



Contents lists available at BioMedSciDirect Publications

International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com



Original Article

A comprehensive comparison of PCR based assay versus microscopy & culture in the diagnosis of tuberculosis in different clinical specimens

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ARTICLE INFO

Keywords:

Comparison of microscopy
Culture & PCR in different
Clinical specimens: Tuberculosis

ABSTRACT

Background: The laboratory diagnosis of tuberculosis ranges from simple microscopy, culture to complex molecular techniques. Rapid diagnosis is important since conventional techniques have limitations. **Aims:** To evaluate the efficacy of PCR using genus specific and species specific primers versus microscopy and culture in the diagnosis of tuberculosis in different clinical samples. **Methods:** A total of 125 clinical samples were processed for the detection of Mycobacterium tuberculosis by smear, culture & PCR in the diagnosis of tuberculosis. **Results:** The overall sensitivity, specificity, PPV and NPV of PCR were 75 %, 88%, 62.5% and 93.42% respectively. PCR done on non tuberculous patients were negative thus showing the specificity of 100%. PCR sensitivity in pulmonary & extra pulmonary clinical samples were 83.33% and 71.42% respectively a relatively higher value than other tests. Out of eight smear positive-culture positive (SP-CP) & twelve smear negative -culture positive (SN-CP) specimens, PCR detected 87.50 % & 66.66% respectively. **Conclusions:** This study showed that PCR can serve as a useful complement to clinical diagnosis. PCR can be useful in detecting the cases in extra pulmonary specimens which may be missed by smear & \ or culture. The PCR based assay cannot be used reliably alone as a sole diagnostic tool in the diagnosis of tuberculosis, it has to be performed in conjunction with microscopy & culture methods and interpreted with the clinical settings.

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1. Introduction

Tuberculosis is one of the leading infectious diseases in the world and remains a major public health problem causing considerable morbidity and mortality. [1,2] The dual infection of tuberculosis with HIV & emergence of drug resistance strains have increased alarmingly in recent years , adding to the existing burden.[3] Several methods are available for the laboratory diagnosis of tuberculosis ranging from simple microscopy to complex molecular biological techniques. Although microscopy & conventional Lowenstein Jensen medium (LJ medium) remains the corner stone for the diagnosis of tuberculosis, these methods are less sensitive & slow respectively. The isolation, identification and drug susceptibility testing can take several weeks or longer. This can affect the treatment by either delaying it or causing inappropriate empiric therapy for tuberculosis to subjects without mycobacterial infections.

Therefore, timely identification of tuberculosis is important because of the need to make decisions regarding management such as initiation of antituberculous therapy, isolation and prophylaxis. The outcome for the patient could be improved if rapid, simple & reliable tests are available. During the past 20 years, several molecular methods have acquired a greater relevance in the field of laboratory diagnosis of tuberculosis. These methods are able to potentially reduce the diagnostic time from weeks to days. [4,5,6,7]

The present study was carried out to evaluate the role of PCR based assay using both genus specific & species specific primers in the detection of tuberculosis in different clinical specimens. We also aimed to compare the results of PCR v/s microscopy & culture.

2. Materials & Methods:

A total of 125 clinical samples were included in our study. All the samples were subjected to AFB staining, culture & PCR.

One hundred clinical specimens were obtained from patients with a strong clinical suspicion of tuberculosis from different

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hospitals in & around Mangalore. The samples included 16 pulmonary and 84 extra pulmonary specimens. Of the 16 pulmonary specimens, 12 were BAL, 2 gastric lavage, 1 bronchial washing and 1 tracheal aspirate. The 84 extra pulmonary specimens consisted of 38 pleural fluid, 23 ascitic fluid, 7 synovial fluid, 5 pus, 4 CSF, 4 FNAC (lymph node) and 3 bone marrow aspirate.

A total of 25 clinical samples obtained from non tuberculous subjects were used as negative controls. The control samples included 10 pulmonary specimens & 15 extra pulmonary specimens. The 10 pulmonary specimens included 6 BAL and 4 bronchial washings obtained from chronic asthmatics & chronic bronchitis patients. The 15 extra pulmonary specimens included 4 ascitic fluid, 4 pleural fluid and 7 CSF. Ascitic fluid was obtained from 4 cases of portal hypertension and pleural fluid from 4 cases of nephrotic syndrome. CSF samples were obtained from 3 cases of congenital hydrocephalous, 2 cases of febrile seizures & 2 cases of aseptic meningitis.

Processing of specimen:

Depending upon the nature of specimens, appropriate concentration methods were employed.

Sterile body fluids were centrifuged, where as specimen containing mucous like BAL, bronchial washings, tracheal aspirates, gastric washings were digested & decontaminated by using Modified Petroff's (4% NaOH) method [8,9]. Sediment thus obtained were subjected to all the three tests, i.e smear preparation, inoculation onto the LJ medium & for PCR.

