



## Molecular Characterisation of HIV Associated Tuberculosis Compared to Conventional Methods

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### Abstract:

**Background:** Early diagnosis of pulmonary tuberculosis is difficult using conventional diagnostic method. Acid-fast bacillus (AFB) culture and smear microscopy are alone inadequate to diagnose of human immunodeficiency virus (HIV) -associated Tuberculosis (TB). In both test low sensitivity is a major drawback. Advances in molecular techniques, which rapidly identify Mycobacterium DNA in sputum, may overcome these obstacles.

**Objective:** Molecular characterisation of HIV associated tuberculosis compared to conventional methods.

**Study Design:** A cross-sectional study design.

**Setting:** King George's Medical University, Lucknow, Uttar Pradesh and Dolphin (PG) Institute of Biomedical and Natural Sciences, Dehradun, Uttarakhand,

**Method:** The present study showed the laboratory finding of 250 sero-positive HIV patients carried out of department of Microbiology, Medical University, Lucknow and Dolphin (PG) Institute of Biomedical and Natural Sciences, Dehradun, Uttarakhand. Their HIV status confirmed by Enzyme linked immune Sorbent Assay (ELISA) test in antiretroviral therapy (ART) centre Medical University, Lucknow. The study consisted with HIV-positive patient, clinically diagnosed for tuberculosis cases. All sputum samples subjected to AFB smear microscopy, culture and drug-susceptibility testing by 1% proportion method on Lowenstein-Jensen (LJ) medium and polymerase chain reaction (PCR).

**Results:** Among 250 HIV positive sputum samples 48 (19.2%) were smear positive 54 (21.6%) were culture positive and 58 (23.4%), PCR positive. PCR was found high sensitive and specific as a diagnostic tool compare to smear microscopy and culture.

**Conclusion:** The present study shows the major advances in molecular techniques, which rapidly identify Mycobacterium DNA in sputa with in comparison to other methods.

**Key words:** Mycobacterial culture, polymerase chain reaction, acid-fast bacilli, Lowenstein-Jensen medium.

## Introduction

TB is a leading cause of death, mainly in the developing countries where co-infection with HIV constitutes significant public health problems. During the last decade, there has been a dramatic change in the laboratory approach to TB diagnosis in the developing world. This change began with the realization that acid-fast bacillus smear microscopy alone was totally inadequate to deal with the problems of HIV associated TB and drug-resistant TB.<sup>[1]</sup>

Direct smear examination with Ziehl-Neelsen (ZN) staining for the diagnosis of TB as employed but its low sensitivity is a major drawback. Major advances in molecular techniques, which rapidly identify Mycobacterium (deoxyribonucleic acid) DNA in sputa, may overcome these obstacles.

The discovery of the polymerase chain reaction (PCR) as a means to generate detectable signals from as little as a single molecule of target DNA has led to the development of extremely powerful and rapid diagnostic tests for a number of infectious agents.<sup>[2]</sup>

In this study, the PCR technique was used to diagnose TB in HIV patients. The sensitivity and specificity of this technique were compared to those of the usual diagnostic techniques like AFB smear microscopy and culture.

## Material and Methods

### Study Design and Setting

A cross-sectional study was conducted at Department of Microbiology, King George's Medical University (KGMU), UP, Lucknow and Dolphin (PG) Institute of Biomedical and Natural Sciences, Dehradun, Uttarakhand.

### Study Population

The study consisted of 250 HIV positive patients diagnosed for AFB pulmonary tuberculosis (PTB) of both sexes and between the age group of <10 to 50 > years at the time of interview and were about to be registered for treatment. Their HIV status was confirmed by using Enzyme linked immunosorbent assay (ELISA) test in antiretroviral (ART) Center, KGMU, Lucknow UP India. Patients were

excluded if they were having any cardiac and metabolic problems and not willing to participate in the study.

### Samples:

5ml sputum from each patient were collected for each case and controls.

### Data collection

Personal interview and clinical examination

Interviews were conducted using structured questionnaire to collect the data on clinical history. Patients were thoroughly examined by medical doctors in the hospital.

### Assessment of clinical outcomes

Clinical outcomes were assessed including fever, cough, expectoration, chest pain breathlessness, wheezing, haemoptysis, dyspnoea, night sweat, loss or improve of appetite and weight loss/gain.

