CONCURRENT EVALUATION: CONVENTIONAL VS RAPID TECHNIQUES FOR DIAGNOSIS OF TUBERCULOSIS IN A TERTIARY CARE CENTRE OF PUNJAB

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ABSTRACT

BACKGROUND

Mycobacterium Tuberculosis (TB) is an infectious human pathogen causing serious public health problem worldwide. India is among the highest TB burden countries. A fast and accurate diagnosis is necessary to control and eliminate Tuberculosis (TB).

The aim of the study was to evaluate the results of Mycobacterium Tuberculosis by conventional versus the rapid methods of diagnosis in a tertiary care centre in Punjab.

MATERIALS AND METHODS

This study was done in the Microbiology Department of a tertiary care centre in Punjab to look for results of clinical samples tested for TB using real time PCR (Targeting 16S rRNA gene), Ziehl-Neelsen (ZN) staining and culture on Lowenstein Jensen (LJ) medium. Few samples were also subjected for liquid culture using BACTEC Micro MGIT system. This study included all samples tested for TB from July 2015 to May 2016.

RESULTS

A total of 320 samples were tested for TB using smear microscopy, culture and PCR [including 36 pulmonary samples (11.25%) and 284 (88.75%) extrapulmonary]. These included 106 Cerebrospinal Fluid (CSF), 52 Pleural fluid, 51 Tissues, 24 Ascitic fluid, 26 Urine, 27 Broncho-Alveolar Lavage (BAL), 25 Pus and 9 Sputum samples. Smear microscopy was positive in a total of 8 samples out of 320 (2.5%) which included 3 sputum, 1 BAL, 2 pleural fluid, 1 tissue and 1 ascitic fluid samples, while culture was positive for 12 samples (3.75%) which included 3 sputum, 2 pus, 2 BAL, 2 pleural fluid, 2 tissue and 1 ascitic fluid sample. The TB-PCR results were positive in a total of 9 (28.75%) samples out of 320. Liquid culture was used on 36 of these samples and was positive in 8 samples (22.22%). Out of the 8 samples reported positive, 3 samples were positive both by PCR and culture too, while only 1 sample positive by smear microscopy.

CONCLUSION

PCR and MGIT are very rapid and accurate diagnostic tool for early detection of TB, particularly for EPTB. But for maximum recovery of Mycobacteria, both conventional and rapid methods should be incorporated.

KEYWORDS

Tuberculosis (TB), Ziehl-Neelsen (ZN) Staining, Lowenstein Jensen (LJ) Medium, Polymerase Chain Reaction (PCR), BACTEC Micro MGIT (Mycobacterium Growth Indicator Tube) System, Extrapulmonary TB (EPTB).


BACKGROUND

Tuberculosis (TB) remains a major global health problem and causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the Human Immunodeficiency Virus (HIV). As per WHO Global TB Report 2015, out of the estimated global annual incidence of 9.6 million TB cases, 2.2 million were estimated to have occurred in India.

While pulmonary tuberculosis is the most common presentation, Extrapulmonary Tuberculosis (EPTB) is also an important clinical problem. The term EPTB has been used to describe isolated occurrence of tuberculosis at body sites other than the lung.

Smear microscopic examination by the Ziehl-Neelsen (ZN) staining is a rapid and cheap method to detect acid fast bacilli, but it has low sensitivity and specificity. Amongst many different culture media devised for growing the tubercle bacillus, egg-based media are among the best known solid media used for isolation of M. tuberculosis in particular Lowenstein Jensen (LJ) medium, as these media tend to yield high number of positives from direct clinical specimens, because it is less inhibitory to the mycobacteria. Culture using LJ medium though more sensitive than microscopy and also highly specific is difficult, tedious and delays diagnosis by 6 - 8 weeks. Culture remains the gold standard for diagnosing tuberculosis. However, the disease most often
remains undiagnosed and even worse untreated. Major difficulty is with extrapulmonary samples, which are associated with low sensitivity of acid-fast bacillus smear and culture. The diagnosis of extrapulmonary tuberculosis is especially challenging for various reasons like lack of adequate sample amount or volumes, paucibacillary nature of specimens yielding very few bacilli and lack of an efficient sample processing technique, universally applicable on all types of extrapulmonary samples.

