Original Article

PCR for *M. tuberculosis* in tissue samples

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Abstract

Background: The presence of granuloma, visualized in histopathology for diagnosing tuberculosis in tissue samples, is not a specific finding. Moreover, histopathological examination of tissue sections needs one to two weeks for final reporting. A rapid and sensitive method is therefore needed for detection of *Mycobacterium tuberculosis* in these paucibacillary tissue samples.

Methodology: A PCR-assay specific for IS6110 was evaluated for 104 different tissue samples in comparison to histopathology that was considered gold standard.

Results: PCR showed 74.1% sensitivity and 96.1% specificity. False positive and false negative results were observed in three (2.88%) and seven (6.73%) samples, respectively. Positive agreement between histopathology and PCR was observed as 0.737, indicating substantial good agreement between two tests.

Conclusions: PCR can be used for early diagnosis of tuberculosis in tissue samples that can help to initiate timely anti-tubercular treatment and prevent progression to irreversible changes.

Key Words: tissue samples, tuberculosis, PCR, histopathology

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Introduction

Tuberculosis, a leading cause of death, infects more than a third of the world's population [1]. Conventional methods for the diagnosis of tuberculosis include smear and culture for *Mycobacterium tuberculosis*. Ziehl-Neelson staining for acid-fast bacilli requires 10^4-10^6 bacilli/ml of tissue or fluid specimens to give a positive result [2,3]. Although culture for mycobacterium is more sensitive, it still needs 10^1-10^2 bacilli/ml of sample for the diagnostic yield and requires two to four weeks for the growth of *M. tuberculosis*. Diagnosis of tuberculosis from tissue samples is usually made by histopathological examination (HPE) that depends on the presence of granulomatous inflammation and caseous necrosis. It needs high expertise and the final reporting takes more than one week. A diagnostic method that is less time-consuming and at the same time has high sensitivity and specificity is therefore desirable.

Nucleic acid amplification (NAA) tests represent a major advance in the diagnosis of TB [4]. With the use of amplification systems, nucleic acid sequences unique to *M. tuberculosis* can be detected directly in clinical specimens, offering better accuracy than AFB smear and greater speed than culture [5]. Advanced molecular methods such as polymerase chain reaction (PCR), a type of nucleic acid amplification system, have shown very promising results for early and rapid diagnosis of the disease due to its detection limit of one to ten bacilli in various clinical samples [6]. Various targets have been used for detecting mycobacterial DNA such as IS6110, 65KD heat shock protein, MPB 64, 38KD protein and ribosomal RNA [7]. IS6110 has been proved to be a good target because of the presence of multiple copies of this insertion sequence (1-20) in most strains of *M. tuberculosis* complex [8,9]. Most of the earlier studies have compared PCR with histopathology from a formalin-fixed tissue using the same sample but formalin is known to cause alterations in DNA, if kept for a long time [10,11]. This study was conducted to determine the efficiency of PCR for detecting *M. tuberculosis* DNA in tissue specimens collected in normal saline and its comparative evaluation with HPE which was considered as gold standard.

Materials and Methods:

Study design and settings

This comparative study was conducted between January 2006 and March 2008 in the department of

microbiology, in collaboration with department of pathology, in a tertiary care centre in South India.

Sample size

One hundred and four tissue samples were processed both for PCR and HPE.

Collection of samples

Tissue samples for PCR were sent in normal saline and for histopathology in 10% formalin. All the samples were kept at 4°C before processing for PCR. All samples for PCR were processed in the microbiology department under the guidance of a senior microbiologist (Kiran Chawla). All the steps of PCR were performed in separate rooms to minimize the chance of carry-over of templates. All histopathology slides were reviewed by two senior pathologists after processing.

Processing of samples for PCR

All the tissue biopsies were homogenized in pestle and mortar followed by centrifugation at 6,000 rpm for 10 minutes. The supernatant was discarded and 3 ml of tris-buffer was added to the pellet obtained.

