Evaluation of Microscopy, Culture and PCR Assay in the Diagnosis of Extrapulmonary Tuberculosis

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Extra pulmonary tuberculosis presents a diagnostic dilemma for both physicians as well as for clinical microbiologists. The laboratory diagnosis of tuberculosis ranges from simple microscopy, culture to complex molecular assays. To evaluate the sensitivity, specificity and turnaround time of microscopy, culture and PCR in the diagnosis of Extrapulmonary tuberculosis & to evaluate the use of PCR in the early diagnosis of Extrapulmonary tuberculosis. A total of 71 samples patients with strong clinical suspicion of extra-pulmonary tuberculosis were processed and evaluated by ZN staining, fluorescent microscopy, LJ culture, BacT Alert culture and PCR. The positivity rates by microscopy, LJ culture, BacT Alert culture and PCR were 11.26%, 8.45%, 14.08% and 14.08% respectively. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of both staining methods was 50%, 92.3%, 37.5% and 95.2% respectively. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of BacT Alert culture was 83.3%, 92.3%, 50% and 98.4% respectively. The recovery rate was higher by BacT Alert culture (90.9%) compared to LJ culture (63.63%). The mean turnaround time for culture positivity was 36.3 days with LJ culture and 14.6 days with BacT Alert culture. The sensitivity, specificity, positive predictive value and negative predictive value of PCR assay was 66.66%, 90.76%, 40% and 96.72% respectively. PCR has high sensitivity, specificity, substantial level of agreement with BacT Alert culture and shorter turnaround time. Therefore, use of PCR in combination with other diagnostic modalities is a useful tool to detect additional EPTB cases which may be missed otherwise.

Keywords: Extrapulmonary tuberculosis, diagnostic methods.
various genes for rapid detection of *M. tuberculosis* complex with encouraging results. The aim of this study was to evaluate the sensitivity, specificity and turnaround time of microscopy, culture and PCR in the diagnosis of Extrapulmonary tuberculosis and to evaluate the use of PCR in the early diagnosis of Extrapulmonary tuberculosis.

**MATERIALS AND METHODS**

Patients with strong clinical suspicion of extrapulmonary tuberculosis willing to participate after informed consent were included in the study. The cases already on Anti-tubercular therapy or had been confirmed as having tuberculosis was excluded from study.

A total of 71 samples were included in our study & were processed and evaluated by ZN staining, fluorescent microscopy, LJ culture, BacT Alert culture and PCR. The 71 clinical specimens included in this study were pus (15), endometrial biopsy (14), lymph node aspirate (10), peritoneal fluid (9), pleural fluid (7), tissue (5), CSF (5), synovial fluid (3), urine (3) as shown in the Table 1.

**Processing of clinical samples**

Sterile body fluids were centrifuged, where as pus, urine specimen were digested & decontaminated by using Modified Petroff’s (4% NaOH) method. Tissue & lymphnode aspirates were homogenized using sterile tissue homogenizer. The Sediment thus obtained were subjected to ZN staining, fluorescent microscopy, LJ culture, BacT Alert culture and PCR.

Auramine rhodamine stain was used in fluorescent microscopy, In house prepared Lowenstein Jensen medium were used for solid culture media. and for liquid culture method, BacT/Alert MP culture media (BioMerieux,) were used by following manufacturer’s instructions.

**Polymerase Chain Reaction**

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

The DNA was extracted by spin column method (Qiagen tissue extraction kit)DNA was extracted from 71 clinical samples, *M. tuberculosis* standard strain (H37RV). 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. The species specific primer amplified a 245 base pair nucleotide sequence in IS 6110 present in strains of the *M. tuberculosis*

The sequences of the species specific primers were:

Forward: 5’ CGT GAG GGC ATC GAG GTG GC 3’
Reverse: 5’ GCG TAG GCG TCG GTG ACA AA 3’

The conditions were:

Initial delay: 94° C for 5 minutes.
94° C for 2 minutes
68° C for 2 minutes
72° C for 2 minutes

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.

**RESULTS**

The positivity rates by microscopy, LJ culture, BacT Alert culture and PCR were 11.26%, 8.45%, 14.08% and 14.08% respectively as shown in Table 2.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of both staining methods was 50%, 92.3%, 37.5% and 95.2% respectively as shown in Table 3. The mean turnaround time for culture positivity was 36.3 days with LJ culture (range 5 to 49 days) and 14.6 days with BacT Alert culture (range 4 to 21 days). The use of BacT Alert culture has reduced the mean detection time by 2.5 times when compared to LJ culture.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of BacT Alert culture was 83.3%, 92.3%, 50% and 98.4% respectively as shown in Table 3. BacT Alert culture was positive in additional 5 samples when compared to LJ culture. The recovery rate was higher by BacT Alert culture (90.9%) compared to LJ culture (63.6%).

