuberculosis, 15% of the cases of extrapulmonary tuberculosis (EPTB), and 2% of all cases of tuberculosis (TB).[1] The prevalence of this disease continues to increase in developing countries including India.[2] Pott's disease is commonly associated with poor outcomes because of delays in diagnosis due to various causes. The morbidity and mortality of this disease continue to pose a challenge to the treating physicians and surgeons.[3] Paraplegia is the most serious complication, develops when cases remained undiagnosed or untreated until late in the natural history of the disease. Although Pott's disease may be suspected on the basis of clinical and radiological tests, a definitive diagnosis of *Mycobacterium tuberculosis* infection requires laboratory tests. The conventional bacteriological methods are based on Ziehl–Neelsen (ZN) microscopy and culture of the bacteria. ZN microscopy is fast, inexpensive, and highly specific for acid fast bacilli (AFB) detection but has poor sensitivity.[4] Mycobacterial culture remains the gold standard for diagnosis, but it is time-consuming and takes 4-8 weeks to generate definitive results.[5,6]

The polymerase chain reaction (PCR) method has significantly improved the microbiological diagnosis of TB since the last decade. Studies reveal that the sensitivity of PCR ranges from 42% to 93% depending on the clinical specimens.[7-10] The PCR-based amplification of the bacterial genome has the potential to conquer the limitations of conventional methods and establish itself as a rapid, sensitive, and effective method of detecting DNA of *M. tuberculosis* in different clinical specimens from both respiratory and non-respiratory sites.[11,12]

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Most studies from laboratories around the world have described the use of the *IS6110* primer sequence to target the *IS6110* insertion element of *M. tuberculosis*.[13] Multiple copies of the *IS6110* insertion element are present in the genome of *Mycobacterium tuberculosis* complex (MTBC) species. This increases the sensitivity and specificity of PCR based diagnostics.[12,14,15] The aim of this study was to evaluate the efficacy of PCR *IS6110* and to compare this technique with BACTEC culture and ZN microscopy as diagnostic techniques for Pott's disease.

MATERIALS AND METHODS

Patients and Specimens

A total of 65 clinico-radiological suspected cases of Pott's disease were enrolled for the study (after obtaining an informed consent) between January 2008 and August 2011. All subjects were tested for HIV infection before biopsy/CT-guided fine needle aspirate (FNA) of the spinal lesion. Specimens were either pus from an abscess or tissue bits were obtained either during surgery or by CT-guided FNA. Tissue specimens were initially grind in a mortar with three drops of normal saline whereas pus specimens were used as such. The specimens were divided into two half; the first half was used for ZN microscopy and BACTEC culture and the second half was used for PCR.

Inclusion Criteria

1. Clinico-radiological suspected cases of Pott's disease that underwent either open biopsy or CT guided aspiration at our institute.

Exclusion Criteria

1. Those subjects who did not give consent for biopsy or CT-guided aspiration
2. Biopsy was diagnosed to other pathology such as malignancy, etc.

Clinical Microbiological Methods

Microscopic smears were made and stained using the ZN stain according to standard laboratory procedures.[16] Culture was done on radiometric BACTEC 12B vials. The vials were incubated and interpreted as per the Becton Dickinson (BD, Sparks, MD, USA) manual instructions.[17] The *p*-nitro-α-acetylamino-β-hydroxy propiophenone test was performed to identify and differentiate MTBC from non-tubercular *Mycobacterium* in all grown isolates.[17]

DNA Extraction from Pus Specimens

Genomic DNA was extracted from pus specimens as per the method described by Van Sooligen *et al.*[18] 200 µl specimens were incubated along with 200 µl TE buffer (Tris–EDTA, pH = 8.0). Bacteria were lysed for 30 min at 95°C, followed by enzymatic degradation of the cell walls with lysozyme at a final concentration of 20 g/ml at 37°C for 30 min and 10% sodium dodecyl sulfate with proteinase K (10 mg/ml) at 65°C for 20 min. CTAB (cetyl trimethyl ammonium bromide)—NaCl (70 µl) was used for purification of extracted genomic DNA at 65°C for 20 min. The extracted DNA was again purified by a mixture of chloroform and isoamyle alcohol (ratio 24:1) and precipitated by 70% ethanol. Further DNA was dissolved in TE (pH 8.0) and stored at −20°C until further analysis.

