

Evaluation of nested PCR targeting IS6110 of *Mycobacterium tuberculosis* for the diagnosis of pulmonary and extra-pulmonary tuberculosis

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Abstract

The purpose of this study was to compare sensitivity and specificity of the PCR from smear and culture in the diagnosis of suspected cases of pulmonary and extra pulmonary tuberculosis. This study was carried out on 140 specimens obtained from suspected cases of tuberculosis. The various specimens collected from these patients included 74 sputum, 38 endometrial biopsies, 16 CSF and 12 gastric aspirates. All the specimens were tested by ZN staining, culture was on L-J medium and PCR was performed for targeting IS6110 sequence. Out of 140 patients, 61.4% were suffering from pulmonary tuberculosis and 38.5% from extra-pulmonary tuberculosis. In these 140 patients, 40 (28.5%) were ZN smear positive for AFB, 48 (34.2%) were culture positive for AFB and 104 (74.2%) were sensitive to nested PCR for *Mycobacterium tuberculosis*. We observed significant difference in sensitivity of PCR for smear positive and negative cases, and also for culture positive and negative cases.

Keywords: Tuberculosis; Pulmonary tuberculosis; Extra-pulmonary tuberculosis; Nested PCR for tuberculosis.

Introduction

Tuberculosis is globally still a leading cause of adult mortality arising from a single infectious agent and 21% of the world's TB-infected population is in India (RNTCP 2008). The conventional method for diagnosing tuberculosis using clinical samples by the acid-fast bacilli (AFB) smear has low sensitivity and specificity and culture for *Mycobacterium tuberculosis* complex is time consuming. (Grange 1984) Under the RNTCP guideline laboratory diagnosis of tuberculosis relies on microscopic examination of smears and culture of specimens (Nandgopal *et al* 2010). Smear microscopy although rapid and inexpensive but lacks sensitivity. It can detect AFB, if the smear contains ≥ 1000 bacilli which means that specimen must have $\geq 10,000$ bacilli per ml. Laboratory AFB culture method is gold standard and sensitive (10-100 viable bacilli are required) but because a long period of about 6-8 weeks is required, most clinical and therapeutic decisions have to be made before laboratory results become available (Prasad *et al* 2001). ZN smear provides preliminary diagnostic information within an hour suffer from several limitation such as low sensitivity and specificity especially in extra-pulmonary tuberculosis manifestation. Serology and other newer techniques are not widely used due to high cost, low sensitivity and specificity or both. Thus, there is a definite need for a rapid, highly sensitive and specific method for quicker diagnosis of *M. tuberculosis*. Nowadays Nucleic acid amplification technologies such as PCR are revolutionizing the detection of infectious

pathogens such as *M. tuberculosis*. Amplification technology offers the potential for the diagnosis of tuberculosis in a few hours with a high degree of sensitivity and specificity. Polymerase chain reaction has been found to be useful for rapid diagnosis of tuberculosis from variety of clinical specimens. Many laboratories around the globe are using primers focused on IS6110 sequence of *Mycobacterium* genome. IS6110 is an insertion sequence specific for *Mycobacterium tuberculosis*. The aim of the present study was to evaluate sensitivity and specificity of polymerase chain reaction targeting IS6110 insertion site in diagnosis of pulmonary and extra-pulmonary tuberculosis. We also aim to compare the results of PCR tests with those of conventional ZN (Ziehl Neelsen) stained acid fast bacilli microscopy and culture on L-J medium.

Materials and Methods

The present study was carried out on 1231 patients attending outpatient and inpatient departments of JNMC, AMU, Aligarh, who were suspected to be suffering from tuberculosis. Out of 1231, 140 were selected for this study on the basis of radiological diagnosis and other investigations. The clinical samples collected from these patients were 74 sputa, 38 endometrial biopsies, 16 CSF and 12 gastric aspirates. The samples were collected at the first time of contact with the treating physician. A detailed questionnaire and consent was obtained from each patient.

Specimen processing

The specimens were processed by standard method; one portion of the sputum was subjected to routine direct microscopic examination using Ziehl Neelsen method. The rest of the sputum was digested and decontamination by N-Acetyl-L-Cysteine-2%NaOH method (NALC-2%NaOH) and concentrated by centrifugation at 3000g for 20 minute, from the pellet one smear was prepared for Ziehl-Neelsen smear staining and the two Lowenstein Jensen's medium slant were inoculated which were incubated at 37°C for 6-8 weeks. In case of endometrial biopsy or tissue, first it was grind in sterile mortar and pestle, then decontaminated. While CSF directly inoculated on LJ medium and incubated at 37°C for 6-8 weeks. The second part of the sediment was stored at -20°C to be used later for PCR. The inoculated LJ media were examined every second day during the first week and then weekly for upto 6 weeks for presence of growth. The growth if present was identified by standard morphological and biochemical tests.

