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***Research Article***

# OLD TOOLS VS NEW TOOLS IN DIAGNOSTIC FIELD OF TUBERCULOSIS: A COMPARATIVE ANALYSIS

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## ABSTRACT
Tuberculosis is a disease associated with significant morbidity and mortality, more so in a developing nation like India. Most often it remains undiagnosed and therefore untreated. The gold standard for diagnosis of tuberculosis is by microscopic detection of Mycobacterium or its isolation from the specimen. But because of the poor performance of these conventional methods on extrapulmonary specimens, one has to opt for molecular methods like PCR. However, the cost per test and lack of treatment monitoring by PCR are the disadvantages in a developing country like ours. Therefore, a study was done comparing the conventional methods with PCR. It is a retrospective study conducted over a period of one year, in which a total of 114 samples sent for smear, culture & PCR to the microbiology laboratory of a tertiary care hospital in northern India were included. Sample was first concentrated by Petroff's method and then with the concentrate obtained was inoculated on Lowenstein Jensen medium for culture and rest was used to make smear which was stained by Ziehl Neelsen stain. Another sample was sent to Dr. Lal Path Lab for Real Time PCR. Out of 114 samples, 88 (77.2%) were negative for Mycobacterium by all three methods. 26 (22.8%) samples were positive by PCR, out of which 24(92.3%) were extrapulmonary samples and 2 (7.7%) were pulmonary samples. Out of the 2 pulmonary samples, 1(50%) which was positive by PCR was also positive by conventional techniques; rest 25 samples (mainly extrapulmonary) were negative by conventional techniques. Conventional methods of microscopy & culture remain gold standard for diagnosis of pulmonary tuberculosis & monitoring of treatment. However, due to paucity of bacillary load & uneven distribution of bacilli in extrapulmonary samples, one has to count on PCR. In conclusion, molecular methods like PCR work complementary to conventional methods rather than stand alone superior in the field of tuberculosis diagnosis.

***Keywords:*** *Tuberculosis, Zeihl Neelsen Staining, Lowenstein Jensen Medium, Real Time PCR*

## INTRODUCTION
Tuberculosis is a disease associated with significant morbidity and mortality, more so in a developing nation like India (WHO, 2002). Diagnostic process of tuberculosis initiates with a high clinical suspicion, and is supported through the use of various diagnostics (Foulds and O'Brien, 1998; Kar *et al.*, 2003). The only rapid test for presumptive diagnosis of tuberculosis is smear examination of the patient's specimen for acid-fast bacilli (AFB). Culture remains the gold standard for diagnosing tuberculosis (Heifets, 2000). However, the disease most often remains undiagnosed and, even worse, untreated (Aggarwal *et al.,* 1999). Major difficulty is with extrapulmonary samples, which are associated with low sensitivity of acid-fast bacillus (AFB) smear and culture (Aggarwal *et al.,* 1999). The diagnosis of extrapulmonary tuberculosis is challenging for a number of reasons: the lack of adequate sample amounts or volumes; the apportioning of the sample for various diagnostic tests (histology/cytology, biochemical analysis, microbiology, and PCR), resulting in non uniform distribution of microorganisms; the paucibacillary nature of the specimens yielding very few bacilli ; and the lack of an efficient sample processing technique universally applicable on all types of extrapulmonary samples (Chakravorty *et al.,* 2005). Poor performance of these conventional methods on extrapulmonary specimens demands for more sensitive and specific techniques. Molecular methods like nucleic acid amplification using polymerase chain reaction (PCR) have the potential for the diagnosis of tuberculosis in a few hours with a high degree of sensitivity and specificity (Brisson-Noel *et al.,* 1989). Many studies have demonstrated the value of PCR in the diagnosis of extrapulmonary tuberculosis, including pleural effusion and lymphadenitis (Baek *et al.,* 2000; Ersoz *et al.,*

1998; Goel *et al.*, 2001; Pfyffer *et al.*, 1996; Singh *et al.*, 2000; Verma *et al.*, 1995). However, the presence of PCR inhibitors in clinical samples hampers the use of amplification techniques with full confidence and ease, (Pfyffer *et al.*, 1996; Singh *et al.*, 2000; Burkardt, 2000; Clarridge *et al.*, 1993; Honore-Bouakline *et al.*, 2003), and there is a pressing need for a robust, reproducible, and uniform method of inhibitor removal from clinical specimens (sputum, fluids, and tissues). Also, the cost per test and lack of treatment monitoring by PCR are the disadvantages in a developing country like ours. Therefore, this study was undertaken to compare the conventional methods with modern diagnostic techniques like PCR.

