Comparison of conventional diagnostic modalities, BACTEC culture with polymerase chain reaction for diagnosis of extra-pulmonary tuberculosis

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Abstract

This study attempts to determine and compare the sensitivity, specificity and turnaround time of conventional diagnostic modalities, BACTEC culture and polymerase chain reaction test for diagnosis of extra-pulmonary tuberculosis. One hundred clinically suspected patients of extra-pulmonary tuberculosis were studied. Specimens from these patients were processed for Ziehl-Neelsen (ZN) stain and culture for M. tuberculosis was done with inoculation on Lowenstein-Jensen (LJ) medium as well as the BACTEC MGIT 960 TB system. All the samples were processed for PCR amplification with primers targeting 123 bp fragment of insertion element IS6110 sequence of M. tuberculosis complex. Out of the hundred extra-pulmonary samples processed, 5% were positive by ZN staining, 15% were positive by both LJ culture and BACTEC MGIT 960 TB culture and 70% were positive by PCR. Those samples positive by ZN smear, LJ culture and BACTEC culture were all found to be 100% positive by PCR. The mean detection time for M. tuberculosis was 23.13 days by LJ Medium culture, 9.86 days by BACTEC MGIT 960 TB culture and less than one day by PCR. PCR as a diagnostic tool is more sensitive and useful in diagnosis of extra-pulmonary form of tuberculosis when compared to conventional methods or BACTEC culture by definitely shortening the time with early initiation of anti-tubercular treatment and can prevent disease progressing towards irreversible tissue damage.

Key words: BACTEC MGIT 960 TB system, Extra-pulmonary tuberculosis, IS6110 sequence, LJ Medium, PCR, ZN staining

Tuberculosis can potentially involve any system or organ in the body. While pulmonary tuberculosis is the most common presentation, extra-pulmonary tuberculosis (EPTB) is also an important clinical problem but it is a milder form of disease in terms of infectivity. The risk of extra-pulmonary tuberculosis increases with immunosuppression. Lymphadenitis is the most commonly occurring form of extra-pulmonary tuberculosis followed by pleural effusion, bone and joint, geni-
touinary tuberculosis, miliary tuberculosis, tuberculous meningitis, abdominal tuberculosis and others. However, the EPTB most often remains undiagnosed and even worse, untreated. In India, EPTB forms 10 to 15 percent of all types of tuberculosis. With the global rise in Human Immuno-deficiency Virus (HIV) infection, EPTB accounts for more than 50% of all cases of tuberculosis among HIV positive patients.

An accurate diagnosis of tuberculosis is desirable before the start of anti-tuberculosis therapy (ATT). Conventionally smear microscopy with Ziehl-Neelsen (ZN) staining is the most widely used rapid method in most laboratories for diagnosis in developing countries. However, the ZN smear technique has several limitations such as low sensitivity and specificity, especially in extra-pulmonary cases. The classical approaches to diagnosis have been based on microscopy and culture. The gold standard, culture in Lowenstein–Jensen (LJ) medium, though sensitive is laborious, takes 4-8 weeks and should be followed by identification tests.

A fully automated, high-capacity (960-tube volume), non-radiometric and non-invasive system incorporating Mycobacterial Growth indicator tube system (MGIT 960 TB) contains a modified Middlebrook 7H9 broth in conjunction with a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with ruthenium pentahydrate) in an atmosphere of 10% CO₂. The system automatically records bacterial growth every 60 minutes, based on O₂-sensitive fluorescence. This is reliable, has shorter turnaround time and can be used with minimum infrastructure and technical skills.

Several studies have been done to detect Mycobacterium tuberculosis in diagnosis of extra-pulmonary tuberculosis by amplifying different DNA sequences by Polymerase Chain Reaction (PCR) test with ensuing results.

In this study, an attempt has been made to assess the utility of BACTEC culture and PCR test for diagnosis of extra-pulmonary tuberculosis by comparing them with conventional diagnostic modalities.

Materials and methods

The study protocol was approved by Institutional Ethics Committee. The study was conducted between October 2010 and November 2011.