Polymerase chain reaction

DNA extraction from specimens

Different methods were used for extraction of DNA from different specimens. In sputa, gastric aspirates and endometrial biopsies, one aliquot of the sediment obtained after NALC-2%NaOH decontamination was kept frozen at -20°C was used as a source of DNA. In case of CSF it was directly used for DNA extraction.

PCR amplification

Amplification of 123 bp DNA segment belonging to IS 6110. The extracted DNA was amplified by two step PCR assay (GeNei Bangalore). In the first step, 220 bp DNA segment was amplified by external primer. The amplification carried out in 12 µl final volume, in which 8.22 µl amplification premix (reaction buffer with MgCl₂, dNTPs and *Mycobacterium tuberculosis* complex specific external primer) 0.33 µl Taq DNA polymerase, 0.5µl uracil DNA glycosylase and 3 µl DNA template. The amplification was carried out in a thermocycler (Minitaq). After initial denaturation at 94°C for 5 minute, 35 amplification cycles were performed within thermocycler. Each cycle consisted of denaturation at 94°C for 2 min. annealing of primer at 68°C for 2 min and primer

extension at 72°C for 1.30 min. after complete amplification of 220 bp of DNA segment, the amplified product was used as DNA template for amplification of 123 bp segments. 15 µl of master mix (buffer with MgCl₂, dNTPs and *Mycobacterium tuberculosis* specific internal primer) and first PCR product was added in PCR tube, and amplification was carried out in a thermocycler. After initial denaturation at 94°C for 5 minute, 35 amplification cycles were performed within thermocycler. Each cycle consisted of denaturation at 94°C for 2 min annealing of primer at 68°C for 2 min and primer extension at 72°C for 1.30 min.

Analysis of amplified product

The nested or second PCR products were analyzed by gel electrophoresis in 2% agarose (Sigma-MO USA) prepared in Tris-Borate EDTA buffer containing 0.5 g/ml of ethidium bromide (Sigma-MO USA). The gel was examined in a gel documentation system (Bio-Red) for a 123 bp using standard molecular marker. Presence of a single band equivalent to 123 bp was taken a positive result when negative control gave no reaction. All the false negative and doubtful results were retested.

Statistical analysis

All the data were recorded on standardized case report form and data were analyzed using SPSS v.13 (SPSS Inc. USA).

Results

We examined 1231 suspected tuberculosis patients, out of which 140 were selected for the study taking into consideration the clinical findings and other investigation performed for the clinical diagnosis and 20 healthy non-tubercular patients were also included in the study. Out of 140 patients 61.4% were suffering from pulmonary tuberculosis and 38.5% extra-pulmonary tuberculosis. Most of these patients were of 21-30 age and mostly females. Our study shows that 40 samples were smear positive for AFB, 48 were culture positive and 104 were sensitive to PCR for *Mycobacterium tuberculosis* (table 1). The highest PCR positivity was for sputum 75.6% followed by CSF 75%, endometrial biopsy 73.6% and gastric aspirate 66.7%. Among 20 healthy controls, all the samples were negative for smear as well as culture, while only 1 out of these 20 was PCR positive for *Mycobacterium tuberculosis* (table 2).

Table 1. Comparison of sensitivity of smear, culture and PCR test for different samples.

Specimens	No. of specimens	Smear		Culture		PCR	
		Positive	Negative	Positive	Negative	Positive	Negative
Sputum	74	40	34	42	32	56	18
Endometrial biopsy	38	0	38	2	36	28	10
CSF	16	0	16	0	16	12	4
Gastric aspirate	12	0	12	4	8	8	4
Total	140	40(28.5%)	100(71.4%)	48(34.2%)	92(65.7%)	104(74.2%)	36(25.7%)

Table 2. Specificity of different tests conducted on 20 non-tubercular healthy patients.

Test performed	Results		Specificity (%)
	Negative	Positive	
ZN smear	20	0	100%
LJ medium	20	0	100%
PCR	19	1	95%

In this study, we also evaluated the sensitivity of PCR with two most widely used conventional methods for diagnosis of tuberculosis. The sensitivity of PCR for smear positive specimen was 90% while for smear negative specimen was 68%. The PCR sensitivity for LJ culture positive specimen was 95.8% while for culture negative was 63%. Sensitivity for smear negative and culture positive specimen was 100%. Sensitivity for smears as well as culture negative specimen was 64.4% (table 3).