### Assessment of bacteriological outcomes

Bacteriological outcomes were assessed by RNTCP guidelines, 2006 which included AFB smear examination and grading, AFB culture and drug susceptibility test.<sup>[3]</sup>

All specimens were carried to the accredited Intermediate Reference Laboratory (IRL) at the Department of Microbiology, King George Medical University, Lucknow where further processing was done.

### Specimen collection

The diagnosis of TB was done in accordance with the RNTCP guidelines 2006. At the time of enrollment, from three sputum samples each patient were collected in properly labelled screw capped, sterile disposable plastic bottles after oral gargling with normal water. Thus, there were three samples: SPOT, EARLY MORNING and SPOT.<sup>[3]</sup>

Specimens contained mucous or mucopurulent material with minimum amounts of oral or nasal material into the McCartney bottles and volume was of approximately 5ml.

### AFB smears examination and grading

The AFB smear examination was carried out by direct microscopy using the Zheel Neelson (ZN) method. The sputum smear result was examined and interpreted according to the AFB grading.<sup>[4]</sup>

### AFB culture and drug susceptibility test

Culture examination was done on all diagnostic specimens regardless of AFB smear positivity. Sputum specimens from each patient were processed with sodium hydroxide (Noah) method by modifying Petroff's procedure and cultured on LJ slopes.<sup>[5]</sup>

All inoculated LJ drug and control media were incubated at 37°C. All cultures were examined 48-72 hours after inoculation to detect gross contaminants. Thereafter, cultures were examined weekly, up to eight weeks on a specified day of the week. Typical colonies of *M. Tuberculosis* were rough, crumbly, waxy, non-pigmented (buff colored) and slow-growers, i.e., Only appeared two to three weeks after inoculation. The colony was confirmed by ZN staining. Detection time for other than *Mycobacterium tuberculosis* (MOTT) was 25 days. *M.tb* positive strains were culture negative when they grew on p-Nitro benzoate (PNB) containing medium. Only a few colonies of non-tuberculous *Mycobacterium* (NTM – often pigmented, with smooth morphology or PNB positive) were grown as visible colonies on PNB containing medium<sup>[4]</sup>.

Drug resistance was expressed in proportion method, where a strain was considered to be drug resistant if the number of colonies that grew on a drug containing medium was 1% or more of the colonies that grew on a control drug free medium. The control (drug free) medium showed good growth at least 50 to 100 colonies.

Petroff's method<sup>[6]</sup> was used for decontamination of the samples. Sputum samples were treated with equal volume of 4 percent NaOH for 30 min. After washing with saline twice, the pellet was used for smear preparation. The remaining pellet was used for DNA extraction.

### Extraction of DNA

DNA isolation from clinical specimens was performed as previously described.<sup>[7]</sup> Briefly, samples pellet was suspended in 200µl of TE buffer and boiled for 10 min. Equal volumes of lysis buffer (Tris 10 mm, EDTA 2 mm, NaCl 0.4 M and triton x-100, 0.5%) pH 8.0 was added and vortexed thoroughly. Tubes were incubated at 65°C for two hours after adding 10µl of proteinase K (10 mg/ml). Samples were again boiled for 10 min to inactivate the proteinase K, followed by DNA purification with phenol: chloroform (24:1 v/v) and then by chloroform alone. The aqueous phase was finally transferred in 2.5 volumes of chilled ethanol and sodium acetate (0.3 M final concentration) was added. Tubes were kept in –20°C overnight for precipitation of DNA. Samples were centrifuged at 12,000 g for 15 min at 4°C and DNA pellets were washed with 70 percent ethanol by centrifugation at 12,000 g for 10 min and allowed to air dry.

### Amplification of mycobacterial DNA

All steps of sample processing, were carried out in separate rooms to avoid the carry over the contamination. A pair of oligonucleotide primers with insertion sequence *IS6110* amplifying a 123 bp fragment specific of *M. tuberculosis* complex, was used in this study; Primer 1:5'CCTGCGA GCGTAGGCGTCCG3' (forward primer) and Primer 2:5', CTCGTCCAGCGCCGCTTCGG3' (reverse primer) were used. (Bangalore Geni, Bangalore, India). Amplification was carried out in a final volume of 20 µl containing 10mM TrisHCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 250 µM of each dNTPs, 50 pico moles of each primer and one unit of Taq DNA polymerase (reagents from Bangalore Geni, Bangalore). 2 µl of sample DNA was added to 20 µl of reaction mixture. A positive control having a DNA sample from a standard strain of *M. tuberculosis* was included in every batch of experimental. Amplification cycles were performed on automated thermal cycler (Techne, UK). Amplification protocol: The cycling profile used, i.e., initial denaturation at 94°C for 10 min