Study group

One hundred samples, which included 37 cerebrospinal fluid (CSF), 29 ascitic fluid, 24 pleural fluid, 6 fine needle aspiration cytology (FNAC), 2 synovial fluid, one each pericardial fluid and urine were taken from patients with clinical suspicion of extra-pulmonary tuberculosis. Patients who fulfilled three of the five following criteria were included in the study.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Criteria</th>
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<tbody>
<tr>
<td>1</td>
<td>Clinical</td>
<td>Fever and other constitutional symptoms for 2 weeks not responding to antibiotics</td>
</tr>
<tr>
<td>2</td>
<td>Biochemical</td>
<td>CSF: Proteins &gt; 60mg%, Sugar &lt; 2/3rd of blood sugar</td>
</tr>
<tr>
<td>3</td>
<td>Pathological</td>
<td>Body fluids: Lymphocytosis &gt; 60%; Tissue samples: Histopathology showing granulomatous picture</td>
</tr>
<tr>
<td>4</td>
<td>Radiological</td>
<td>Chest X-ray/CT scan showing tubercular changes</td>
</tr>
<tr>
<td>5</td>
<td>Supportive evidence</td>
<td>family history of tuberculosis</td>
</tr>
</tbody>
</table>

All cases already on ATT or had been confirmed as having Pulmonary Tuberculosis (PT) were excluded from the study. The control group comprised of 20 patients who were diagnosed with non-tuberculous lymphadenitis and cases of pyogenic meningitis were taken.

Processing of samples for culture

Extra-pulmonary samples were collected by the treating physician under strict aseptic condition and sent to the lab. In case of anticipated delays in processing, they were stored at 4°C, for maximum of 4 days. The portion of the sample to be processed for PCR was stored at -20°C.

No decontamination with 4% NaOH was done, since the samples were drawn from 'sterile' areas. The samples were concentrated by centrifuging at 4000 rpm for 20 minutes. For every sample, smear microscopy and culture on both LJ and BACTEC MGIT 960 TB media were done.

In house prepared LJ media was used for primary isolation of Mycobacterium tuberculosis. Two loops full of concentrated deposit were inoculated on the entire surface of two LJ media slants in a pre-sterilized inoculation hood under aseptic con-
ditions. The date of inoculation was noted. The slopes were incubated at 37°C for a maximum of 8 weeks. The slopes were inspected daily in the first week and twice a week from second week onwards for any growth or contamination. In case of any growth of Mycobacteria, the date of first appearance of colonies was noted and the slopes were further incubated for further growth. In case of any contamination, the slopes were removed.

A BBL MGIT tube (from Becton Dickinson Microbiology systems, Sparks, MD, USA), containing 7 ml of Middlebrook 7H9 broth was used. Lyophilized MGIT PANTA (containing Polymyxin B, Azlocillin, Nalidixic acid, Trimethoprim and Amphotericin B) was reconstituted with MGIT growth supplement (containing Oleic acid, Albumin, Dextrose, Catalase, Polyoxymethylene stearate) and 0.8 ml of this was added to MGIT tube prior to sample inoculation. About 0.5 ml of the sediment obtained after decontamination and concentration, was added to this MGIT tube and the tubes were incubated inside the BACTEC MGIT 960 TB instrument for 6 weeks. The growth in the MGIT tubes was automatically detected by the instrument which is able to continuously monitor the fluorescence due to growing Mycobacteria once in every 60 minutes. The instrument indicates any growth automatically by flashing a red light to indicate instrument positive tubes. If no growth occurs at the end of 6 weeks, it indicates negative by flashing a green light. MGIT positive tubes as well any negative tubes which had some deposit in them were processed by biochemical tests. Growth on LJ Media as well as in MGIT tubes was confirmed by acid fast stain and standard biochemical tests.

Biochemical test

The mycobacterial isolates obtained in culture were subjected to limited biochemical testing for species characterization by carrying out a Nitrate Reduction Test and absence of growth on LJ medium with para-nitrobenzoic acid.5,6 Quality control was carried out using the strain M. tuberculosis, H37Rv as a positive control, and a reagent control without organisms as negative control. No attempt was made at further speciation.

Polymerase Chain Reaction (PCR)

DNA extraction

In our study, sonication method was used to release the DNA from cells7. All specimens sampled at 10,000 g for 15 minutes was be centrifuged followed by the removal of supernatant. Then 100 ul of molecular grade water was added to the pellet and vortexed thoroughly. Following heat killing at 95°C for 25 minutes, sonication was done for 15 minutes at room temperature, followed by centrifugation at 13,000 g for 5 minutes. The supernatant was collected in the fresh tube. 3μl of DNA was used for the reaction.