The Mycobacterium Growth Indicator Tube (BBL MGIT) contains modified Middlebrook 7H9 broth base in conjunction with a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with ruthenium pentahydrate) in an atmosphere of 10% CO2. This compound is sensitive to the presence of dissolved oxygen in the broth. As the microorganisms grow in the media oxygen gets depleted, allowing the fluorescence to be detected automatically over time. When supplemented with MGIT Growth Supplement and PANTA, it provides an optimum medium for growth of a majority of mycobacterial species.(6)

The PCR is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Many reports have described the application of PCR to the diagnosis of pulmonary TB from sputum examination, but there are few from India more so in extrapulmonary cases from Central and Northern India. The molecular based diagnosis by PCR technique is faster and sensitive. Since the conventional techniques have limitations, rapid techniques like Polymerase Chain Reaction (PCR) is increasingly in use as a tool for early TB diagnosis.

Introduction of rapid methods like PCR and MGIT presents a major advance in early diagnosis of TB, which is essential for prompt treatment and for the control of disease transmission. In view of this, the present study was undertaken to determine the diagnostic utility of Real Time PCR and MGIT with conventional diagnostic modalities like ZN staining and LJ culture.

MATERIALS AND METHODS

This study was conducted from July 2015 to May 2016 in the Mycobacteriology and Molecular Lab of a tertiary care hospital in Northern India. A total of 320 clinical samples were tested for Tuberculosis (TB) using conventional microbiological techniques of diagnosis like ZN staining and culture on LJ medium (according to the standard procedure) and with rapid techniques using Real Time PCR assay (targeting 16S rRNA gene). Also for few samples BACTEC Micro MGIT system was also used.

Processing of the Samples

Clinical samples (both Pulmonary and Extrapulmonary) like sputum, BAL, CSF, pleural fluid, ascitic fluid, pus, urine, lymph nodes, biopsies and other tissues from clinically suspected cases of TB were received at our laboratory in sterile containers. Two samples were received from each patient. One sample was processed for AFB smear and culture, while the second sample was processed for Real Time PCR.

For AFB smear and culture, the samples were first homogenised and concentrated using Petroff’s method (4% NaOH solution). This mixture was homogenised by allowing it to stand at room temperature for 15 - 20 minutes. After this step, phosphate buffer was added and mixed well. The specimen was then centrifuged at 3,000 rpm for 15 - 20 minutes. After centrifugation, a portion of sediment was directly inoculated onto LJ medium slopes; the other portion was used for preparation of direct smear for ZN staining. Fluid specimens from sterile sites such as CSF, pleural fluid, etc. were centrifuged and a portion of sediment was directly inoculated in LJ medium without prior treatment (Petroff’s method). The tissue biopsy specimens were minced and homogenised in a sterile homogeniser and a portion of the homogenate was directly inoculated onto LJ medium slopes and other portion was used for making smears for ZN staining. The LJ medium bottles were incubated at 37˚C in the incubator. Culture readings were monitored weekly and discarded as negative in case of no growth at the end of 8 weeks.(11)

DNA extraction was performed on all the received samples by Genosen’s DNA extraction mini kit (Genome diagnostics Pvt. Ltd., New Delhi, India). TB DNA real time amplification was done by Genosen’s Mtb/MOTT (rotor gene) Real TIME PCR kit (Genome Diagnostics Pvt. Ltd., New Delhi, India) as per the manufacturer’s instructions. PCR Assay Based on DNA Coding for 16S rRNA for Detection and Identification of Mycobacteria in clinical samples was used.

In addition, clinically suspected cases of TB, which showed negative results in PCR for mycobacterium tuberculosis were also tested for MOTT using the same kit and results were evaluated accordingly.

Few samples were processed by the BACTEC Micro MGIT liquid culture system. A lyophilized vial of BBL MGIT PANTA (containing Polymyxin B, Azlocillin, Nalidixic acid, Trimethoprim and Amphotericin B) antibiotic mixture was reconstituted with 15 mL of BACTEC MGIT Growth Supplement (containing Oleic acid, Albumin, Dextrose, Catalase, Polysyoxyethylene stearate) and 0.8 mL of this was added aseptically into the 7 mL MGIT tube prior to sample inoculation. After that 0.5 mL of the digested, decontaminated and concentrated specimen suspension was added to the MGIT tube and incubated at 37˚C in the incubator for 42 days. Tubes are read daily starting from the second day of incubation in the BACTEC Micro MGIT Fluorescence reader one by one for interpretation. Tubes showing reading above 14 mark up to 20 are considered as positive, whereas readings 1 - 13 are considered as negative. Positive tubes are stained for acid-fast bacilli for ruling out bacterial contamination.(12)

The study was initiated after due approval by Institutional Ethical Committee and samples collected after informed written consent.