Gabbet's cold staining was done on these smears using standard techniques. [10] Cultures were examined for growth twice weekly for the first 2 weeks & once weekly thereafter, up to 8 weeks. The positive cultures grown were amplified by genus and species specific primers.

Polymerase Chain Reaction:

Extraction of DNA, amplification & detection were done in physically separate areas.

The DNA was extracted by CTAB method (Cetyl Trimethyl Ammonium Bromide). [11] DNA was extracted from clinical samples, M. tuberculosis standard strain (H37RV) and culture isolates. Each step of the extraction protocol was performed inside bio safety cabinet, using protected tips and dedicated pipettes at room temperature.

PCR amplification of DNA :

The primers used for the assay were based on the published sequence. [11,12]

Two set of primers- genus specific and species specific were used for the assay.

The genus specific primers amplified a 383 base pair fragment of a gene that codes for a 65 kDa protein present in all species of mycobacteria.

The sequences of the genus specific primers were:

Forward primer: 5' GAGATCGAGCTGGAGGATCC 3'

Reverse primer: 5' AGCTGCAGCCCAAACCTGTT 3'

The species specific primer amplified a 123 base pair nucleotide sequence in IS 6110 present in strains of the M.tuberculosis complex.

The sequences of the species specific primers were:

Forward primer: 5' CCTGCGAGCGTAGGCGTCCG 3'

Reverse primer: 5' CTCGTCCAGCGCGCTTCGG 3'

Preparation of master mix solution:

The reaction mixture was prepared using dedicated pipettes in a separate area free from contamination with bacterial cultures and amplified products.

Genus specific: The amplification of 65 KDa gene was done using TB1 & TB2 primers.

PCR was performed in a 25 µL reaction volume of 1X PCR buffer (10mM Tris HCl, pH 9, 1.5 mM MgCl₂, 50mM KCl & 0.01% gelatin), 0.2 mM dNTP each, 0.5 µM primers each (forward & reverse), 0.7 U/Taq polymerase, 3 µl of template DNA & 11.75 µl of DNAase and RNAase free double distilled water.

Species specific: The amplification of 123 bp nucleotide sequence in IS 6110.

PCR was performed in a 25 µl reaction volume of 1X PCR buffer (10mM Tris HCl, pH9 1.5mM MgCl₂, 50mM KCl & 0.01% gelatin). 0.2 mM dNTP each, 0.5 µM primers each (forward & reverse), 0.3 U/Taq polymerase, 3 µl of template DNA & 11.9 µl of DNAase and RNAase free double distilled water.

Amplification of DNA:

DNA amplification by PCR was performed with a total reaction volume of 25 µl by using model Biorad Gene Cycler. The contents were well mixed with vortex and subjected to thermocycling as follows:

For Mycobacterium (genus specific) the conditions were:

Initial delay: 94°C for 5 mins

940^c for 90 Seconds

570^c for 90 Seconds

720^c for 90 Seconds

Final delay: 720^c for 5 minutes

} 30 Cycles

For *M.tuberculosis* complex (species specific) the conditions were :

Initial delay: 94° C for 5 minutes.

94° C for 2 minutes

68o C for 2 minutes

72° C for 2 minutes

} 30 Cycles

Final delay: 72° C for 5 minutes

Detection of Amplification products:

PCR products were detected on 1.5% agarose gel in 1X TE buffer containing ethidium bromide at 10µg/ml concentration under ultra violet illumination.

Quality Control:

The quality of the amplification was monitored by the simultaneous testing of a positive & negative controls in each set of samples tested.

Positive amplification control: *M. tuberculosis* standard strain H37RV

Negative amplification control: Reaction mixture without sample

Negative processing control: TE buffer

Interpretation:

The molecular weight markers, positive control, negative control & samples were observed for the appropriate bands.

When a single band of 383 bp & 123 bp were obtained, it was inferred that the sample was positive for mycobacterium species & *M. tuberculosis* complex respectively.

3. Results:

A total of 125 clinical samples were subjected to AFB staining, culture and PCR.

We compared the results of 100 clinical samples obtained with a clinical suspicion of tuberculosis and other 25 clinical samples obtained from non tuberculous patients. (Table 1)

Culture was considered as the gold standard for the diagnosis of tuberculosis

Species specific PCR in the smear positive-culture positive (SP-CP) samples and smear negative- culture positive (SN-CP) samples showed a positivity rate of 87.5% and 66.66% respectively. In case of smear positive-culture negative (SP-CN) and smear negative-culture negative (SN-CN) the positivity rate was 71.42% and 5.48% respectively. (Table 2)

When a combination of smear and culture results were analyzed, in the smear &/or culture positive and smear &/or culture negative specimens, the PCR showed a sensitivity of 74.07% and a specificity of 94.5%. (Table 3)

In the pulmonary specimens, the sensitivity & specificity of the smear examination is 83.33% & 37.5% respectively. In contrast the PCR showed the sensitivity, specificity, PPV & NPV of 83.33%, 62.5%, 62.5% & 83.33% respectively.

