Development of a TB-PCR Kit for the Diagnosis of Tuberculosis

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Abstract

More people die of tuberculosis (TB) in India than due to any other infectious disease. The early diagnosis of pulmonary TB (PTB) and extra-pulmonary TB (EPTB) is the most important step that will help in controlling the transmission of TB in the community. As the conventional techniques lack sensitivity, specificity or are too lengthy, it is important to develop a test which is rapid, sensitive and specific. A test based on polymerase chain reaction (PCR) fulfills all these criteria and hence a PCR test was developed at RMC for the rapid diagnosis of tuberculosis. After successful validation of the test, both in PTB and EPTB, and development of an efficient DNA extraction method, a diagnostic kit was developed in collaboration with JONAKI, BRIT. The kit, now launched in the market, will be useful in early diagnosis of TB.

Introduction

Tuberculosis caused by Mycobacterium tuberculosis (MTB), is a public-health problem of global importance. According to the World Health Organization, more than 8 million people develop TB each year. In the last decade, TB has reemerged as one of the leading causes of death, killing nearly 3 million people annually. India holds one-fifth of the global burden of TB with more than 350,000 deaths every year. The emergence of HIV infection and a rising prevalence of multi-drug resistant (MDR) tuberculosis have threatened the effectiveness of standard chemotherapy such as Directly Observed Treatment (DOTS). Though, most of the total TB population is contributed by PTB, almost 10-15% of total cases are EPTB.

An aspect of tuberculosis control that clearly needs improvement is the speed with which the diagnosis is confirmed. This is especially true with pulmonary tuberculosis, as these patients are responsible for the respiratory transmission of tuberculosis. Though the conventional method, AFB smear microscopy, has high positive predictive value, it lacks the sensitivity because of requirement of at least \(10^4\) bacilli in the spectrum sample for positivity. Culture technique is sensitive and is considered to be the Gold standard, but is labour intensive and takes 3-6 weeks because of extremely slow growth rate due to long generation time (12-18 hr) of mycobacteria. Tuberculin test is another widely used supportive diagnostic test that lacks specificity whereas serological test were found to be unsatisfactory both in sensitivity and specificity\(^1\).

The invention of Polymerase Chain Reaction (PCR), a Nobel Prize winning technology by Kary Mullis (1993) finally provided an exciting boost in the diagnosis of tuberculosis. As this technique offered amplification of small amount of DNA it was extensively evaluated for the detection of MTB from clinical samples\(^1\). A number of published reports have exhibited quite good sensitivities and specificities for the detection of MTB from sputum samples for the diagnosis of PTB.
Many of the PCR tests for *MTB* detection, described in the literature, are based on amplification of IS6110 sequences that is believed to be restricted to members of the *MTB* Complex. The presence of multiple copies of this element in the majority of *MTB* strains undoubtedly enhances the sensitivity of PCR. The discovery of occasional *MTB* strains lacking IS6110 in India however implies the possibility of a few false negative results in the test with IS6110-based PCR. To overcome these problems, a PCR test was developed in our laboratory targeting a house keeping gene of *MTB* for the 38 kDa protein, involved in phosphate transport.

![Flowchart](image)

Fig. 1: Development of TB PCR kit- Steps involved
The PCR conditions were optimized using standard MTB DNA. The analytical sensitivity and specificity of the test were established. Different DNA extraction protocols were standardized and compared for extraction efficiency. Standardized protocols were then used on clinical samples such as sputum from pulmonary TB cases and abdominal biopsies and Cerebro Special Fluids from patients with extra-pulmonary TB for validating the PCR test. After satisfactory validation, it was decided to the test market, in a kit form, jointly by RMC and JONAKI, BRIT. The flow chart in Fig. 1 illustrates various steps involved in development and commercialization of TB-PCR kit.

Methods

A) Standardization of PCR test

A primer pair KD1 and KD2 (KD1 5’ – CCAAGCAAGATCCCGAGGGCT – 3’, KD2 5’ – TTGATGATCGGGTAGCCGTCC – 3’), targeting 340bp segment of the 38kDa gene of MTB was designed and blast search was performed to confirm its specificity. PCR was standardized using the primer pair and DNA from a standard strain, MTB H37Rv, grown in laboratory. The analytical sensitivity of the PCR test was found to be 10fg which is equivalent to 3 bacilli (Fig. 2). The test was proved to be specific only to MTB complex strains and did not give amplification with various bacterial DNA as well as human DNA (Fig. 3).

B) PCR test validation for pulmonary as well as extra-pulmonary tuberculosis

The PCR test was validated for PTB (168 sputum samples from TB patients) as well as EPTB (50 abdominal biopsies from suspected abdominal TB cases and 60 CSF samples from TB meningitis cases). The DNA was extracted using phenol-chloroform method for sputum samples and abdominal biopsies whereas only proteinase K with boiling treatment was used for CSF samples. The test was always performed in duplicate and one of the samples is shown in Fig. 3.

Fig. 2: Sensitivity of KD1 and KD2 PCR test

Fig. 3: Specificity of KD1 and KD2 PCR test
the test samples was spiked with positive control DNA to look for the inhibition. Amplicons were visualized in ethidium bromide stained agarose gel. For CSF samples the sensitivity was further increased using Southern hybridization and ECL detection using biotinylated internal probe whereas dot-blot hybridization was performed for abdominal biopsies using radioactive internal probe.

