ROLE OF POLYMERASE CHAIN REACTION AS A DIAGNOSTIC TOOL IN PULMONARY TUBERCULOSIS

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ABSTRACT

The rapid diagnosis of infectious diseases that represent a public health problem, like tuberculosis, is a challenging problem. The sensitivity of Polymerase Chain Reaction (PCR) makes it a potential diagnostic test for detection of *Mycobacterium tuberculosis (M. tb)* in samples with low bacillary load. A cross sectional study was carried out to determine the efficiency of PCR as compared to other routine diagnostics like smear microscopy and culture, amongst sputum samples and blood samples. Total 250 blood and sputum samples were collected from clinically suspected cases of pulmonary tuberculosis among HIV/AIDS patients. Sputum samples were stained with ZN stain, then decontaminated and cultured on LJ medium (gold standard) and PCR, whereas blood samples were processed only for PCR. Out of 250 cases, 49 were AFB smear positive (19.6%), 56 (22.4%) culture positive and PCR from sputum was positive in 69 (27.6%). The overall sensitivity and specificity of the PCR assay were 91.5% and 86.0% respectively and the positive and negative predictive values were 87.0% and 91.0% respectively. It also identified 13 smear negative and culture-negative cases as positive, whereas in case of blood sample (n=250), only 15 (6.0%) were PCR positive. The higher amount of TB positivity by PCR compared to culture & smear emphasize the role of PCR as a first line diagnostic tool in detection of pulmonary tuberculosis in sputum samples in HIV population as PCR is rapid, sensitive and cost effective compared to conventional diagnostic methods.

KEY WORDS: Polymerase Chain Reaction, Pulmonary tuberculosis, Sputum

INTRODUCTION

Tuberculosis (TB) is a leading cause of death, mainly in the developing countries where co-infection with HIV constitutes significant public health problems. India is home to over 3.4 million TB patients - about one-fifth of the global figure - making it the most TB prevalent country. [1] TB kills an estimated 1.7 million people each year. One third of the HIV-positive population worldwide is co-infected with *Mycobacterium tuberculosis (M. tb)* and accounts to about 14 million people worldwide. [2]

A HIV-positive person infected with *M. tb* has a 50-60% lifetime risk of developing TB disease as compared to an HIV-negative person who has only a 10% risk. It is estimated that 40% of the adult population of India harbors *M. tb.* [3]

Diagnostic process of tuberculosis starts with a clinical suspicion, early diagnosis followed by adequate treatment is essential to prevent both morbidity and mortality. The conventional technique of direct smear examination with Ziehl-Neelsen staining (ZN) is cheap and easy to perform, but its low sensitivity and sputum samples on consecutive day's makes procedure slow and non-compliant. Though culture is gold standard for diagnosis of TB because of slow growth rate of most

mycobacteria, isolation, identification and drug susceptibility testing can take several weeks. [4] Hence, a need for an alternative test as sensitive and specific as culture but rapid is PCR. PCR has the potential to be a cost-effective alternative,

provided the diagnosis can be determined with one sputum examination, more so in HIV patients who has insufficient sputum production and low bacillary load. PCR may reduce delay both in diagnosis and in the start of treatment.

PCR permits the direct detection and identification of infectious agents in clinical specimens. Several studies have investigated different PCR systems and performed clinical studies based on PCR [5,6,7] as a diagnostic test for tuberculosis and reported widely differing results with respect to specificity and sensitivity. While sensitivity of microscopy is 60–70% in culture positive respiratory material, the sensitivity of PCR is 90–100% and 60–70% on smear positive culture positive and smear negative culture positive respiratory samples respectively. [8] The value of AFB-microscopy in HIV-positive suspects has been criticized, while its sensitivity would become insufficient. Reports have shown that reduced smear and culture sensitivity may be true for patients in an advanced stage of HIV/AIDS only, while where few bacilli are present in sputum.

The purpose of this study was to redefine the role of PCR as compared to other routine diagnostics like smear microscopy and culture, amongst sputum samples from a pool of highly probable tuberculosis suspects in HIV positive patients referred to a tuberculosis clinic.

MATERIALS & METHODS

A cross-sectional study was carried out in the Department of Microbiology, CSM Medical University, Lucknow, India during the period of September 2006 to September 2009. A total of

250 blood and sputum samples (Figure 1) from clinically suspected cases of pulmonary tuberculosis among HIV/AIDS patients attending to Anti Retroviral Treatment (ART) center, Department of Medicine, CSM Medical University, .

