A STUDY OF THE DIAGNOSIS OF PULMONARY TUBERCULOSIS AND POLYMERASE CHAIN REACTION

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ABSTRACT

The sensitivity of Polymerase Chain Reaction (PCR) makes it a potential diagnostic test for detection of M. tuberculosis in samples with low bacillary load. To assess the efficiency of PCR as compared to routine diagnostics in detection of M. tuberculosis from sputum samples of suspects referred to a tuberculosis clinic and those identified during a morbidity survey. Respiratory samples (sputum with or without saliva) from 144 individuals were examined by PCR, using MPB64 primers, culture and microscopy. 97 samples were from suspects referred to a tuberculosis clinic, 26 were from suspects identified during a morbidity survey and 21 were from patients with diseases other than tuberculosis. Study was conducted blind. Total cases considered to be positive for tuberculosis by all criteria was 71. PCR detected 98% of ‘culture positive’, 97% of ‘smear positive, culture positive’, and 100% of ‘smear negative’ culture positive samples. PCR was also positive for 86% of smear negative samples, from tuberculosis suspects diagnosed on the basis of other routine diagnostics and supporting clinical evidence. Seventeen samples were positive only by PCR but based on clinical parameters only 7 were considered as true positives. The sensitivity of PCR was 91.5% compared to 51% for smear microscopy and 68% for sputum culture. This was due to the fact that PCR could pick up bacterial DNA even from saliva mixed sputum specimens, which are generally not considered appropriate for microbiology. The specificity of PCR (86%) was found to be lower than other diagnostic tests mainly due to lack of a suitable gold standard to assess its efficiency. This is an important limitation in evaluation of the test.

Key words: Tuberculosis, Polymerase Chain Reaction, MPB64 primers.

1. INTRODUCTION

Diagnostic process of tuberculosis initiates with a high clinical suspicion, and is supported through the use of various diagnostics¹-². 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 final confirmatory laboratorydiagnostic for tuberculosis³. The need for more sensitive and specific techniques thus become obvious. Nucleic acid amplification using the principle of polymerase chain reaction (PCR) has the potential for the diagnosis of tuberculosis in a few hours with a high degree of sensitivity and specificity⁴. The potential of PCR as a diagnostic test for tuberculosis has been investigated in a large number of studies⁴-¹⁴. 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 respectively⁴. The limitations of PCR have also been discussed². The overall reported sensitivity of PCR ranges from 58% to 100%. Sensitivity is reported to be higher in smear-positive samples (95% to 100%) than in smear-negative samples (46 to 63%). 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 primers². The purpose of this study was to determine the efficiency of PCR as compared to other routine diagnostics like smear microscopy and culture, amongst sputum/saliva samples from a pool of highly probable tuberculosis suspects referred to a tuberculosis clinic and from symptomatic who were identified during a morbidity survey in a slum.

¹ January –March 2016
2. METHODOLOGY

2.1 Research design

A total of 144 respiratory samples (sputum with or without saliva) from as many individuals were tested. Of these, 123 samples were from individuals suspected of having pulmonary tuberculosis, and 21 samples were from hospital patients having a disease other than tuberculosis. The latter samples were controls for all the investigations carried out on the test samples. Of the 123 samples, 97 samples were taken from 97 highly probable tuberculosis suspects who were referred to or who presented at a tuberculosis clinic. Diagnostic and treatment decisions were made by site physicians according to the Revised National Tuberculosis Control Program (RNTCP) guidelines. Single overnight sample was used for culture and PCR examination, after it had been examined for smear microscopy at the tuberculosis clinic. Twenty six samples were collected from 26 chest symptomatics identified during a morbidity survey carried out in a slum. These individuals reported a productive cough with or without sputum for over three weeks along with one or more cardinal signs of tuberculosis like low grade fever in the evening, weight loss and chest pain. Samples from these individuals were collected by holding health camps (n=12), by referring symptomatics to a nearby municipal clinic (n=1), or through the collection of samples by health workers (n=13).