PCR was positive in 10 samples. The sensitivity, specificity, positive predictive value and negative predictive value of PCR assay was 66.66%, 90.76%, 40% and 96.72% respectively.
The mean turnaround time for ZN staining, fluorescent staining, LJ culture, BacT Alert culture & PCR are 40 minutes, 30 minutes, 36.3 days, 14.6 days & 5 hours respectively as shown in Table 4.

**Statistical analysis**

Statistical analysis was done using ‘SPSS 22’ software and the sensitivities, specificities, positive predictive values, negative predictive values were calculated considering LJ culture as gold standard. Cohen’s kappa value was also obtained to assess the reproducibility and level of agreement between the PCR assay and other diagnostic tests employed.

In our study, the level agreement between BacT Alert and PCR was ‘substantial’ with a kappa value of 0.767 whereas ‘moderate’ level of agreement between LJ culture and PCR with kappa value of 0.441

**DISCUSSION**

Tuberculosis remains a major health problem in the developing countries in the world especially in India. In clinical practice the diagnosis of EPTB is difficult because of its non-specific, misleading and variety of clinical manifestations. The microscopy and culture are still the methods of choice for the diagnosis of tuberculosis in most of the microbiological laboratories.

The sensitivity of microscopy and culture on LJ media are low in EPTB owing to its paucibacillary nature. 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. Recent introduction of liquid culture has reduced the time taken for culture.

### Table 1. Distribution of Extrapulmonary tuberculosis samples (N = 71)

| Samples                  | Number |
|--------------------------|--------|
| Pus                      | 15     |
| Endometrial biopsy       | 14     |
| Lymph node aspirate      | 10     |
| Peritoneal fluid         | 09     |
| Pleural fluid            | 07     |
| CSF                      | 05     |
| Tissue                   | 05     |
| Synovial fluid           | 03     |
| Urine                    | 03     |

### Table 2. Positivity rate of individual diagnostic method employed in detection of M tuberculosis (N = 71)

| SI No | Diagnostic Method                  | Positive | Positivity rate (%) |
|-------|------------------------------------|----------|---------------------|
| 1     | Ziehl Neelsen Stain                | 8        | 11.26               |
| 2     | Fluorescent stain (Auramine Rhodamine Stain) | 8        | 11.26               |
| 3     | Lowenstein Jensen Medium (Solid Culture) | 6        | 8.45                |
| 4     | Bact Alert (Liquid Culture)        | 10       | 14.08               |
| 5     | Polymerase Chain reaction          | 10       | 14.08               |

### Table 3. Sensitivity, Specificity, Positive predictive value (PPV), Negative predictive value (NPV) of individual diagnostic method

Reference: Lowenstein Jensen culture as Gold Standard

| SI No | Diagnostic Method                  | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|-------|------------------------------------|-----------------|-----------------|---------|---------|
| 1     | Ziehl Neelsen Stain                | 50              | 92.3            | 37.5    | 95.2    |
| 2     | Fluorescent stain (Auramine Rhodamine Stain) | 50              | 92.3            | 37.5    | 95.2    |
| 3     | Bact Alert (Liquid Culture)        | 83.3            | 92.3            | 50      | 98.4    |
| 4     | Polymerase Chain reaction          | 66.7            | 90.8            | 40      | 96.7    |
positivity and also increased the rate of isolation of *M. tuberculosis*\(^ {11,12}\). The role of PCR in early diagnosis of EPTB has been evaluated with the hope of shortening the time required for diagnosis of EPTB\(^ {13,14}\). Out of 71 samples processed, 16 samples (22.5%) were positive by one or more methods employed for the detection of acid fast bacilli. Microscopy showed the positivity of 11.26% by both the methods (ZN and fluorescent staining method). A study done by Sudhindra KS *et al* also reported equal positivity rates of ZN and fluorescent staining method\(^ {8}\). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of both ZN staining and fluorescent staining were 50%, 92.3%, 37.5% and 95.2% respectively which is much higher when compared to studies done by others.

Among 8 cases which were positive by ZN staining and fluorescent microscopy, 2 were also positive by both culture methods and PCR.