Lucknow were enrolled for the study. The study was approved by the ethical committee of our university. Written informed consent was obtained from all the participants

Fig. 1 Schematic representation of distribution of blood and sputum samples (n=250).
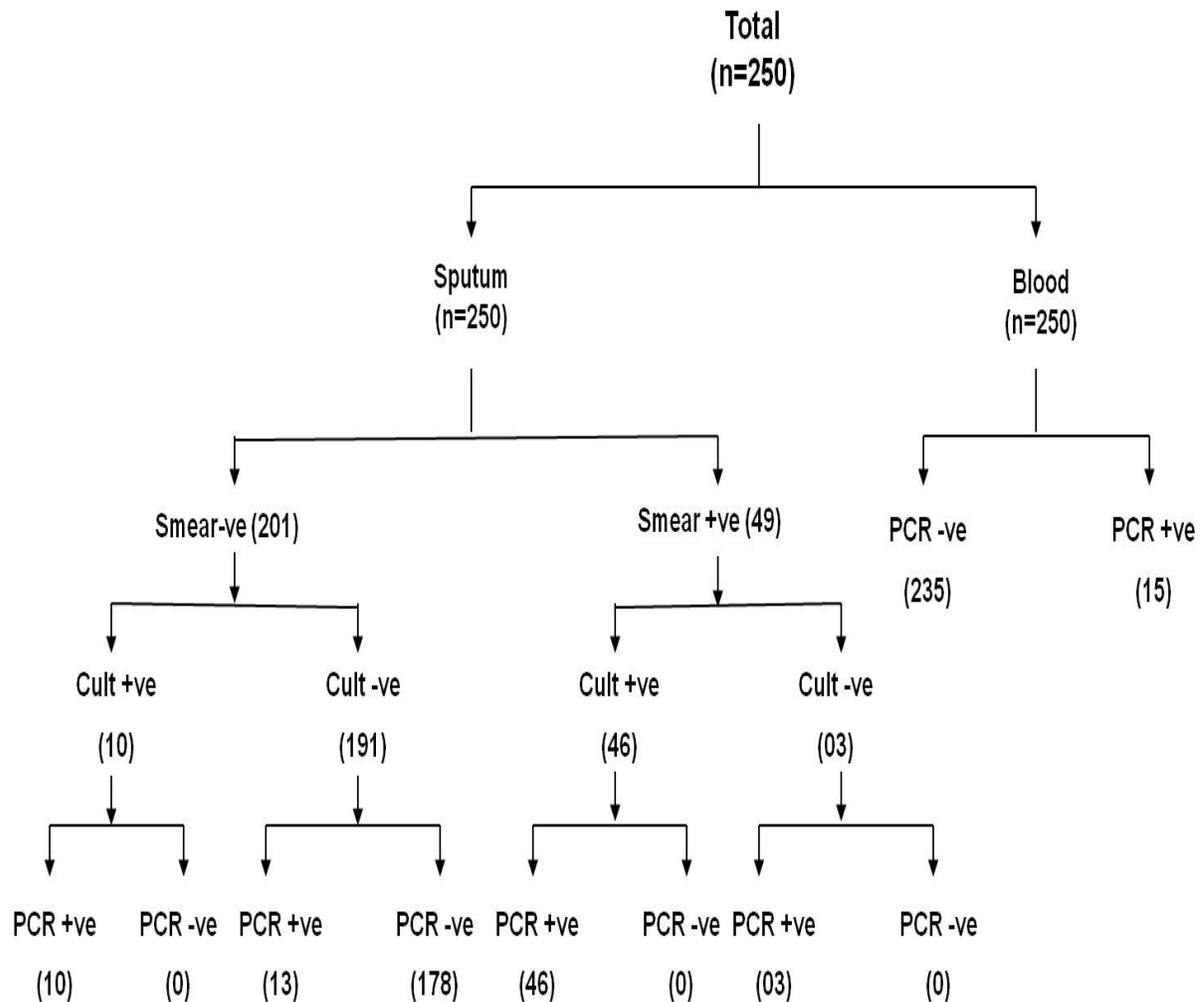
Each patient was interviewed individually for treatment history, socioeconomic status, risk factors for TB as well as social behavior in pre-designed proforma. The patients included were those with; chronic cough for more than one month's duration, positive mantoux test with 5 Tuberculin Unit (0.1mL) (area of in duration > 5mm was taken as positive) and presence of one of the radiographic chest findings consistent with pulmonary tuberculosis i.e. hilar lymphadenopathy, pulmonary infiltrates or cavity formation in the upper lobes or in the apical segments of lower lobes. No discrimination was made on account of age, sex or demography. Sputum samples were processed for ZN staining [9] according to the Revised National Tuberculosis Control Program (RNTCP) manual and Acid Fast Bacilli (AFB) culture and PCR. Blood samples were processed for PCR.