followed by 35 cycles, each consisting of three steps: (i) denaturation at 94°C for 60 sec, (ii) annealing at 60°C for 60 sec, and (iii) primer extension at 72°C for 60 sec. Final extension was done at 72°C for 10 min. Detection of amplified products: Amplified products were resolved electrophoretically on 2 per cent agarose (Bangalore Geni, Bangalore, India) containing ethidium bromide (0.5 µg/ml) and visualised on 260 nm wavelength UV transilluminator (Bangalore Geni, Bangalore, India).<sup>[8]</sup>

### Statistical Analysis

The data collected was entered into Microsoft Excel and checked for any inconsistency. The descriptive statistics such as percentages were calculated. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the test were calculated. All the analysis was carried out by using SPSS 15.0 version STATA 12.0 version.

### Results

A total of 250 HIV positive patients were recruited, in which 60.8% were males and 39.3% were females. The most frequent age group in the present study was 31-40 years consisting of 39.2% patients, followed by 29.6% patients in the age group of <41 years. Majority of the patients were

Muslims (53.6%). It was found that the majority of 56.4% were smokers. (Table 1).

**Table-1:** Age and gender of the patients

Age in years	No. (n=250)	%
Age in years		
<10	10	4.0
11-20	18	7.2
21-30	38	15.2
31-40	98	39.2
41-50	74	29.6
>50	12	4.8
Gender		
Male	152	60.8
Female	98	39.2

The AFB positivity was observed to be in 19.2% of the patients, however, culture positivity was found to be 21.6%. The PCR positivity was 23.4%. There was 1.6% scanty in AFB test (Table-2).

**Table-2:** AFB smears, AFB culture, and AFB PCR findings from sputum samples (n = 250)

Test results	AFB		Culture <sup>1</sup>		PCR	
	No	%	No	%	No	%
Positive	48	19.2	54	21.6	58	23.4
Negative	202	80.8	196	78.4	192	76.8
1+positive	26	10.4	-	-	-	-
2+positive	12	4.8	-	-	-	-
3+positive	06	2.4	-	-	-	-
Scanty	04	1.6	-	-	-	-

<sup>1</sup>Including 4 MOTT

The sensitivity of AFB smear was low then sensitivity of AFB PCR and specificity of both AFB smear and AFB PCR were almost similar.

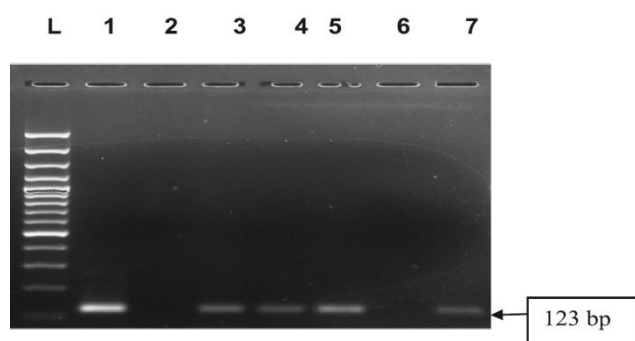
Similarly, PPV+, PPV- and accuracy of both AFB smear and PCR tests were also similar (Table-3,fig.1).

**Table-3:** Comparison of sensitivity and specificity of AFB smear and PCR against AFB culture

	AFB Culture		Total
	Positive	Negative	
AFB smear			
Positive	48	0	48
Negative	6	196	202
Total	54	196	246
AFB PCR			
Positive	56	1	57
Negative	1	188	189
Total	57	189	246

AFB smear: Sensitivity=88.9%, Specificity=100%, PPV+=100%, PPV- =97%, Accuracy=97.6%.