All the 144 samples, whether overnight or spot collections, were examined by routine smear microscopy, culture and PCR. The data were compared with available clinical information. Radiological data was available from 61 subjects. The study was conducted blind. Quality of samples submitted Of the 144 sputum samples, 45 were mixed with saliva. All the samples were processed for bacteriological investigations, namely smear, culture and PCR.

2.2 Sample processing, culture and PCR

Samples were processed using either Petroff’s method or N-acetyl-L-cysteine-sodium hydroxide method\(^{15}\). A small amount of the processed pellet was used for culture on Löwenstein Jensen (LJ) medium. For PCR, DNA was extracted, by incubating the remaining pellet in extraction buffer (1mg/ml proteinase K in 10mM Tris-HCl pH8.0, 1mM EDTA, 10% SDS). Proteinase K was inactivated by heating at 100°C for ten minutes. PCR was done using MPB64 primers (Sigma Aldrich/ Bangalore Genei) which are specific for Mycobacteria of the tuberculosis complex\(^{16}\). Amplification reaction was typically performed in a 50µl reaction mix containing 0.25nmoles of forward and reverse primers, 2mM final concentration of dNTP, 1.5 U of Taq polymerase in 1x buffer and proteinase K digested sample. The sequence of the forward and reverse primers used were 5’-TCCGCTGCGAGTCGTCGCTC-3’ and 5’-GTCCGTGTGAGTCTAAGCCA-3’. Forty cycles of amplification were performed using an initial denaturation step of 95°C for five minutes, followed by denaturation at 95°C for one minute, annealing at 55°C for one minute and extension of 72°C for one minute. A final extension was carried out at 72°C for seven minutes. The 0.2Kb amplified fragment was detected on a 2% agarose gel through ethidium bromide staining. DNA from M. tuberculosis strain H37Rv was routinely used as a positive control. Appropriate negative controls were set up for each sample. 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 twelve weeks. Characterization of Mycobacteria was done at the Corporation laboratory by primary differential tests for atypical Mycobacteria.

3. STATISTICAL ANALYSIS
As no single gold standard was available for comparison of the performance of the individual tests, an analysis of results was done using a variety of standards. Efficiency of microscopy, culture and PCR in terms of sensitivity, specificity, positive predictive value and negative predictive value was done using the gold standards of culture for the culture positive samples and smear microscopy, combined microbiological data, response to ATT, chest radiographic findings and clinical follow up data for culture negative samples.

1. RESULTS

A total of 144 samples, one from each subject (97 from a tuberculosis clinic, 26 identified during a morbidity survey in a slum community and 21 from cases having chest diseases other than tuberculosis) were examined by smear microscopy, culture on LJ slants, and PCR using primers specific for MPB64. Results for sputum smear microscopy and PCR required less than 48 hours whereas results for culture were available 4-8 weeks later.

PCR results amongst samples positive by culture and smear microscopy

Thirty five percent (50/144) samples tested positive by culture. Two culture positive samples were Mycobacteria Other Than Tuberculosis (MOTT), identified as M. scrofulaceum and M. intracellulare. PCR and microscopy were negative for both samples identified as MOTT and positive for 98% (47/48) of the remaining samples. There were 36 samples that were positive by Ziehl Neelsen staining. PCR was positive for 94% (34/36) of these samples. Thirty two samples were positive by microscopy and culture (S+C+). PCR was positive for 97% (31/32) of these samples (Table 1).