In the extra pulmonary specimens, the sensitivity & specificity of the smear examination is 21.42% & 97.2 % respectively. In contrast the PCR showed the sensitivity, specificity, PPV & NPV of 71.42%, 91.6%, 62.5% & 94.28% respectively. (Table 4)

4. Discussion:

Tuberculosis even today, remains a major health problem in the developing countries in the world especially in India. The microscopy and culture are still the methods of choice for the diagnosis of tuberculosis in most of the microbiological laboratories. Detection of acid fast bacilli by conventional microscopy is simple and rapid but lacks adequate sensitivity (sensitivity ranges from 20-80%). [13] The limit of detection is that, sample should contain atleast 10,000 bacilli/ml. Moreover, a positive result with this test doesn't discriminate between mycobacterium species. Cultivation of *M.tuberculosis* is considered as the gold standard in the diagnosis of tuberculosis. This gold standard lacks sensitivity and is negative in specimens from majority of paucibacillary cases. This poses great dilemma for comparing gene amplification methods which are vastly more sensitive but have danger of false positivity due to contamination. The culture has a sensitivity of 80- 93% & specificity of 98%, but result becomes available only after several weeks. [14,15] This can affect the treatment by either delaying it or causing inappropriate empirical therapy for tuberculosis to subjects without mycobacterial infections. Several reports on the successful use of the PCR for the detection of *M.tuberculosis* complex in clinical specimens have been published. [16,17]

In our study we subjected all clinical samples to AFB staining, culture and PCR. AFB staining was done using Gabbets cold staining method, as it is was easy to perform, economical & no significant differences in the results in comparison with Ziehl Neelsen staining as reported by different studies. [18,19,20] We included wide range of clinical specimens both pulmonary and extra pulmonary in our study.

Extra pulmonary tuberculosis presents a diagnostic dilemma for both physicians as well as for clinical microbiologists. The extra pulmonary tuberculosis is on the increase worldwide and is now beginning to emerge from the shadows of its senior cousin. In our study, 84 extra pulmonary & 16 pulmonary specimens were included. Among pulmonary specimens, sputum was not included in our study because tubercle bacilli if present are usually multibacillary in comparison to other pulmonary specimens, whereas in extra pulmonary it is usually paucibacillary. The poor

Table 1: Results of smear, culture and PCR tests conducted on different clinical specimens

Tests performed	Suspected cases of Tuberculosis (100)					Negative controls (25)			
	Total samples	Positive	Results Negative	Sensitivity	Specificity	Total samples	Positive	Results Negative	Specificity
Microscopy	100	15	85	40%	91.3%	25	0	25	100%
LJ Culture	100	20	80			25	0	25	100%
PCR Genus specific	100	26	74	85%	88.75%	25	0	25	100%
PCR Species specific	100	24	76	75%	88.75%	25	0	25	100%

Table 2: Comparison of Positivity of PCR with smear and culture for *Mycobacterium tuberculosis complex* (species specific)

Test Results	Total	PCR result		Positivity
		Positive	Negative	
Smear positive-culture positive	8	7	1	87.5%
Smear negative-culture positive	12	8	4	66.66%
Smear positive-culture negative	7	5	2	71.42%
Smear negative-culture negative	73	4	69	5.48%
Total	100	24	76	24

Table 3: Validity of PCR when compared with combination of smear and / or culture

PCR		Smear and /or culture positive		Smear and/ or culture negative	
Positive (24)		20		4	
Negative (76)		7		69	
Predictive values (%) of PCR					
Sensitivity	Specificity	PPV	NPV	Positive likelihood ratio	Negative likelihood ratio
74.07%	94.5%	83.33%	90.78%	12.98	0.273

Table 4: Detection of tuberculosis by PCR assay v/s smear, culture in pulmonary & extra pulmonary specimens

Tests performed	Pulmonary (16)			Extra pulmonary (84)			
	Positive	Results Negative		Positive	Results Negative	Positive	Negative
Microscopy	10	6		5			79
LJ culture	6	10		14			70
PCR genus specific	8	8		18			66
PCR species specific	8	8		16			68
Predictive values of PCR species specific primers				Predictive values of PCR species specific primers			
Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
83.33%	62.5%	62.5%	83.33%	71.42%	91.62%	62.5%	94.28%

performance of conventional microbiological techniques in extra pulmonary specimens has stimulated the increased use of PCR assays in the laboratory diagnosis of tuberculosis. Therefore, the utility of PCR was ascertained for paucibacillary cases. We used both genus specific and species specific PCR primers to increase the specificity and sensitivity of the assay. We were able to evaluate the utility of PCR test by comparing with smear microscopy and culture results in the specimens obtained from clinically suspected tuberculosis patients and non tuberculous patients.