Our study showed least positivity rate by LJ culture i.e. 8.45%. The low positivity rate may be attributed to paucibacillary nature of the disease and the sampled site may not represent the site of active infection\(^ {13,14,15,16}\). Low positivity rates were also reported by Chhina D *et al* (2.1%)\(^ {17}\), Ajantha GS *et al* (5%)\(^ {18}\), Sharma K *et al* (11.3%)\(^ {19}\) and Siddiqui MAM *et al* (15%)\(^ {20}\).

BacT Alert had positivity rate of 14.08% which is much higher than reported by Angeby Kak *et al* (3.44%)\(^ {21}\), Carricaga A *et al* (4.07%)\(^ {22}\) and Piersimoni C *et al* (7.07%)\(^ {23}\). PCR was positive in 10 samples accounting for 14.08% of positivity rate. In studies by Hajia M *et al*\(^ {12}\), Pednekar SN *et al*\(^ {24}\) and Chawla K *et al*\(^ {25}\) reported higher positivity rates of 41%, 53% and 74% respectively.

One lymph node aspirate was positive by BacT Alert and PCR but failed to grow on LJ media. This could be due to non uniform distribution of bacilli in the aliquots apportioned for the diagnostic tests\(^ {26,27}\). Two samples were PCR negative but culture positive and later diagnosed as Non tuberculous mycobacteria (NTM). Three samples were negative by both culture methods and PCR. The reason for smear positive but culture and PCR negative could be the availability of small quantity of sample for processing.

In our study sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of BacT Alert culture was 83.3%, 92.3%, 50% and 98.4% respectively which

### Table 4. Mean Turn Around Time (TAT) of individual diagnostic method

| S. No. | Diagnostic Method                                      | TAT   |
|-------|--------------------------------------------------------|-------|
| 1     | Ziehl Neelsen Stain                                    | 40 minutes |
| 2     | Fluorescent stain(Auramine Rhodamine Stain)            | 30 minutes |
| 3     | Lowenstein Jensen Medium(Solid Culture)                | 36.3 days |
| 4     | Bact Alert(Liquid Culture)                             | 14.6 days |
| 5     | Polymerase Chain reaction                              | 5 hours   |

![Fig.1. Gel documentation picture of samples positive by PCR assay (LAD - Ladder 100 bp, PC- positive control, NC- negative control)](image-url)
correlates with study by Martinez MS et al who reported sensitivity of 87.7% and specificity of 99.2%.

The percentage of isolation of *M. tuberculosis* by BacT Alert and LJ culture from synovial fluid was 33.3%, whereas in lymph node it was 30% by BacT Alert compared to 20% by LJ culture. In pleural fluid, percentage of isolation by BacT Alert was 28.57% whereas isolation by LJ was none; however, in pus samples, isolation percentage was higher by LJ culture (20%) compared to BacT Alert (13.33%). Endometrial biopsy, peritoneal fluid, tissue samples, CSF and urine samples did not yield growth of *M. tuberculosis*. Since the number of synovial fluid samples processed were less, the isolation percentage by both culture methods in present study is high. In study by Ghadage et al has reported maximum isolation of *M. tuberculosis* by LJ culture in pus (33%), followed by pleural fluid (26.3%), fine needle aspiration biopsy (25%) and CSF (12.5%)²⁹.

BacT Alert culture was positive in additional 5 samples when compared to LJ culture. The recovery rate was higher by BacT Alert culture (90.9%) compared to LJ culture (63.63%). Though BacT Alert had higher recovery rate, in one case of suspected Pott’s spine only LJ culture was positive. This underlines the need to use the combination of liquid and solid media especially in the diagnosis of EPTB.

PCR was positive in 10 samples. The sensitivity, specificity, positive predictive value and negative predictive value of PCR assay was 66.66%, 90.76%, 40% and 96.72% respectively. These results are comparable to studies by Oberoi A et al and Siddqui MAM et al.

PCR was positive in 2 samples (endometrial biopsy, lymph node aspirate) where both the staining techniques and both the culture methods were negative. This could be due to paucibacillary nature and high sensitivity of PCR.

However in one case of suspected Pott’s spine, only LJ culture was positive but other methods failed to detect *M. tuberculosis*. This false negative PCR result could be due to nonuniform distribution of bacilli in the aliquots apportioned for the diagnostic tests, ineffective extraction of DNA or the presence of PCR inhibitors.