Table 1: Efficiency of PCR amongst samples positive by other diagnostic tests

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Sample Description (i)</th>
<th>Number (ii)</th>
<th>PCR positivity</th>
<th>PCR Efficiency* (iii/ii x 100) (iv)</th>
</tr>
</thead>
</table>
a. Culture positive samples | 50 | |
| Culture positive M.tuberculosis | 48 | 47 | 98 |
| MOTT | 2 | |
| Smear positive samples | 36 | 34 | 94 |
| Smear positive culture positive | 32 | 31 | 97 |
| Smear positive culture negative | 4 | 3 | 75 |
b. Smear negative samples | 28 | |
| Smear negative culture positive | 16 | 16 | 100 |
| Smear negative culture negative | 12** | 8 | 66.6 |
c. Persons (control) with respiratory disease other than tuberculosis(sputum and X-ray negative) | 21 | 0 | |

PCR data of smear negative samples from tuberculosis

Twenty nine individuals whose samples were negative by smear microscopy were diagnosed as having tuberculosis by other routine diagnostics. Of these one did not respond to anti-tuberculosis treatment and was excluded from the data presented in Table 1. PCR was positive for 86% (24/28) of these samples. Fifty seven percent (16/28) of these smear negative samples were positive by culture (SC+). PCR was positive for 100% of these samples.

3 January –March 2016
PCR data of smear negative samples from individuals diagnosed as not having tuberculosis

In addition, PCR was positive for 17 individuals who were declared not to have tuberculosis using the diagnostic paradigm of the RNTCP. Thirteen 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. Seven individuals took anti-tuberculosis treatment (ATT) subsequently, one HIV positive individual with recent history of extra-pulmonary tuberculosis was dead. Three individuals were immediate family contacts of tuberculosis cases, two of whom were on antituberculosis treatment. The remaining two individuals were highly probable cases of which one had prominent cervical lymph nodes suggestive of tuberculosis and the other had previous history of tuberculosis. On follow up, one submitted a sample for PCR. Figure-1 shows that within a period of six months after being diagnosed as not having tuberculosis, the PCR profile of the individual had changed markedly, suggesting an increase in the bacterial load in the sputum. Only seven of the 17 cases i.e. those who took ATT subsequently were considered as true cases of tuberculosis, even though the other six could be rated as highly probable cases of tuberculosis.

Table-2 summarizes the final diagnosis of tuberculosis made in 71 cases following a diagnostic review consisting of response to ATT/ clinical follow up / information from X-ray/sputum investigations.

PCR data on controls with diseases other than tuberculosis

PCR, culture examination and smear microscopy were performed on 21 individuals with respiratory diseases other than tuberculosis. These cases were as follows, two each of asthma, pneumonia and cancer lung, one each of post CABG, COPD and lung abscess and 12 with cough and cold. All samples from these individuals had tested negative for tuberculosis by all three diagnostics. No PCR positivity being the expectation for this group, the PCR results could be interpreted as 100% efficient (Table-2 & Fig.1).

![PCR profile of respiratory sample](image.png)

**Fig. 1:** PCR profile of respiratory sample from suspect at time of first reporting to the microscopy centre (Lane 2) and after six months (Lane 3). MW represents DNA molecular weight marker.
Table 2: Final diagnosis of tuberculosis patients

<table>
<thead>
<tr>
<th>Tuberculosis patients</th>
<th>Diagnostic review</th>
<th>Final response to ATT &amp;/or positive on X ray/microbiological data and clinical follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATT and/or of TB patients</td>
<td>Description</td>
<td>Number</td>
</tr>
<tr>
<td>Smear positive</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>Smear negative</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td>PCR positive</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>72</td>
<td>48 (67.6%)</td>
</tr>
</tbody>
</table>

Comparative efficiency of PCR to routine diagnostics like microscopy and culture

Table 3 presents the comparative efficiency of PCR to diagnostics like microscopy and culture. The sensitivity, specificity, positive and negative predictive values for each of the diagnostics was compared using the gold standards of smear microscopy, culture, and combined microbiological data along with chest radiographic findings and information on clinical follow up. Of the 144 samples, 48 were confirmed on the basis of culture and response to ATT, while 23 culture negative samples were confirmed on the basis of response to ATT, microbiological data and on clinical follow up. PCR was the most sensitive diagnostic with a sensitivity of 91.5% as against that of culture (68%) and microscopy (51%). However, its specificity was only 86% when compared to sputum microscopy (100%) and culture (97%) (Table-3). Comparison of PCR to conventional methods using McNemars test ($\chi^2 = 5.26$, df=1, P showed a significant difference.