Our study shows the microscopy has a sensitivity of 40% & specificity of 91.3%. In comparison, the PCR showed a higher sensitivity of 75% & specificity of 88.75%. PCR tests done on non tuberculous patients showed a specificity of 100% (Table 1) .Our findings were consistent with other studies done on different clinical specimens which showed the sensitivity & specificity of PCR ranging from 60-100% & 77-100 % respectively.[21,22,23]

Further in our study, when the combination of smear & culture results were analyzed, PCR could detect only 15 samples out of 20 culture positives (both SP-CP & SN-CP) (Table 2) .The false negativity of PCR results could be attributed to non homogeneous distribution of bacilli, presence of amplification inhibitors in the samples, mutations in the regions for primer annealing or truncations in the genes targeted for amplification.[24] Out of 7 SP-CN, PCR tested positive for 5 samples. Culture negativity could be either due to the presence of non viable mycobacteria in the samples as the subjects were receiving ATT or the culture may have missed some true positive cases due to use of the harsh Petroff's method. It has been recommended that for each sample, decontamination should be performed by the gentler N-acetyl-L-cysteine method with close attention paid to total time of exposure rather than the harsh sodium hydroxide method. The two media, one egg based and another agar based should be used to maximize the chances of isolation.[25]

In 73 samples, negative by either smear or culture, PCR test was able to detect 4 positive cases (which included 2 lymph node, 1 bone marrow and 1 pus). PCR could have amplified M.tuberculosis DNA because the organism present was too less to grow in culture or host defenses would have rendered the mycobacteria non cultivable. These were not likely to represent false positive results as PCR repeatedly done on these samples was positive. These samples belonged to highly suspected cases of tuberculosis & also histopathological examination of these samples was consistent with tuberculosis. All these patients also responded well to antitubercular treatment. Thus when a combination of smear and culture results were analyzed, in the smear &/or culture positive and smear &/or culture negative specimens, the PCR showed a sensitivity of 74.07% and a specificity of 94.5% (Table 3). Various studies have shown a sensitivity of 55-95% in the culture positive and 100% in both smear and culture positive clinical specimens.[26,27,28]

In specific studies of pulmonary and extra pulmonary specimens, sensitivities of PCR ranged between 20-94%.[29,30] Our findings were consistent with these studies done on pulmonary and extra pulmonary specimens. In our study there was no significant difference in the sensitivity of the smear and PCR in case of pulmonary specimens. But in extra pulmonary specimens, the sensitivity of the PCR was higher (71.42%) when compared to the smear examination (21.42%). Also the high NPV of PCR in extra pulmonary specimens could be useful for clinicians.

The genus specific PCR & species specific PCR were positive in 26 & 24 clinical specimens respectively. Two clinical specimens (1 bone marrow aspirate and 1 pus aspirate) which were positive by genus specific PCR were tested negative by species specific PCR. Both these clinical specimens belonged to patients who tested positive for HIV. This could probably suggest that the infection might have been caused by atypical mycobacteria as repeated testing of these samples with species specific PCR yielded negative results. The cultures from these patients also grew mycobacteria. The PCR performed on these cultures were also positive by genus specific PCR and not by species specific PCR. It is known that the incidence of atypical mycobacteria infections are on the rise in HIV infected patients. Approximately, 90% of the mycobacterial infections in patients with AIDS involve either MAC or M.tuberculosis, various other NTM cause the remaining 10% of the infection.[31]

Our study could have been improved by incorporating:

1. Fluorescent staining method which is more sensitive than cold staining.[21]
2. Use of Middle brooke liquid culture medium which could have increased the sensitivity of the culture.[25]
3. Use of internal controls which would have detected amplification inhibitors present in the clinical samples.[32]

It is important that any PCR based assay include a specific positive internal control to allow proper evaluation of DNA preparation and amplification. Quality control of the PCR mix and the performance of the amplification itself is mandatory if routine PCR is to replace culture for the diagnosis of tuberculosis.

4. Conclusion:

This study showed that PCR can serve as a useful complement to clinical diagnosis. PCR should be performed in conjunction with microscopy & culture. PCR based assay cannot be used reliably alone in the diagnosis of tuberculosis, because it misses a substantial number of tuberculosis cases especially in SN-CP samples. [24,26,27] PCR can be useful in detecting positive cases in extra pulmonary specimens which may be missed by the smear and/or culture.

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