DNA Extraction

All the homogenized tissues were centrifuged again at 6,000 rpm for 10 minutes and to the resultant pellet, 250 µl of lysis buffer I and 20 µl of proteinase K was added (provided in the kit from Bangalore Genei, Bangalore, India). Then after mixing by vortexing, all the samples were kept in dry bath at 90°C for 20 to25 minutes and then centrifuged at 10,000 rpm for 10 minutes. To 200 µl of supernatant, 200 µl of lysis buffer II (containing internal control at the concentration of 10 µl/ml) was added in a 1.5 ml Eppendorf tube (Axygen Scientific, Union City, California) and incubated at 70°C for 10 minutes. Next 200 µl of 96-100% ethanol was added and mixed by vortexing. This mixture was added to a spin column placed in a 2-ml collection tube and centrifuged at 6,000 rpm for three minutes. The spin column was kept in a new 2-ml collection tube and washed twice with wash buffer (provided in the kit) and final centrifugation was performed at 14,000 rpm for two minutes to ensure complete removal of the wash buffer. Then the spin columns were kept in a 1.5-ml tube and 100 µl of pre-warmed (50°C) elution buffer (provided in the kit) was added. After incubating at room temperature for five minutes, it was centrifuged at 10,000 rpm for two minutes to elute the DNA. The DNA samples were kept at -20°C until further analysis.

Polymerization of DNA

Two-step nested PCR was performed by commercial kit method from Genei Bangalore (India) for IS6110 of *M. tuberculosis* in PTC-200 Peltier Thermocycler Inc., USA.

Analysis of amplified products

Amplified DNA underwent electrophoresis using 1.5% agarose gel at 120 volts for one hour and the resultant bands were interpreted by UV transillumination. A product of 123 bp was indicative of infection with *M. tuberculosis* and an amplified product of 340 bp was used as an internal control.

Histopathological examination

All the tissues received for histopathology were fixed in 10% formalin, embedded in paraffin, cut to 5-µm thick sections, and stained with hematoxylin-eosin (H&E) and gabbet's stain before microscopic examination. Presence of typical caseating granulomas and/or Langerhans cells after H&E staining or presence of acid-fast bacilli after gabbet's staining indicated proof of tubercular involvement of the tissue.

Table 1. Distribution of various tissue samples showing results of histopathology and PCR for *M. tuberculosis*

TISSUE SAMPLE	TOTAL NO. (n=104)	HPE+ PCR+	HPE+ PCR-	HPE- PCR+	HPE- PCR-
Synovial	36	1	4	1	30
Vertebral	25	9	0	0	16
Soft tissue	15	4	0	1	10
Bone	11	2	2	0	7
Genital	7	2	0	0	5
Renal	6	1	0	1	4
Intestinal	2	0	0	0	2
Lymph node	2	1	1	0	0

Results:

Distribution of different tissues processed in this study along with the results of PCR and HPE is given in table 1. Out of 104 samples, 20 (19.23%) were positive by both PCR and histopathology and 74 (71.15%) showed negative results by both tests. False positive and false negative results were observed in 3 (2.88%) and 7 (6.73%) samples respectively.

Considering HPE as gold standard, PCR has shown 74.1% sensitivity (with 95% Confidence Interval [CI] 53.4; 88.1) and 96.1% specificity (with 95% CI, 88.2; 98.9). Overall positive and negative predictive value of PCR was observed as 86.9% (with 95%CI, 65.3; 96.6) and 91.4% (with 95% CI, 82.5; 96.2) respectively. Positive agreement between HPE and PCR was observed as 0.737 indicating substantial good agreement between two the tests ($p < 0.05$). The results shown by all the samples are shown in table 2.

Table 2: Comparison of PCR with histopathology results for total 104 tissue samples

	PCR Positive	PCR Negative
Histopathology Positive	20	7
Histopathology Negative	3	74

Discussion:

Histopathological diagnosis of tuberculosis is usually observed in the presence of granulomatous inflammation and caseous necrosis. In the absence of caseous necrosis, the final diagnosis depends only on the granulomatous inflammation. However, it is not specific for tuberculosis as it can be present in a variety of other conditions such as sarcoidosis, syphilis, leprosy, Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, and pneumoconiosis [12]. Therefore, to confirm the diagnosis of tuberculosis, either acid-fast staining or culture of tissues for *M. tuberculosis* must be performed. Both of these tests have poor sensitivity because of paucibacillary tissue samples. Salian *et al.* [13] have shown only 24.5% (13/53) and 25% (15/60) positivity by culture and acid-fast staining of histological specimens respectively. Many tissues that are sent for histopathology are rarely sent for culture as it is a very laborious and time-consuming procedure. Recent molecular techniques such as PCR have high sensitivity and specificity for diagnosing tuberculosis [14,16]. Using serially diluted *M. tuberculosis* DNA, as little as 8 fg of mycobacterial DNA (~2 bacilli) could be detected in a TB-PCR assay of cultured bacteria, although the sensitivity is less for clinical samples because of the presence of human genomic DNA [17]. The turn-around time for giving the diagnosis is less (within 48 to 72 hours) for PCR. Li *et al*. [7] have shown that in 59% (68/115) of patients where granulomatous inflammation was observed in tissue samples but without demonstrable acid-fast bacilli on Ziehl-Neelson staining, tissue PCR helped to make the final diagnosis of tuberculosis. Various studies conducted in the past have shown different sensitivities for PCR with the same sequences (IS6110) ranging from 61-83% using different tissue samples. Chakravorty *et al*. [18] noticed sensitivity of PCR using pleural tissue and lymph nodes at 75% and 69.2% respectively. Cheng *et al.* [2] observed the sensitivity of PCR at 81.2% for all tissues and Cegielski *et al.* [19] at 80% for pericardial tissue. In the present study PCR has shown 74.07% sensitivity and 96.1% specificity, which correlates well with the study done by Park *et al*. [11]. False negative results were observed in 6.73% samples. False negative results for tissue PCR are mainly due to uneven distribution of mycobacteria in tissue samples, inadequate samples sent for PCR, presence of extensive necrosis in the tissues, and presence of inhibitors [18,20]. The submission of quality specimens is very important for getting reliable results [21]. In our study, the possibility of the presence of inhibitors was eliminated by using internal controls (beta-globin gene) for running PCR. Occasionally in paucibacillary tissue samples, false negative results can be due to loss of DNA during extraction [22]. Other reports indicate that the IS6110 gene can be absent in the case of a few strains that can also give false negative results, or it may be the presence of mycobacteria other than *M. tuberculosis* [23]. In 2.88% samples, PCR showed positive results but results for HPE were negative for tuberculosis. False positive results in for PCR testing are usually due to carry-over contamination [9]. In the present study, different steps of PCR (DNA extraction, pre-PCR mixing, PCR, and post-PCR gel documentation) were conducted in different rooms, reducing the chances of contamination. These three false positive cases were initiated on antitubercular treatment (ATT) because of high clinical suspicion of tuberculosis and all cases responded very well with significant clinical improvement. After good response to ATT, it is debatable whether these three cases truly represented false positive results. The probable explanation in these cases is that the disease was still developing and well-developed granuloma had not yet formed, but the presence of mycobacterial DNA in tissues could still be easily detected by PCR at the early stage. The present study differs from earlier tissue PCR studies as here tissue samples were preserved in normal saline and not in 10% formalin as the latter is known to cause alterations in DNA if kept for a longer time [10,11].

There is disagreement over whether antitubercular treatment should be started in cases of tuberculosis diagnosed by PCR; however, treatment should be initiated as early as possible for extrapulmonary tuberculosis to prevent irreversible events. The present study has shown good correlation between results of PCR and HPE. We noticed that if tissue PCR produces positive results when performed in careful, clean and uncontaminated conditions and the clinician suspects tuberculosis, ATT can be started. On the other hand, if PCR shows negative results when tuberculosis is strongly suspected, PCR results should be correlated with HPE before starting the treatment.

PCR also has some limitations. As it fails to distinguish between live and dead bacilli, its relevance must be judged in light of the overall clinical picture in cases where the patient has received anti-tubercular treatment recently, reactivation tuberculosis, or asymptomatic infection.[13]. This sophisticated technique is limited by the need for a suitable infrastructure and the high cost of the test.

To conclude, tissue PCR is a rapid, sensitive and specific test that can be used for early diagnosis of extrapulmonary tuberculosis. Though HPE is a cost-effective method, early PCR results can enable clinicians to start treatment in advance to prevent irreversible sequelae associated with morbidity of the disease.

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