Table 3: Comparative sensitivity, specificity, predictive value and efficiency of PCR to routine diagnostics.

<table>
<thead>
<tr>
<th>Diagnostic</th>
<th>Sensitivity(%)</th>
<th>Specificity(%)</th>
<th>Positive predictive value</th>
<th>Negative predictive value</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>51(36/71)</td>
<td>100(73/73)</td>
<td>100(36/36)</td>
<td>67.5(73/108)</td>
<td>76(109/144)</td>
</tr>
<tr>
<td>Culture</td>
<td>68(48/71)</td>
<td>97(71/73)</td>
<td>96(48/50)</td>
<td>75.5(71/94)</td>
<td>83(119/144)</td>
</tr>
<tr>
<td>PCR</td>
<td>91.5(65/71)</td>
<td>86(63/73)</td>
<td>87(65/75)</td>
<td>91(63/69)</td>
<td>89(128/144)</td>
</tr>
</tbody>
</table>

Report on utility of PCR amongst inappropriate respiratory samples

Saliva mixed sputum specimens constituted 31% (45/144) of all respiratory samples. Thirty-one of these were negative by all diagnostics while seven of them were positive by routine diagnostics. Of the latter, only one was positive by microscopy while the remaining 6 belonged to individuals diagnosed on the basis of radiological examination. Four of these seven samples were also positive by culture and five by PCR. In addition, seven saliva samples were positive by PCR. Thus microscopy, culture, radiological examination and PCR could diagnose one, four, six and eight cases respectively of those individuals whose respiratory samples were considered as inappropriate for microbiological processing.

RESULTS AND DISCUSSION

The need for an efficient tuberculosis diagnostic becomes evident from the fact that for every patient of tuberculosis who can be detected using microscopy, nine have to be screened using indirect methods due to the
low sensitivity of microscopy\textsuperscript{[17]}. This is the primary impetus for a world wide effort for developing new tools
to diagnose tuberculosis. The use of a molecular technique like PCR for the laboratory detection of
Mycobacteria in respiratory and other tissue samples from tuberculosis suspects has thus attracted enormous
attention. The present study demonstrates the utility and limitations of PCR. Among the total of 144 specimens
studied, the sensitivity of smear, culture and PCR was 51%, 68% and 91.5% respectively. Smear microscopy
was positive in only 67% of the culture positive samples. In comparison, PCR was positive in 98% and it could
detect 83% of the smear negative cases that were only radiologically positive. This aspect has great potential in
the laboratory diagnosis of tuberculosis, particularly in paucibacillary cases. However, its overall specificity
was only 86% when compared to smear and culture. PCR was negative in all negative controls and did not
show any cross reactivity with the two MOTT isolates, which indicate good specificity of the primers used.
This study has also indicated that PCR can be a useful tool in those who are not able to expectorate a proper
sputum sample. Out of 45 such samples, PCR was able to detect 12 positives while the routine diagnostic tests
were positive in only seven. Three of the additional seven cases detected by PCR were considered as true
positives by the clinicians. In one of the subjects who had persistent chest symptoms and whose sample was
available for PCR at the time of first presentation and on follow up after 6 months, a dramatic increase in bacillary load could be detected by PCR. 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. The
primers MPB64 used in this study proved to be specific and should hold promise for the future. However,
studies with larger numbers need to be taken up in order to validate these results. In this context, a recent study
examined the cost-effectiveness of polymerase chain reaction versus Ziehl-Neelsen smear microscopy for
diagnosis of tuberculosis in a high-burden, resource-starved environment. The study demonstrated that costs per
correctly diagnosed case were US dollar 41 and dollar 67 for smear microscopy and PCR, respectively. When
treatment costs were included, including treatment of culture-negative cases, PCR was found to be most cost-
effective at dollar 382 versus dollar 412\textsuperscript{[18]}.

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