## MATERIALS AND METHODS

A retrospective study was done in the department of microbiology in a tertiary care hospital in Northern India. A total of 1775 samples (including both pulmonary and extrapulmonary samples) for tuberculosis were received in a year from 1st January, 2012 to 31st December, 2013 in the microbiology laboratory, but out of these only 114 samples were sent for smear, culture & PCR, which were included in the study. Out of 114 samples, 3.51% (4) were pulmonary samples and 96.49% (110) were extrapulmonary samples. The percentage distribution of various extrapulmonary samples like CSF, pleural fluid, pus, urine, bone marrow, tissue, peritoneal fluid, gastric lavage and lymph node was 23.68%, 22.80%, 13.16%, 11.40%, 9.65%, 7.02%, 0.88% and 0.88% respectively (as shown in figure1).

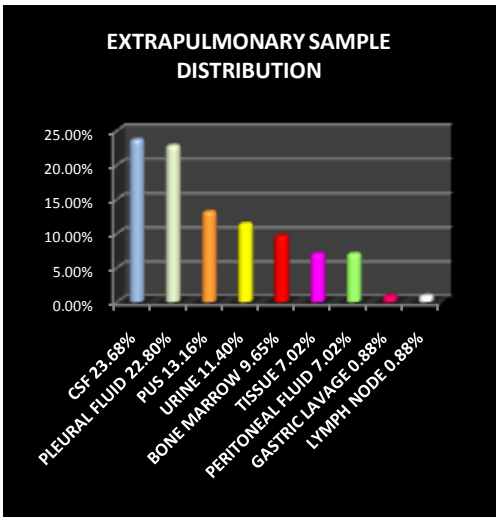


**Figure 1: Percentage distribution of extrapulmonary samples**

Two duplicate samples were received from each patient. One sample was processed in the microbiology laboratory for the AFB smear and culture, while another sample was sent to Dr. Lal Path Lab for Real Time PCR. For AFB smear and culture, the samples were first homogenized and concentrated using Petroff's method (Winn *et al.*, 2006), in which steps followed were as follows. First, 4% NaOH solution was mixed in equal parts with the specimen (NaOH serves as decontamination agent). This mixture was homogenized by allowing it to stand at room temperature for 15-20 minutes, swirling the tube periodically. After this digestion –decontamination step, phosphate buffer (pH 6.8) was added up to the top ring in the tube and mixed well. The phosphate buffer makes strong shift in pH less likely and also serves to "wash" the specimen, to dilute and neutralize toxic substances, and reduce the specific gravity of the specimen so that centrifugation is more effective in the sedimentation of organisms. The specimen was then centrifuged at 3,000 × g for 15-20 minutes. After centrifugation, supernatant was carefully decanted into a jar with phenolic disinfectant and a portion of sediment was directly inoculated onto Lowenstein Jensen medium slopes; the other portion was used for preparation of direct smear for Ziehl Neelsen staining (Winn *et al.*, 2006). The tissue biopsy specimens were minced and homogenized in a

sterile homogenizer and a portion of the homogenate was directly inoculated onto Lowenstein Jensen medium slopes and other portion was used for making impression smears for Ziehl Neelsen staining. The Lowenstein Jensen medium bottles were incubated at 37˚C in the incubator. Culture readings were taken every week, and discarded as negative in case of no growth at the end of 8 weeks. At Dr. Lal Path lab, where another duplicate sample was sent, they used MYCOSURE Real Time –PCR assay which is an in house developed Real time PCR assay for qualitative detection of Mycobacterium. This test includes 3 targets of which 2 are for Mycobacterium tuberculosis complex (IS6110 & MPB64) & 1 target for Mycobacterium genus (16sRNA). Limit of detection of this assay is 1-10 mycobacteria per PCR. This test does not differentiate between the mycobacteria species.