Sputum sample processing, culture and PCR
Sputum samples were processed using either Petroffs's method or N-acetyl-L-cysteine-sodium hydroxide method. For culture morning sputum samples of three consecutive days were collected in sterile, wide mouthed plastic bottles. After a standard N-acetyl-L-cystein sodium hydroxide digestion, decontamination [10], sputum was used for microscopy and culture in Lowenstein-Jensen medium. Culture results were monitored at one, two and four weeks and reported positive if growth was found after five to six weeks. Positive cultures were confirmed by microscopy for AFB. Cultures were declared negative if there was no growth by 12 weeks. A small amount of the processed pellet was used for culture on Lowenstein-Jensen (LJ) medium. For PCR, 3

ml of blood was collected aseptically in a heparinized container and stored at 4°C till further processing. DNA purification from whole blood was done. Positive and negative controls were run with each batch. A specific oligonucleotide primer based on insertion element IS6110 specific to _M. tb_ (synthesized by Bangalore Gennei, India) was used. [11] The sequence of the forward and reverse primers used was 5'-TCCGCTGCCAGTCGTCTTCC-3' and 5'-GTCCTCGCGAGTCTAGGCCA-3'respectively.

Amplification reactions were performed in a final volume of 50µl containing 10mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 50 picomoles/ul of each primer, 0.25 mM of each deoxynucleoside triphosphate (dATP, dGTP, dTTP and dCTP) and 0.5µl of _Taq_ DNA polymerase (Bangalore Gennei, India). 5µl of extracted DNA was added to 20µl of PCR mixture with a positive displacement pipette. Amplification mixtures were subjected to 40 cycles of amplification on automated progene thermo cycler (Techne, UK) using an initial denaturation step of 95^0 C for 120 seconds, 1 cycle of 95^0 C for 20 seconds, 45^0 C for 360 seconds and 72^0 C for 120 seconds followed by denaturation at 95^0 C for 20 seconds, annealing at 62^0 C for 30 seconds and extension at 72^0 C for 1 minute. A final extension was carried out at 72^0 C for 7 minutes. The aliquot of 5µl PCR product of each sample was detected by 0.8% agarose gel (w/v) electrophoresis after staining with ethidium bromide (0.5 mg/ml, Sigma) along with 100 bp DNA ladder (Bangalore Genie, India) and PCR products of controls. A constant current (50V) was maintained for 90 minutes. Multiple amplified DNA was visualized under UV transilluminator (264 nm) light in positive samples.

Statistical analysis
Efficiency of sputum and blood PCR in terms of sensitivity, specificity, positive predictive value and negative predictive value in comparison to microscopy and culture was done. Using culture as gold standard for the culture positive samples and smear microscopy, combined microbiological data, response to ATT, chest radiographic findings for culture negative samples.

RESULTS
Sputum and blood samples were collected from 250 clinically suspected patients of pulmonary tuberculosis. Sputum samples were processed for ZN staining and AFB culture (Gold standard) and blood samples were processed for PCR. When PCR was performed, 69 (27.6%) samples showed a band typical in size (123 bp) to the target gene of _M. tb_ complex (_IS 6110_) as indicated by the standard DNA marker. 72.4% samples were negative. Out of 250 cases, 183 (73.2%) were males and 67 (26.8%) were females. Mean age of the patients was 34.7±7.9 years (range 7 -65 years) and median age was 35 years. Male to female ratio was 2.7:1.

PCR results amongst samples positive by culture and smear microscopy
Twenty two percent (56/250) samples tested positive by culture and PCR positive for 100% (56/56) of these remaining samples (Figure 2). There were 49 samples that were positive by ZN staining. PCR was positive for 100% (49/49) of these samples. Forty six samples were positive by microscopy and culture (S+C+). PCR was positive for 100% (46/46) of these samples.