The mean turnaround time for ZN staining was 40 minutes as compared to fluorescent staining (30 minutes). The mean turnaround time for culture positivity was 36.3 days with LJ culture (range 5 to 49 days) and 14.6 days with BacT Alert culture (range 4 to 21 days). The use of BacT Alert culture has reduced the mean detection time by 2.5 times when compared to LJ culture. PCR mean detection time was 5 hours. Thus, PCR reduces detection time when compared with culture. PCR provides additional information when positive microscopy results are combined with PCR results and it also differentiates *M. tuberculosis* from NMT.

The Cohen’s kappa statistics was applied to assess the reproducibility and the level of agreement between the PCR assay and other diagnostic tests. In our study, the level agreement between BacT ALERT and PCR was ‘substantial’ with a kappa value of 0.767 whereas ‘moderate’ level of agreement between LJ culture and PCR with kappa value of 0.441.

The ability of PCR to detect even few organism from clinical sample makes it very attractive diagnostic tool in the diagnosis of EPTB. In the present study, PCR had sensitivity of 66.7%, specificity of 90.76% and short detection time (5 hours). The sensitivity of PCR from clinical samples reported from different studies varies between 55% and 90%, which is much higher when compared to any other test used in the diagnosis of EPTB. This makes PCR a valuable screening test, especially when limitations of conventional diagnostic modalities have negative impact on patient care. Though PCR has been reported to have high sensitivity and specificity, it has few drawbacks. It is very expensive, needs expertise and proper standardization and risk of false negative and false positive results.

**CONCLUSION**

PCR has high sensitivity, specificity, substantial level of agreement with BacT ALERT culture and shorter turnaround time; hence in the era of evidence based clinical practice, it adds meaningful evidence to the results of conventional method employed in the diagnosis of EPTB, to rule-in or rule-out the disease. Therefore, use of PCR in combination with other diagnostic modalities helps to provide maximum information to clinicians in the diagnosis of EPTB.
REFERENCES