DNA Extraction from Tissue Specimens

DNA extraction from tissues was done with Hipura™ genomic DNA extraction kit according to the manufacturer's protocol. Tissue specimens were mechanically homogenized in liquid nitrogen. Briefly, the isolation of *Mycobacterium* DNA from clinical specimens was done by spin-column procedure and harvested by centrifugation. After harvesting the bacterial cell wall, it was degraded by lysozyme (20 g/ml) and Proteinase K (20mg/ml). Lysis was followed by the binding of DNA to silica-gel membrane of the Hielute miniprep spin column. Two rapid wash steps removed trace salt and protein contaminations. DNA was next eluted in an elution buffer provided with the Hipura™ MB505 bacterial genomic DNA miniprep purification spin kit, Himedia laboratories Private limited, India.

PCR Amplification

The amplification reaction was performed on a final volume of 20 µl for each specimen. The reaction mixture contained 10 µl Pyrostart Fast PCR Master mix 2X (dNTP, Taq polymerase with $MgCl_2$,), 1 µl (10 pmoles) of each primer, 3 µl water (nuclease free) and 5 µl of extracted genomic template DNA according to Fermentas India. The oligonucleotide primers[14] used were forward and reverse: 5′-CCT GCG AGC GTA GGC GTC GG-3′ and 5′-CTC GTC CAG CGC CGC TTC GG-3′, respectively (SBS Gentech Co. Ltd). These primers amplified a target fragment (123 bp) from the repeated insertion sequence *IS6110* of MTBC.

The PCR amplification was done in a thermal cycler (MJ Research, PTC-100, GMI, Inc., USA). In brief, the initial denaturation was done at 94°C for 5 min. Further, all 35 cycles were proceeded by each cycle at 94°C/2 min of denaturation, 68°C/2 min for annealing, and 72°C/1 min for extension followed by a final extension at 72°C for 7 min was carried out.

An aliquot (10 µl) from the PCR-amplified product was analyzed in 2% agarose gel through electrophoresis

in Tris–acetate EDTA (TAE) buffer for 40 min at 95 V. The gel was stained with ethidium bromide and visualized on the UV transilluminator. The presence of a 123-bp fragment indicated a positive test with respect of positive control [Figure 1]. Each PCR series had one positive control (50-100 pg H37 Rv DNA) and one negative control (RNAs and DNAs free water) interpreted with the specimens to monitor cross-contamination.

Ethics Approval

This study was approved by 41 institutional ethics committee "A-04 PGI/IMP/EC/41/28/2/2008."

Statistical Analysis

The final diagnosis was established by using the results of ZN microscopy, BACTEC culture, PCR and correlated with clinical-radiological response of anti tubercular treatment (ATT). The test tool's efficiency was calculated as ((total number of positive/total number of analyzed cases) ×100. Sensitivity (Tp/(Tp + Fn)) × 100 and specificity (Tn/(Tn + Fp)) × 100 were also determined. In addition, the positive predictive value was calculated as (Tp/(Tp + Fn)) × 100, negative predictive value was calculated as (Tn/(Tn+Fp)) × 100 (Abbreviations used in above formula: Tp = total number of true positives; Tn = total number of true negative; Fp = total number of false positive, Fn = total number of false negative). The positive concordance between the PCR and performed microbiological tests was assessed using the kappa coefficient (K^c) where >0.75, excellent agreement; ≤0.75, fair; ≥0.4, and <0.4, good to poor agreement agreements).[19] The significance level was determined by the Chi-square (χ^2) test with the help of the SPSS 15.10 version. The significance of difference was taken as the significance value (P<0.05).

RESULTS

Patient Characterization

Of the 65 cases, 36 (55.4%) were males and 29 (44.6%) females. The mean age was 40.7 years and ranged from 12 to 78 years. 25 (38%) of all patients gave history of ATT intake and 40 (62%) subjects gave a history of fever. Pain was the most significant symptom, although the level of pain varied with the severity of the disease. 26 (40%) cases had severe, 24 (37%) moderate and 15 (23%) had mild pain. Serologic tests for HIV were positive in 2 (3%) patients; both were on antiretroviral therapy.