PCR amplification and documentation

Amplification of DNA was performed with primers IS-F-5′-CTTCGAGGCTAGGCTCGG-3′ and IS-R ‘CTCGTCCAGCGCCGCTTCGG-3′, to amplify 123 bp fragment of insertion element IS6110 of M. tuberculosis complex (BioServe Biotechnologies India Pvt. Ltd., Hyderabad, India). Briefly PCR was carried out in 25 μL volume, using 300μM dNTPs, 10pM of each primer and 1ul of Taq polymerase, followed by template. Conditions followed were initial denaturation at 95°C for five minutes, followed by 35 cycles at 94°C for one minute, 63°C for one minute, 72°C for one minute, and a final extension at 72°C for 7 minutes. The amplified products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide at 90 V for 30 minutes. Resultant bands were interpreted by UV transillumination. A product of 123 bp was indicative of the presence of M. tuberculosis and the results were documented (Fig 1). Quality control was done by including a negative and a positive control with every batch of samples. Throughout the PCR processing, the three room procedure and other recommended stringent precautions were followed and the results were evaluated in the light of the performance of appropriate positive and negative controls, to avoid cross-contamination and false positive reactions.

![Fig 1. PCR amplification of 123bp region in IS6110 gene of M. tuberculosis on 2% agarose gel. Lane 1: Positive Control (PC), Lane 2: Negative Control (NC), Lane 3, 4, 6, 7, 8: Positive for IS 6110 sequence, Lane 5: Negative Test, Lane 9: 100 bp DNA marker (Sigma Co., USA).](image-url)
Results

In our study, 100 (90 adult and 10 pediatric) patients with clinically suspected extra-pulmonary TB were included. The age ranged from 1 year to 70 years with 79% of patients being less than 50 years of age with a male to female ratio of 2.03:1. Most of the samples received were of CSF (37), followed by ascitic fluid (29), pleural fluid (24), FNAC (6), synovial fluid (2) and pericardial fluid (1) and urine (1). Five patients in the study were HIV infected.

Five of hundred samples (5%) were positive for AFB by ZN staining. The lymph nodes aspirates showed a high proportion of positivity (33%) for AFB.

Both LJ medium and BACTEC MGIT 960 TB culture detected M. tuberculosis growth in 15 samples. The mean turnaround time for culture positivity was 23.13 days with LJ medium and 9.86 days by BACTEC MGIT 960 TB culture.

The sensitivity of PCR was found to be highest regardless of the type of specimen. The sensitivity of culture on LJ medium or by BACTEC MGIT 960 TB was much lower than sensitivity of PCR with all the specimens.

The sensitivity of smear examination for AFB by either ZN staining was much lower than sensitivity of culture by either method or PCR with all the specimens.

Of the 5 cases which were positive by microscopy, all 5 (100%) showed growth on LJ medium. In addition, the LJ medium could detect 10 out of 85 (11.7%) cases which were negative by microscopic examination.

Of the 5 cases which were positive by microscopy, all 5 (100%) showed growth on BACTEC MGIT 960 TB. In addition BACTEC MGIT 960 TB could detect 10 out of 85 (11.7%) cases which were negative by microscopic examination.

Of the 5 samples that showed presence of AFB on microscopy, all 5 (100%) were positive by PCR. In addition to this, PCR could detect 65 cases out of 95 (68.4%) which were negative by microscopic examination.

Out of 15 cases which showed growth on LJ medium, all 15 (100%) showed growth by culture using BACTEC MGIT 960 TB system.

Out of 15 cases which were positive by culture on LJ medium, all 15 (100%) were positive by PCR. In addition, PCR could detect 55 out of 85 (64.7%) which were negative by culture on LJ medium.

Out of 15 cases which were positive by culture on BACTEC, all 15 (100%) were positive by PCR. In addition, PCR could detect 55 out of 85 (64.7%) which were negative by culture on BACTEC.

The PCR is found to be the most sensitive (70%), specific (100%) and most rapid method for the diagnosis of extra-pulmonary tuberculosis.