RESULTS

A total of 320 samples were tested for TB using smear microscopy, culture and RT PCR (including 36 pulmonary samples (11.25%) and 284 (88.75%) extrapulmonary). The sample distribution is shown in Figure 1. These included 106 CSF, 52 pleural fluid, 51 tissues, 24 ascitic fluid, 26 urine, 27 BAL, 25 pus and 9 sputum samples. Smear microscopy was positive in a total of 8 samples out of 320 (2.5%) which included 3 sputum, 1 BAL, 2 pleural fluid, 1 tissue and 1 ascitic fluid samples, while culture was positive for 12 samples (3.75%) which included 3 sputum, 2 pus, 2 BAL, 2 pleural fluid, 2 tissue and 1 ascitic fluid sample (Table 1). The TB PCR results were positive in a total of 92 samples out of 320 (28.75%) (Table 2). The samples detected positive by ZN smear and LJ
culture were all positive by PCR too. In addition to the above-mentioned results, 3 samples also tested positive for MOTT. These included 1 tissue, 1 pleural fluid and 1 BAL sample. However, these samples were not further analysed for species identification.

Liquid culture was used on 36 of these samples and was positive in 8 samples (22.22%). Out of the 8 samples reported positive, 3 samples were positive both by PCR and culture too, while only 1 sample was positive by smear microscopy.

The mean time to detection for MTB was 28.81 days (7-48 days) by LJ media, 9.66 days (2-39 days) by MGIT and less than 1 day by PCR.

<table>
<thead>
<tr>
<th>Type of Specimen</th>
<th>Number of Specimen</th>
<th>Smear Positive (%)</th>
<th>Culture Positive (%)</th>
<th>PCR Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary Specimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum</td>
<td>09</td>
<td>03 (33.3)</td>
<td>03 (33.3)</td>
<td>04 (44.4)</td>
</tr>
<tr>
<td>BAL</td>
<td>27</td>
<td>01 (3.7)</td>
<td>02 (7.4)</td>
<td>07 (25.9)</td>
</tr>
<tr>
<td>Extra-Pulmonary Specimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>106</td>
<td>-</td>
<td>-</td>
<td>31 (29.2)</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>52</td>
<td>02 (3.8)</td>
<td>02 (3.8)</td>
<td>12 (23.0)</td>
</tr>
<tr>
<td>Tissue</td>
<td>51</td>
<td>01 (1.9)</td>
<td>02 (3.9)</td>
<td>17 (33.3)</td>
</tr>
<tr>
<td>Ascitic fluid</td>
<td>24</td>
<td>01 (4.1)</td>
<td>01 (4.1)</td>
<td>09 (37.5)</td>
</tr>
<tr>
<td>Urine</td>
<td>26</td>
<td>-</td>
<td>-</td>
<td>04 (15.3)</td>
</tr>
<tr>
<td>Pus</td>
<td>25</td>
<td>-</td>
<td>2 (8.0)</td>
<td>08 (32)</td>
</tr>
<tr>
<td>Total</td>
<td>320</td>
<td>08 (2.5)</td>
<td>12 (3.75)</td>
<td>92 (28.75)</td>
</tr>
</tbody>
</table>

Table 1: Type of Specimens and Positivity of Polymerase Chain Reaction, Culture and Smear

<table>
<thead>
<tr>
<th>Number of Samples (%)</th>
<th>Total Samples</th>
<th>PCR Positive</th>
<th>Culture Positive</th>
<th>Smear Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>320 (100)</td>
<td>92 (28.75)</td>
<td>12 (3.75)</td>
<td>08 (2.5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Total Number of Samples including Polymerase Chain Reaction, Culture and Smear Positive Sample