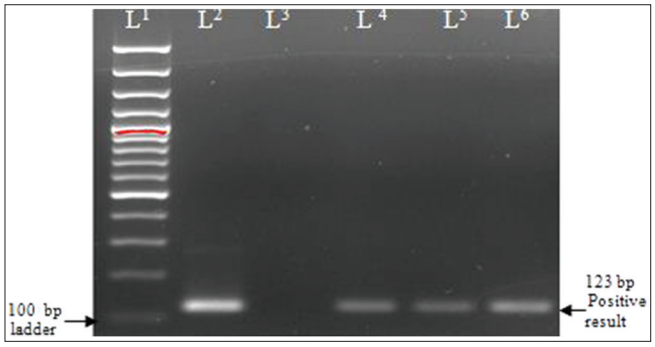

Figure 1: Result of *PCR IS6110* for detection of M.TB Complex in 2% agarose gel. Lane1 (L1) ladder 100 bp, L2 positive and L3 negative control, L 4,5,6, showed amplified 123-bp-positive specimens

Efficiency of ZN Microscopy, BACTEC Culture, and PCR *IS6110*

In all 65 cases, it was possible to arrive at the final diagnosis using the collective results of all the performed tests and clinical response was seen to the standard four drugs ATT regimen or modified ATT regimens as per standard protocols. Of the 65 specimens, 24 (37%) cases were positive on ZN microscopy and 32 (49.2%) cases were positive on BACTEC culture. Thirty-eight (58.5%) specimens were found positive either on ZN microscopy or on BACTEC culture for AFB. All these obtained culture isolates were confirmed as MTBC by the mentioned biochemical test. The PCR *IS6110* was positive in 42/65 (65%) specimens in this study. PCR was alone positive in seven (26%) specimens in 27/65 which were negative by both conventional bacteriological techniques. The results of all 3 tests considered (ZN microscopy, BACTEC culture and PCR *IS6110*), the 45 (69.2%) specimens turned out to be positive out of 65 specimens.

Sensitivity of PCR *IS6110* Against ZN Microscopy and BACTEC Culture

Analysis of PCR results among specimens that were positive and negative by conventional bacteriological methods showed that 24 specimens were positive on ZN microscopy, and out of these 23 (96%) were positive on PCR. Among 41 negative specimens by ZN microscopy, 19 (46.3%) of these specimens were positive on PCR. Further, BACTEC cultures were positive in 32 specimens, and out of these 30 specimens were positive on PCR. Again among 33 negative specimens by BACTEC culture, 12 (36.4%) were positive on PCR [Table 1]. Thus, the total 38/65 (58.5%) specimens were positive either on ZN microscopy or on BACTEC culture for AFB, and out of these PCR was positive in 35 (92%) and negative in 3 (8%) specimens. Further 7 specimens were additionally positive on PCR method, where conventional bacteriological tests were found to be negative. Thus, conventional bacteriological methods were positive in 38/65 (58.5%) of specimens where PCR was positive in 42/65 (65%) of specimens.

Sensitivity and Specificity of PCR *IS6110* Against Gold Standard BACTEC Culture and Combined Results of Bacteriological Tests (BACTEC Culture + ZN Microscopy)

The sensitivity of PCR was 94% and specificity was 64% with positive and negative predictive values of 71.7% and 91.3% when compared with the BACTEC culture. The difference was observed to be significant ($P<0.001$). The kappa coefficient for positive agreement was also calculated ($K^c=0.6$) with a fair agreement between PCR and BACTEC culture [Table 2]. However, when PCR assay was compared against combined results of performed bacteriological methods, the sensitivity was 95% and specificity 74.1%. The positive agreement and difference were ($K^c=0.7$, $P<0.001$) which implies a fairly positive agreement [Table 2].

DISCUSSION

Pott's disease still has a large prevalence in developing countries like India. It may occur at any age, from 1 to 80 years.[20] In this study, the age ranged between 12 to 78 years with a mean age of 40.7 years. A male preponderance was noted by us (Female:Male, 1:1.24). This is in keeping with previous observations (Female:Male, 1:1.05 and 1:4.71).[21,22] Pott's disease gradually spreads to adjacent vertebral bodies via the disc space and leads in later stages to collapse of the vertebral body, resulting in progressive paraparesis or quadriparesis depending on the level of involvement. Although Pott's disease is a curable disease, this is only possible if the disease is diagnosed at an early stage and patients are compliant with ATT regimens.[23]

The definitive diagnosis of Pott's disease is still difficult for most clinical laboratories. The reasons include (a) inadequate specimens, (b) paucibacillary nature of the specimens, and (c) presence of inhibitors that undermine the performance of nucleic acid amplification-based techniques. However, the conventional bacteriological detection techniques for *M. tuberculosis* are based on ZN microscopy and culture (LJ medium and BACTEC culture). These are still in widespread use for diagnostic purposes, though they fail to provide the desired sensitivity in the expected number of cases.[13] The PCR test may be particularly useful in the diagnosis of Pott's disease where conventional bacteriological techniques for *M. tuberculosis* are negative. The higher sensitivity and specificity levels makes PCR a valuable tool in the diagnosis of *M. tuberculosis* infections.