Fig. 2 Distribution of HIV associated TB samples.
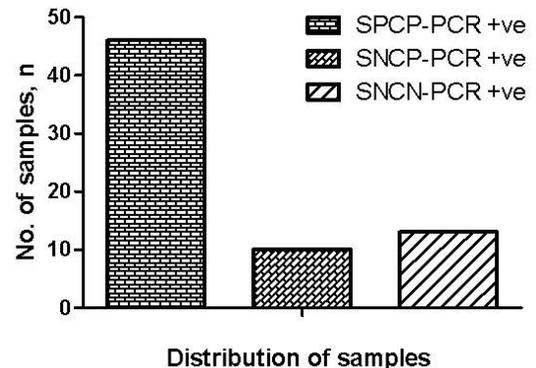
Where-
SPCP-PCR +ve: Smear Positive Culture Positive- PCR Positive
SNCP-PCR +ve: Smear Negative Culture Positive- PCR Positive
SNCN-PCR +ve: Smear Negative Culture Negative- PCR Positive

PCR data of smear negative samples from individuals diagnosed as not having tuberculosis
PCR was positive for 11.4% (23/201) of these sputum samples. Five percent (10/201) of these smear negative samples were positive by culture (S-C+). PCR was positive for 100% of these samples (Table 1).

In addition, PCR was positive for 13 individuals who were declared not to have tuberculosis using the diagnostic paradigm of the RNTCP. Ten such individuals could either be traced subsequently, within a period of one year or clinical information could be obtained within a year of first presenting to the clinic. Ten individuals took anti-tuberculosis treatment (ATT) subsequently in whom chest x-ray had features of pulmonary tuberculosis; two HIV positive individual with recent history of extra-pulmonary tuberculosis was dead (Table 1).

Only eight of the 10 cases i.e. those who took ATT subsequently were considered as true cases of tuberculosis, even though the other five could be rated as highly probable cases of tuberculosis.

DISCUSSION
A rapid and accurate diagnosis of tuberculosis is a cornerstone of tuberculosis control strategies.

Table 1. Sputum and blood efficacy for TB detection in HIV patients.

	Sample (sputum)		Positivity of PCR (c)	Efficiency of PCR % (c/b x 100)
	Description (a)	Number (b)		
A.	Culture positive M.tuberculosis	56 56	56	100
B.	Smear positive	49	49	100
	Smear positive culture positive	46	46	100
	Smear positive culture negative	03	03	100
C.	Total sputum sample	250		
	Culture positive PCR positive	56		
	Culture negative PCR positive	13		
	Total PCR positive Culture positive PCR negative	69 00		
D.	Blood PCR positivity	250	15	6
E.	Status of persons negative by smear and culture and positive by PCR	13		
	Started ATT subsequently based on clinical and radiological findings	10	8 (True +ve)	
	Deceased [+]	02		
	Lost to follow up	01		

[+]Immunocompromised with past history of tuberculosis

Figure 3. Gel picture shows the band pattern of a) Blood and b) Sputum in HIV co-infected TB samples.

L=Ladder(100bp) PC= +ve control
NC=-ve control S= Samples (1,2,3,4,5)
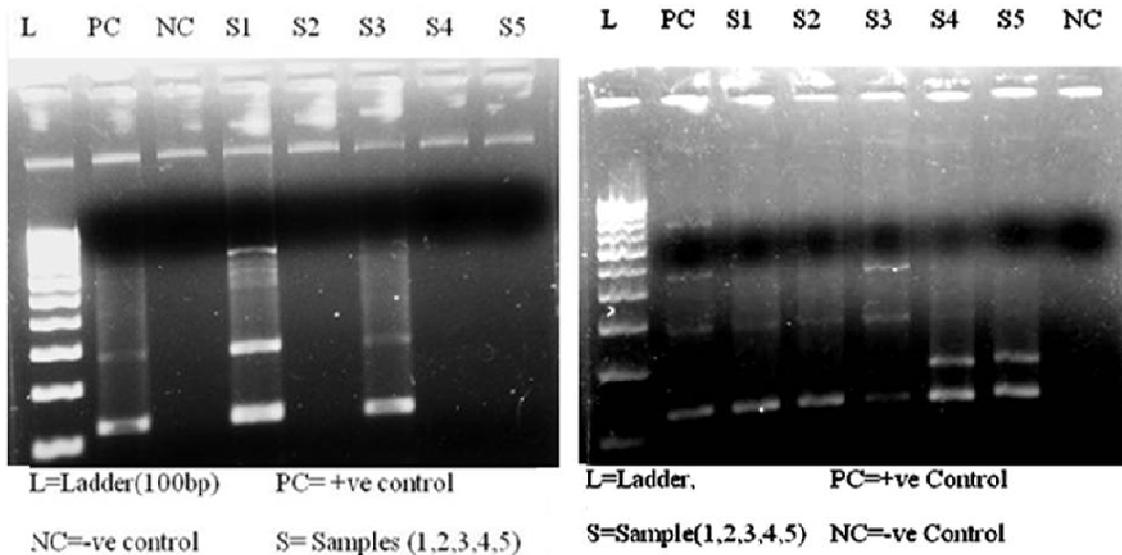
a) Gel picture of blood sample