## RESULTS

Out of 114 samples, 88(77.2%) were negative for Mycobacterium by all three methods. 26 (22.8%) samples were positive by PCR, out of which 24(92.3%) were extrapulmonary samples and 2(7.7%) were pulmonary samples. Out of the 2 pulmonary samples, 1(50%) which was positive by PCR was also positive by conventional techniques; rest 25 samples (mainly extrapulmonary) were negative by conventional techniques.

## DISCUSSION

In the present study, we have tried to compare the efficiency of conventional methods and real time PCR in diagnosing tuberculosis. However, our sample size was small and of uneven distribution, we can derive from our study that for pulmonary tuberculosis, conventional methods like AFB smear and Culture can still be counted upon, but as far as extrapulmonary tuberculosis is concerned one has to look for methods other than conventional methods like PCR for diagnosis. There are many studies that quote differences in the range of sensitivity and specificity for different methods used for diagnosing tuberculosis. In a study by Watterson and Drobniewski (2000), while sensitivity of microscopy was 60-70% in culture positive respiratory material, the sensitivity of PCR was 90-100% and 60-70% on smear positive culture positive and smear negative culture positive respiratory samples respectively. Another study by Noordhoek *et al.*, (1996) reported the overall sensitivity of PCR ranging from 58% to 100%. Sensitivity was reported to be higher in smear-positive samples (95% to 100%) than in smear-negative samples (46% to 63%). A study done in Ludhiana by Oberoi and Aggarwal (2007), showed a significant difference in the sensitivities of these tests, i.e. 73.9% for PCR, 34.78% for ZN smear examination and 52.17% for LJ culture, with no significant difference in their specificities and PCR test sensitivity in pulmonary and extrapulmonary clinical samples was 74% and 78.5% respectively which was significantly higher when compared with those of other tests. In a study by Siddiqui *et al.*, (2013) there was 5% positivity rate by Zeihl Neelsen staining, 15% positivity rate by Lowenstein Jensen medium culture and 70% positivity rate by PCR. Those samples detected positive by ZN smear and LJ culture were all positive by PCR too. Another study by Lima *et al.*, (2008) reported the sensitivity of Ziehl-Neelsen staining, culture on LJ medium and PCR as 54.2%, 67.6% and 77.5%, respectively. All methods were 100% specific. The sensitivity of PCR was lower in specimens with negative results in sputum smear microscopy and culture than in those with positive results (25.6% and 99.0%, respectively). From New Delhi, Negi *et al.*, (2005) showed a significant difference in the sensitivities of different tests, the figures being 74.4% for PCR test, 33.79% for ZN smear examination and 48.9% for LJ culture ($P<0.05$). However, there was no significant difference ($P>0.05$) as far as the specificity of different tests was concerned. PCR test sensitivity in pulmonary and extrapulmonary clinical samples was 72.7% and 75.9% respectively and found to be significantly higher ($P<0.05$) when compared with those of other tests. In many studies, problems with false-positive PCR results, at rates ranging from 0.8% to 30% have been reported. Specificity of PCR results varies between laboratories due to procedural differences, differences in cross-contamination rates and the choice of primers. Also the primary limitation of PCR arises from the absence of a suitable gold standard to assess its efficiency. 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 (Parekh *et al*., 2006).

***Conclusion***

Conventional methods of smear microscopy & culture remain gold standard for diagnosis of pulmonary tuberculosis & monitoring of treatment. However, due to paucity of bacillary load & uneven distribution of bacilli in extrapulmonary samples, one has to count on PCR. To conclude, molecular methods like PCR work complementary to conventional methods rather than stand alone superior in the field of tuberculosis diagnosis.

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