<table>
<thead>
<tr>
<th>Nature of clinical sample</th>
<th>No. of samples</th>
<th>Detection rate in number (%) by different tests</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>ZN staining</td>
</tr>
<tr>
<td>CSF</td>
<td>37</td>
<td>00 (0%)</td>
</tr>
<tr>
<td>Ascitic fluid</td>
<td>29</td>
<td>01 (3.4%)</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>24</td>
<td>02 (8.3%)</td>
</tr>
<tr>
<td>FNAC</td>
<td>06</td>
<td>02 (33.3%)</td>
</tr>
<tr>
<td>Synovial fluid</td>
<td>02</td>
<td>00 (0%)</td>
</tr>
<tr>
<td>Pericardial fluid</td>
<td>01</td>
<td>00 (0%)</td>
</tr>
<tr>
<td>Urine</td>
<td>01</td>
<td>00 (0%)</td>
</tr>
<tr>
<td>Controls</td>
<td>20</td>
<td>Negative</td>
</tr>
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</table>
Table 2: Average turnaround time, sensitivity and specificity of different tests

<table>
<thead>
<tr>
<th>Parameter in study</th>
<th>ZN stain</th>
<th>LJ medium culture</th>
<th>BACTEC MGIT 960 TB</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average turnaround time</td>
<td>20 min</td>
<td>23.13 days</td>
<td>9.86 days</td>
<td>36 hrs</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>5</td>
<td>15</td>
<td>15</td>
<td>70</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>100</td>
<td>100</td>
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</table>

Discussion

This study was undertaken to assess the utility of various diagnostic modalities for diagnosis of extrapulmonary tuberculosis. Also an attempt was made to compare the sensitivity of PCR with the conventional diagnostic techniques and culture using BACTEC MGIT 960 TB system.

Maximum number of patients suffering from extrapulmonary tuberculosis were in the age group of 30 to 39 years (37%) followed by 50-59 years (19%).

In our study, out of 100 cases we could demonstrate AFB in 5 cases (5%) by standard ZN staining. Our findings were nearly correlated with ligazli et al who reported positivity of 4.2%.

Our study showed overall rate of isolation of Mycobacteria on LJ medium to be 15% which falls in between the reported isolation rate by Rodrigues et al and Castro et al of 13% and 16.6% respectively.

Our study showed the isolation rate of Mycobacteria by using BACTEC MGIT 960 TB to be 15%. Rishi et al have reported 21.9% sensitivity rate for isolation of Mycobacteria using BACTEC MGIT 960 TB system.

In this study, out of 15 isolates on LJ medium and BACTEC MGIT 960 TB, none were identified as non-tuberculous (NTM). All of them were identified as belonging to MTB complex. Varying percentage of isolation of MTB and NTM has been reported in literature ranging from 0-9%. The study by Sharma & Mohan showed similarity to our results.

In our study, the overall positivity rate of PCR was found to be 70%. Negi et al and Chawla et al using the same set of primers reported the overall positivity rate to be 77.2% and 74.1%.

The sensitivity of detection of M. tuberculosis in AFB smear positive samples by PCR approached 100%, whereas that in smear negative specimens had a sensitivity of 68.4%. Sekar et al and Negi et al reported 100% positivity of PCR in the smear positive cases.

In our study the sensitivity of BACTEC MGIT 960 TB system in detecting LJ medium positive samples was 100%. Rishi et al reported that BACTEC MGIT 960 TB could pick up 98.07% of LJ medium positive samples.

In our study, the sensitivity of PCR in detecting LJ medium positive samples was 100% and in detecting LJ medium negative samples was 64.7%. Our study is comparable to those of Negi et al and Kesawani et al in PCR positivity in culture positive cases.

In our study, the sensitivity of PCR in detecting BACTEC culture positive samples was 100% and BACTEC culture negative samples was 64.7%. Our study results are in near correlation with the study of Negi et al in which they have reported 97.53% and 34.37% PCR positivity in BACTEC culture positive and BACTEC culture negative samples.

Conclusion

Out of 100 clinically suspected cases of extrapulmonary tuberculosis, 70 (70%) could be diagnosed by PCR, while 15 (15%) could be diagnosed by either of the microbiological methods i.e. LJ media/ BACTEC culture and only 5 (5%) with ZN staining. This difference in the sensitivity rate of PCR, LJ media, BACTEC culture and ZN staining is statistically significant (p<0.05).

Because of this significant difference in the sensitivity rates of ZN staining, LJ media, BACTEC culture and PCR in extra-pulmonary tuberculosis, it appears PCR appears to be a very useful means of diagnosis. These differences between the conventional methods and PCR were also reported by Negi et al and Hajia et al.

In conclusion PCR was found to be the most sensitive (70%), specific (100%) and most rapid method for the diagnosis of tuberculosis, being extremely useful in diagnosis of extra-pulmonary form of tuberculosis, where the yield of conventional methods or BACTEC culture is modest at best. Rapid results by PCR allow quick implementation of treatment regimen.

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Conflict of interest: None

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