L=Ladder, PC=+ve Control
S=Sample(1,2,3,4,5) NC=-ve Control

b) Gel picture of sputum sample

[12,13] The accurate diagnosis of tuberculosis remains difficult and in many cases TB is not detected by conventional microscopy or culture. [14] Sputum smear performing as a quick and easy method for preliminary confirmation of diagnosis has limitations; it is less sensitive, because a large number of bacilli must be present in a specimen for the smear to be positive and also

fails to differentiate between dead and live mycobacteria. [15] In our study, the sputum smear positivity was seen in 19.6% which is similar to Parekh et al. (16) study.

Isolation of Mycobacterium tuberculosis by culture techniques is taken as gold standard for diagnosis. This bacterium grows very slow in culture and may take several weeks for visible growth on conventional solid media. [17] In our study, the culture positivity was seen in 22.4% which is similar to Parekh et al. [16] study.

Serological tests are used as an additional tool among the investigations done for tuberculosis. PCR is the most widely applied alternative rapid diagnostic technique for mycobacterium tuberculosis detection. PCR has enhanced the diagnostic predictability of the disease especially in the extra pulmonary, paucibacillary samples. The present study demonstrates the utility and limitations of PCR. The aim of the present study was to use the polymerase chain reaction (PCR), as a rapid tool for the diagnosis of pulmonary tuberculosis from sputum.

Samples from a total of 56/250 (22.4%) patients were culture positive for any mycobacterial growth by modified Petroff's method on LJ media. Fifty six isolates (22.4%) were typical M. *tuberculosis* strains among all culture positive isolates. By considering both smear positive and smear negative cases together, PCR assay correctly identified 56 positive cultures out of 250 cases.

The present study also identified that 13 smear negative and culture-negative cases as PCR positive from patients of suspected _M. tb_. This result shows that PCR assay is probably more sensitive than the culture by detecting fewer organisms. Similar results were obtained by Oberoi et al. [18] who found that PCR showed the highest sensitivity (73.9%) as compared to other tests.

Out of 13 patients, 8 were considered as clinically true positive patients whose PCR was positive and 2 patients were expired and one lost to follow up and 2 patients were false positive. And in 49 smear positive patients, 3 were (S+C-) and PCR positive patients were false positive amounting PPV of PCR was 87.5%. The higher amount of TB positivity detected by PCR indicates sensitivity of PCR higher than culture and two false positive cases shows its limitation, cannot differentiate live and dead bacilli.

As _M. tb_ also resides in macrophages in the blood. We carried out the PCR using whole blood. This study has also indicated that PCR can be a useful tool in those who are not able to expectorate a proper sputum sample.

The primary limitation of PCR detects DNA and do not differentiate dead and live bacteria. When culture is used as a gold standard in comparison studies, samples containing non-viable mycobacteria may lead to a false positive PCR, thereby misleading clinicians. Though PCR detects bacilli and cannot differentiate non viable bacilli, yet a clinical improvement seen after Anti Koch's Treatment (AKT) in this study suggests that PCR to be more useful test in HIV patient's co-infected with TB.

CONCLUSION
PCR is a rapid, sensitive and efficient diagnostic tool for detecting pulmonary TB in HIV individuals with sensitivity superior to microscopy and specificity equal to culture even in low bacillary load or early stage of tuberculosis. PCR of blood is not as sensitive as PCR of sputum in HIV co-infected TB.

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