In cases of pulmonary tuberculosis, sensitivity of PCR over smear positive specimen was 90%, on smear negative was 60.8%, LJ culture positive specimen was 95.6% and on LJ culture negative specimen sensitivity was 50%. Thus, significant difference was observed in sensitivity of PCR for smear positive and smear negative cases ($p < 0.05$) and also for culture positive and culture negative cases ($p < 0.001$). Among the 12 gastric aspirate 8(66.7%) were positive by PCR but culture was positive in only 4(33.3%) specimens, however none was positive by smear examination (table 1).

For extra-pulmonary tuberculosis specimens, PCR sensitivity over culture positive specimens was found to be 100% while none of the specimen was smear positive. Out of 38 endometrial specimens, 28 (73.6%) were positive by PCR, however, two specimens were culture positive. None of the 16 CSF specimens included in the study were

positive by smear as well as culture but 12 (75%) of these were positive by PCR test (table 1).

Discussion

In India, most of the time the diagnosis of tuberculosis is primarily based on clinical features, histopathology demonstration of acid fast bacilli and isolation of *Mycobacterium tuberculosis*. Nucleic acid amplification method to detect *Mycobacterium tuberculosis* in clinical specimens is increasing used as a tool for TB diagnosis. Application of molecular methods in routine for diagnosing in developing country like ours depends on various factors like high cost and availability of skilled personnel to perform the test. In our study the overall status of nested PCR reaction on suspected tuberculosis cases were found to be 104 (74.2%) positive by nested PCR test while 40 (28.5%) were smear positive and 48 (34.2%) cases were culture positive. PCR amplification method is useful for the rapid detection of *M. tuberculosis* with reported sensitivity of 55 to 95% in the culture positives and 100% in both smear and culture positive clinical specimens (Shawe *et al* 1993, Eisenach *et al* 1991). Similar results were reported by Negi *et al.* in 2005 and they reported PCR positivity 74.4%, while culture and smear sensitivity of 48.9% and 33.7% (Negi *et al* 2005). In another study, PCR positivity of clinical specimens was 100% in both smear and culture positive and 51.8% in

culture positive and 69.4% in culture negative specimens (Banavaliker *et al* 1998). In our study, PCR positivity in smear and culture negative cases were 64.4% and smear as well as culture positive was 94.7%. We also evaluated that PCR test is highly useful diagnostic tool for extra-pulmonary tuberculosis, the PCR positivity in endometrial biopsy was 73.6% and in CSF was 75%. We

observed that 74% of extra-pulmonary cases could be diagnosed by PCR, however, all these were negative by smear examination and only two endometrial biopsies were positive by culture. Thus, PCR method would be much useful in extra-pulmonary tuberculosis patients where diagnosis is a problem.

Table 3. Comparison of sensitivity of PCR test with other tests in pulmonary and extra-pulmonary tuberculosis specimens.

Conventional tests	Pulmonary tuberculosis				Extra-pulmonary tuberculosis				Total PCR positivity		
	Total specimens	PCR positivity			Total specimens	PCR positivity			+Ve	-Ve	Total positivity
		+Ve	-Ve	Total positivity		+Ve	-Ve	Total positivity			
ZN positive (40)	40	36	4	90%	0	-	-	-	36	4	90%
ZN negative (100)	46	28	18	60.8%	54	40	14	74%	68	32	68%
LJ positive (48)	46	44	2	95.6%	2	2	0	100%	46	2	95.8%
LJ negative (92)	40	20	20	50%	52	38	14	73%	58	34	63%
ZN positive LJ positive (38)	38	36	2	94.7%	0	-	-	-	36	2	94.7%
ZN positive LJ negative (2)	2	0	2	0%	0	-	-	-	0	2	0%
ZN negative LJ positive (10)	8	8	0	100%	2	2	0	100%	10	0	100%
ZN negative LJ negative (90)	38	20	18	52.6%	52	38	14	73%	58	32	64.4%

+ Ve = positive; - Ve = negative

There was only one false positive result by PCR test among healthy controls which could be due to the ability of the PCR test to detect very low number and even dead bacteria in a sample. Only two specimen positive for AFB by ZN smear were found to be negative by both culture and PCR test. This could be due to the presence of nonviable *Mycobacterium* in the sample as the patient was receiving antitubercular treatment.

Conclusion

We were able to evaluate the utility of PCR test by comparing it with smear microscopy and culture in a large number of specimens obtained from clinically diagnosed patients. The sensitivity, specificity and speed of molecular test in diagnosis of tuberculosis should encourage the routine use of this test in clinical practices.

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