Previous studies have reported detection rates on ZN staining and microscopy ranging from 14.8% to 28%. Culture of *M. tuberculosis* has been reported as having detection rates of 11.11% to 53% in spinal tuberculosis.[24,25] In this study, we found that 24 (37%) specimens were detected on ZN microscopy and 32 (49.2%) specimens were detected on BACTEC culture. A total of 38 (58.5%) specimens were diagnosed on the

Table 1: Sensitivity of PCR against positive and negative cases on ZN microscopy and BACTEC culture

	No (%)	PCR results (*n*)		Sensitivity of PCR (%)
		Pos.	Neg.	
ZN microscopy positive	24 (36)	23	01	96
ZN microscopy negative	41 (63)	19	22	46
BACTEC culture positive	32 (49.2)	30	02	94
BACTEC culture negative	33 (50.8)	12	21	36.4
ZN microscopy positive with BACTEC culture positive	18 (28)	18	00	100
ZN microscopy negative with BACTEC culture positive	14 (21.5)	12	02	86
ZN microscopy positive with BACTEC culture negative	06 (09.2)	05	01	83
ZN microscopy negative with BACTEC culture negative	27 (41.5)	07	20	26

ZN – Ziehl–Neelsen; PCR – Polymerase chain reaction

Table 2: Sensitivity, specificity, positive/negative predictive value of PCR and their positive correlation (KC) with BACTEC culture and combined results of performed

Test		BACTEC culture							
		Pos.	Neg.	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	K^c	*P* value
PCR *IS6110*	Pos.	30	12	94	64	71.4	91.3	0.60	<0.001
	Neg.	02	21						
		Combined results of all performed bacteriological methods							
	Pos.	35	07	95	74.1	83.3	91	0.70	<0.001
	Neg.	03	20						

PCR – Polymerase chain reaction

basis of these conventional bacteriological methods. This is in agreement with the results reported by Chauhan,[26] who reported 50% positivity by bacteriological methods in Pott's disease. The low sensitivity of the conventional bacteriological method may be due to the absence of bacilli in specimens. The sensitivity of the ZN microscopy and culture methods bears a direct relationship with the concentration of organisms present in the specimens.[27]

In this study, PCR had a 46.3% sensitivity among ZN microscopy negative specimens and 96% sensitivity in positive specimens. Similarly, PCR had a 36.4% sensitivity among BACTEC negative specimens and 94% sensitivity in positive specimens. Our study results suggested that PCR *IS6110* was more sensitive, particularly when microscopy or BACTEC culture were negative. Of the 38 specimens that were positive either on ZN microscopy or on BACTEC culture for AFB, PCR was positive in 35 (92%) specimens. However, the kappa coefficient for positive agreement of PCR with ZN microscopy was (K^c=0.43, P<0.001) slightly good and with the BACTEC culture (K^c=0.6, P<0.001) showed a fine agreement. Finally, the positive agreement of PCR with the combined results of performed bacteriological tests was (K^c=0.7, P<0.001) indicating a fine agreement with statistically significant relation [Table 2].

Standardized studies regarding PCR detection in Pott's disease are lacking. Various studies have documented an increase in diagnostic rates with PCR targeting *IS6110* in specimens of EPTB. Sekar *et al*.[28] reported a 63% positivity rate, Negi *et al*.[6] reported rates of 73%, and Tiwari *et al*.[29] reported a 62% positivity rate among clinical specimens of EPTB. In this study we found that the positivity of PCR *IS6110* was 65% in Pott's disease.

A recent study by Pandey *et al*. demonstrated a sensitivity of PCR of 90% (9 out of 10) and specificity of 100% (12 out of 12) in spinal tuberclosis.[30] This study highlighted PCR as a highly sensitive and specific tool in the diagnosis of Pott's disease as well as other EPTB specimens.

In this study, PCR *IS6110* showed 94% sensitivity and 64% specificity against the gold standard BACTEC culture. Moreover, the overall sensitivity was 95% and specificity was 74% observed against the combined results of ZN microscopy and BACTEC culture [Table 2]. The specificity of PCR was low against the gold standard because five specimens were positive on ZN microscopy and PCR results, but BACTEC culture was negative; this could be due to the presence of nonviable *Mycobacterium* in the specimens as some of the subjects were receiving ATT. Therefore, we also incorporate our data to the PCR *IS6110* which is a useful technique for rapid diagnosis of Pott's disease.

CONCLUSIONS

PCR IS*6110* is a rapid method with a high sensitivity and specificity. It may be chosen for early detection of MTBC strain for an early diagnosis, management, and treatment of Pott's disease especially in high burden countries.

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