AFB PCR: Sensitivity=98.2%, Specificity=99.5%, PPV+=98.2%, PPV- =99.5%, Accuracy=99.2%



**Fig.1** PCR Gel picture of *M.tuberculosis*  
Legnds: Lane 1- marker; 2-7- clinical isolates

### Discussion

The specificity, sensitivity and speed of PCR test in diagnosis of *M.tuberculosis* infection shown in this study should encourage the use of this method in routine diagnosis of TB. We compared the performance of various tests in different clinical samples for diagnosis of TB. PCR showed the highest sensitivity as compared to other tests as reported by others.<sup>[9]</sup>

Among DNA targets, the IS6110 insertion sequence, a *M. tuberculosis* complex-specific sequence which is repeated 10–16 times in the chromosome of *M. tuberculosis*<sup>[10]</sup>, has been the most widely used.

The PCR technique can reduce the diagnosis time and may increase the detection of mycobacteria in

smear-negative TB. However, variations in procedures for in-house PCR could explain the wide variability of sensitivity and specificity reported in several studies.<sup>[11],[12],[13],[14]</sup>In a few samples, ZN smear examination and PCR results were positive but culture was negative; this could be due to the presence of nonviable mycobacteria in the samples as some of the subjects were receiving antitubercular treatment.<sup>[15],[16]</sup>

Smear-based microscopy is the most commonly used approach for pulmonary TB detection, demonstrating 70% sensitivity compared with culture associated with a clinical definition of the disease as the gold standard; however, in HIV-infected patients the smear-based sensitivity might be lower than 40%.<sup>[17],[18],[19]</sup>

There was only one false positive result by PCR test which could be due to the ability of the PCR test to detect very low number and even dead bacteria in a sample which can be present in a symptomatic individual. Current global TB control efforts are based on diagnosis of cases followed by adequate treatment. It is important that diagnosis be established early and efficiently in order to prevent transmission and misdiagnosis. In environments with high prevalence of TB and HIV, better tests and more-efficient diagnostic processes are needed. The sensitivity of PCR reported here compares well to the reported from studies performed in industrialized countries<sup>[20]</sup>.

A study conducted in Lusaka reported a sensitivity of PCR 55%, which is lower than present study being 100%. However, that study used a low-cost “in-house” one-tube nested PCR and a gold standard incorporating both microbiological and clinical data. This demonstrates the need for standardization of reagents and methodology, and particularly for standardization of the gold standard.<sup>[21],[22],[23]</sup>

The aim of this study was to determine the extent to which molecular technology characterized *M.tb* in HIV positive patients by detection of specific gene directly from sputum samples. The performance of PCR test was compared with other conventional methods used in diagnosis of TB mainly ZN and culture on LJ media (gold

standard method). Previous studies shown the success of microscopy is highly variable from 22% to 96% and most authors rate it at round 60% .<sup>[24]</sup> Different smear microscopy results were achieved by ZN 32.7%, ZN 65%,<sup>[25]</sup> ZN 67.6%,<sup>[26]</sup> ZN 50%<sup>[27]</sup>

In a study, the analytical sensitivity of multiplex PCR was found to be 100 fg for 38 kDa gene and 1 fg for *IS6110*. Multiplex PCR, using both the targets, showed highest sensitivity of 81.7%, followed by 69.2% for L-J culture test and 53.3% for AFB smear when clinical diagnosis was considered as a gold standard. The sensitivity of detection of *M. tb* in AFB smear positive and negative samples by multiplex PCR was 93.7% and 67.9%, respectively which are almost similar to the findings of the present study .<sup>[27]</sup>

Assessed the sensitivity of PCR assay over smear microscopy for rapid diagnosis in pulmonary from patients suspected to have tuberculosis. It was observed that 100% of AFB positive and 35.7% of AFB negative pulmonary samples were positive for Mycobacterium DNA detection. Total positivity rates of DNA amplification method in pulmonary samples was 62.16%.<sup>[28]</sup>

Compared four laboratory methods in the diagnosis of pulmonary tuberculosis. The sensitivity of ZN staining, auramine staining, culture on LJ media and PCR was 54.2%, 58.4%, 67.6% and 77.5%, respectively. All four methods presented 100% specificity. The sensitivity of PCR was 50.8% in samples with negative sputum smear microscopy results and 98.8% in those with positive results. The sensitivity of PCR was lower 25.6% in specimens with negative results in sputum smear microscopy and culture ,than in those with positive results 99.0%.<sup>[29]</sup>

### Conclusion

PCR can be considered as an alternative to ZN staining in combination with culture for diagnosis of TB; however, cost-effectiveness studies and operational studies are required to support an evidence-based decision of introducing PCR for TB control in high-burden environments.

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