**DISCUSSION**

In this study, we compared the conventional methods with rapid methods like RT-PCR and MGIT in diagnosing TB. Prompt diagnosis of TB, especially extrapulmonary, remains a challenge always. Prompt diagnosis of TB is absolutely necessary in a developing country like India, where culture on LJ medium is still used as the gold standard for its diagnosis. The culture methods are time-consuming, therefore other evidences like pathological, radiological and clinical acumen are used till date for treating the patient.\(^{13}\)

In our study, PCR for diagnosing TB showed the best diagnostic yield followed by culture and smear. In a study by Siddiqui et al\(^{14}\) there was 5% positivity rate by ZN staining, 15% positivity rate by LJ culture medium and 70% positivity rate by PCR. This is quite similar to our study in which least were positive by ZN staining and maximum by PCR only.

Sample distribution in our study varied greatly as maximum samples were of CSF followed by pleural fluid and tissues, whereas the pulmonary samples were less. So, our study had predominantly extrapulmonary samples. In a study by Muhammad et al\(^{15}\) pulmonary samples predominated as compared to extrapulmonary. We have a 35.7% smear positive by ZN staining, 52.0% by culture and 62.2% by PCR. The high smear and culture positivity could be attributed to the significantly more number of sputum samples as compared to our study.

In a study by Singh et al\(^{16}\) wherein PCR was compared with conventional tests for diagnosis of TB in granulomatous lymphadenopathy, it was concluded that PCR is the most sensitive single technique available to date for the demonstration of MTB in specimens derived from patients with a clinical suspicion of tubercular lymphadenitis. PCR diagnosis of TB was concluded to be a rapid and sensitive method of diagnosis. Our study showed 33% PCR positivity as compared to studies by Muhammad et al and Siddiqui et al\(^{14,15}\) showing 62 - 70%; however, their positivity for smear and culture were also higher as compared to our study. In another study by Patwardhan et al\(^{17}\) which included lymph node specimens, PCR proved highly sensitive (90.1%) test.

In this study, PCR, smear staining and culture positivity were less probably because of lesser number of representative samples. This could have been due to diagnosis based on clinical suspicion rather than on stringent criteria based on clinical acumen, laboratory and radiological investigations.

Though MGIT was also done for TB diagnosis and is considered as a rapid method, but due to its use in very less number of samples in our study the results cannot be compared with PCR and other conventional modalities.

Three of our patients also tested positive for MOTT. This carries a great significance as the NTM are ubiquitous and causes varied diseases, some mimicking those of MTB. However, the treatment given for MOTT entirely differs from the standard anti-tubercular treatment. PCR is found to be a specific test and easily differentiates between the two.

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. Furthermore, the primary limitation of PCR arises from the absence of suitable gold standard to assess its efficiency. Furthermore, samples containing non-viable mycobacteria may lead to a false positive PCR result, thereby misleading physicians. The drawbacks of PCR are its high cost, specific requirement of infrastructure, equipment and expertise. In our study, all the efforts were taken to overcome these limitations by proper preventive laboratory techniques and use of sterilised methods.

At present, India is experiencing an epidemic of TB. Considering the number of cases diagnosed with TB in India, there is an urgent need to use multiple/newer diagnostic modalities for rapid detection of MTB to control the transmission of TB. Molecular methods such as PCR are underutilised and it is becoming imperative to have PCR available at as many health centres as possible. The clinical utility of detecting MTB by PCR is its reduction in the time to detection and its accuracy in detecting the pathogen in AFB smear-negative paucibacillary specimens. PCR is suitable as a public health tool in a country like India, as the tests is rapid and early diagnosis of TB is crucial for prompt treatment and for the control of disease transmission.

CONCLUSION
Conventional methods of smear microscopy and culture remain the gold standard for diagnosing pulmonary TB; however, poor performance of these conventional methods on extrapolmonary specimens demands for more sensitive and specific techniques. PCR is a very rapid and accurate diagnostic tool for early detection of TB, particularly for EPTB. In a country like India with such high burden of TB and limited resources for diagnosing TB, PCR is found to be very valuable for rapid identification and early diagnosis hence necessary for earlier isolation, treatment, improved patient outcome and more effective public health interventions. Also, MGIT proved to be a valuable alternative in settings where PCR is not available. But for maximum recovery of Mycobacteria, both conventional and rapid methods should be incorporated.

REFERENCES