1. http://www.who.int/tb/publications/global_report/en/
2. Lee JY. Diagnosis and Treatment of Extrapulmonary Tuberculosis. *Tuberc Respir Dis* 2015; 78: 47-55.
3. World Health Organization Global Tuberculosis Report 2015. Available from: http://www.who.int/tb/publications/global-TB-report-2015
4. Purohit M, Mustafa T. Laboratory Diagnosis of Extrapulmonary Tuberculosis (EPTB) in Resource-Constrained Setting: State of the Art, Challenges and the Need. *J Clin Diagn Res* 2015; 9: EE01-EE06.
5. Sharma SK, Mohan A. Extrapulmonary Tuberculosis. *Indian J Med Res* 2004; 120: 316-53.
6. Sekar B, Selvaraj L, Alexis A, Ravi S, Arunagiri K, Rathinavel L. The Utility of IS Sequence Based Polymerase Chain Reaction in Comparison to Conventional Methods in the Diagnosis of Extra-Pulmonary Tuberculosis. *Indian J Med Microbiol* 2008; 26: 352-5.
7. Pingle P, Apte P, Trivedi R. Evaluation of Microscopy, Culture and PCR Methods in the Laboratory Diagnosis of Genito-urinary Tuberculosis. *American Journal of Infectious Diseases and Microbiology* 2014; 2:17-21.
8. Sudhindra KS, Prakash RC, Fatima F, Venkatesh VN, Rao A, Vijaynath V. Evaluation of PCR in Early Diagnosis of Tuberculosis. *J Med Res Pract* 2012; 1: 24-28.
9. Arvind N, Chand P, Baliga S, Bharati B, Shenoi S, Saralaya V. A Comprehensive Comparison of PCR Based Assay Versus Microscopy and Culture in the Diagnosis of Tuberculosis in Different Clinical Specimens. *Int J Biol Med Res* 2014; 5: 3763-68.
10. Mercy EG, Aditya T, Amarendra KE, Dan MM. Review on Mycobacterium Tuberculosis. *RRJMB* 2016: 9-17.
11. Lange C, Mori T. Advances in the Diagnosis of Tuberculosis. *Journal of Asian Pacific Society of Respiriology* 2010; 15:220-240.
12. Hajia M, Rahbar M, Amini R. Is PCR assay reliable for diagnosis of extrapulmonary tuberculosis? *Afr J Microbiol Res* 2009; 3:877-881
13. Wim W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, Woods G. Koneman’s Color Atlas and Textbook of Diagnostic Microbiology. 6th ed:2006.1064-1124.
14. Arora VK, Gupta R. Trends of extrapulmonary tuberculosis under revised national tuberculosis control program; a study from south Dehli. *Indian J Tuberc* 2006; 53:73-83
15. Musellim B, Erturan S, Duman ES, Ongen G. Comparison of extrapulmonary and pulmonary tuberculosis case; Factors influencing the site of reactivation. *Int J Tuberc Lung Dis* 2005; 9: 1220-1223
16. Srivastava I, Bhatambare G.S, Deshmukh A.B, Bajpai P, Bajpai T, Singh T. Genital Tuberculosis: Evaluating Microscopy, Culture, Histopathology and PCR for Diagnosis All Play Their Role. *Int J Curr Microbiol App Sci* 2014; 3: 439-445
17. Chhina D, Mohi G, Gupta R, Deeplyall K, Kaur J. Role of gen-probe amplified Mycobacterium tuberculosis Direct Test (AMTD) in the diagnosis of extrapulmonary tuberculosis. *IJPBRS* 2015; 4: 135-143
18. Ajantha GS, Shetty PC, Kulkarni RD, Biradar U. PCR as a Diagnostic Tool for Extra-pulmonary Tuberculosis. *JCDR* 2013; 7: 1012-1015
19. Sharma K, Appannanavar SB, Modi M, Singh M, Sharma A, Varma S. Role of multiplex polymerase chain reaction using IS6110 and Protein b for the diagnosis of extrapulmonary tuberculosis: North India. *Indian J Pathol Microbiol* 2015; 58:27-30
20. Siddqui MAM, Anuradha PR, Nagamani K, Vishnu PH. Comparison of conventional diagnostic modalities, BACTEC culture with polymerase chain reaction for diagnosis of extrapulmonary tuberculosis. *J Med Allied Sci* 2013; 3: 53-58
21. Angeby KA, Werngen J, Toro JC et al. Evaluation of the BacT/ALERT 3D system for recovery and drug susceptibility testing of Mycobacterium tuberculosis. *Clin Microbiol Infect* 2003; 9: 1148 –52.
22. Carriço A, Fonsale N, Vautrin A C, Aubert G. Evaluation of BacT/Alert 3D Liquid Culture System for Recovery of Mycobacteria from Clinical Specimens Using Sodium Dodecyl (Lauryl) Sulfate-NaOH Decontamination. *J Clin Microbiol* 2001; 39:3799-3800
23. Piersimoni C, Scarparo C, Callegaro A Tosi CP, Nista D, Bornigia S et al.Comparison of MB/Bact ALERT 3D System with Radiometric BACTEC System and Lowenstein-Jensen Medium for Recovery and Identification of Mycobacteria from Clinical Specimens: a Multicenter Study. *J Clin Microbiol* 2001; 39: 651-657
24. Pednekar SN, Desai SL, Pol S, Kagal AS, Dharmashale SN, Bharadwaj R S. A Comprehensive Comparison Of Mpb64 Based Pcr Assay Versus Microscopy And Culture In The Diagnosis Of Clinically Suspected Cases
Of Extrapulmonary Tuberculosis. *Int J Recent Advances in Multidisciplinary Research* 2015; 2: 0436-0440

25. Chawla K, Johar R, Vishwanath S, Mukhopadhyay C. Role of PCR in the Diagnosis of Pulmonary and Extra-Pulmonary Tuberculosis. *NJLM* 2015; 4:43-46.

26. Annam V, Kulkarni MH, Puranik RB. Comparison of modified fluorescent method and conventional Ziehl-Neelsen method in detection of acid fast bacilli in Lymph node aspirate. *CytoJournal* 2009; 6:13

27. Chakravorty S, Sen MK, Tyagi JS. Diagnosis of Extrapulmonary Tuberculosis by Smear, Culture, and PCR Using Universal Sample Processing Technology. *J Clin Microbiol* 2005; 49: 4357-4362

28. Martinez MS, Sardinas M, Garcia G, Mederos LM, Diaz R. Evaluation of BacT ALERT 3D System for Mycobacteria isolates. *Journal of Tuberculosis Research* 2014; 9: 59-64.

29. Ghadage DP, Muley VA, Pednekar S, Bhore AV. Evaluation of polymerase chain reaction using primer MPB 64 for diagnosis of clinically suspected cases of extra-pulmonary tuberculosis. *J Sci Soc* 2014; 41:89-93

30. Oberoi A, Aggarwal A. Comparison of the conventional diagnostic techniques, BACTEC and PCR. *JK Sci* 2007; 9:179-181

31. Zakham F, Bazouli H, Akrim M, Lamrabet S, Lahlou O, Elmezibri M et al. Evaluation of conventional molecular diagnosis of mycobacterium tuberculosis in clinical specimens from Morocco. *J Infect Dev Ctries* 2012; 6:40-45.