Various procedures for extracting DNA from clinical samples were evaluated for their extraction efficiency and ability to remove inhibitors. QIAGEN® column extraction method with modifications was found to be the most satisfactory procedure. This extraction protocol was again validated for PTB (sputum samples, n=72). The promising results led to production of a silica column based indigenous TB-PCR prototype kit jointly by RMC and JONAKI, BRIT. The prototype kit was again validated using 110 sputum samples from PTB patients and 72 samples from healthy controls.

Results

Clinical Evaluation

Initial validation of the standardized PCR test performed with 168 sputum samples exhibited 77% positivity in smear positive samples and 10 % sample showed PCR inhibition. (Table 1a). Study and abdominal biopsies showed sensitivity and specificity of 77% and 68% respectively (Table 2a). The detection of the amplicons was very difficult due to presence of large amount of human DNA in PCR reaction; hence the results were confirmed after dot blot hybridization with radioactive internal probe wherever required. In the double-masked study of Tuberculous meningitis (TBM) patients, 73.3% sensitivity observed with ethidium bromide staining was increased to 90% (Table 2, b) by using Southern hybridization and Enhanced Chemiluminescent (ECL) with biotinylated internal probe.

**Evaluation of silica column based extraction procedure and prototype kit**

PCR test performed with DNA extracted from 72 sputum samples using QIAGEN® silica column extraction method, exhibited 87% positivity in culture positive samples with none showing PCR inhibition. Further, 40% clinically diagnosed TB patients also could be detected by the PCR test (Table 1b).

The prototype kit produced jointly by RMC and JONAKI, BRIT when evaluated using clinical samples from TB patients and non-TB controls, showed sensitivity of 84% and specificity of 97%. (Table 3). A small batch of kits was then produced and the kits were given to different hospitals for evaluation.

Table 1: Evaluation of PCR test for pulmonary tuberculosis targeting 38kDa protein gene using KD1 and KD2 primers

<table>
<thead>
<tr>
<th>Clinical samples</th>
<th>DNA Extraction Method</th>
<th>AFB/CultureStatus (Number)</th>
<th>PCR Pos (%)</th>
<th>PCR Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Sputum from TB patients (N=168)</td>
<td>Phenol-chloroform</td>
<td>Pos/Pos (N=36) Neg/Pos(N=50) Neg/Neg (Clinically diagnosed) (N=82)</td>
<td>28 (77%) 28 (56%) 19 (22%)</td>
<td>2(5.5%) 7(14%) 9(10.9%)</td>
</tr>
<tr>
<td>b) Sputum from TB patients (N=76)</td>
<td>QIAGEN silica column method</td>
<td>Pos/Pos (N=14) Neg/Pos(N=17) Neg/Neg (Clinically diagnosed) (N=45)</td>
<td>14 (100%) 13 (76%) 18 (40%)</td>
<td>(0%) (0%) (0%)</td>
</tr>
</tbody>
</table>
After a satisfactory feedback, a bulk production was carried out and a totally indigenous kit which contains both column based DNA extraction as well as PCR reagents was launched in the market in Aug 2009 by BRIT, BARC Fig. 4 a & b.

Discussion

TB-PCR fulfills all the criteria required for being an ideal diagnostic test, in comparison to existing tests for early detection of TB and, hence, justifies being the test of choice. PCR is theoretically capable of amplifying even a single copy of DNA. Further, all types of biological specimens such as sputum, blood, bronchoalveolar lavage, CSF; biopsies, pleural and ascitic fluid are amenable to PCR analysis and, hence, it has been proved to be the successful technique for identification of numerous pathogens in various biological specimens. Thus adaptation of PCR for detection of MTB in uncultured clinical specimens has revolutionized TB diagnosis.

The first target used in for PCR for diagnosis of TB, was a 65 kDa heat shock protein of MTB, but showed cross reactivity due to the conserved nature of this gene throughout all living organisms. Subsequently specific targets for PCR like the antigen 85 complex and IS6110 were evaluated, which

Table 2: Evaluation of PCR test for extra-pulmonary tuberculosis targeting 38kDa protein gene using KD1 and KD2 primers

<table>
<thead>
<tr>
<th>Clinical samples</th>
<th>DNA Extraction Method</th>
<th>Clinical diagnosis</th>
<th>PCR Pos (%)</th>
<th>SEN.</th>
<th>SPE</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laproscopic abdominal biopsies</td>
<td>Phenol- chloroform</td>
<td>HP pos (N=31) HPneg (N=19)</td>
<td>24(77%) 6(31.5%)</td>
<td>77%</td>
<td>68.5%</td>
<td>80%</td>
<td>73%</td>
</tr>
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Table 3: Validation of BRIT-RMC PCR kit for detection of M. tuberculosis

<table>
<thead>
<tr>
<th>Total No. Sputum samples From TB patients (N=110)</th>
<th>BRIT- RMC PCR kit-PCR positives *(%)</th>
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<tbody>
<tr>
<td>Smear +ve &amp; Culture +ve (N=64)</td>
<td>54 (84.3%)</td>
</tr>
<tr>
<td>Smear -ve &amp; Culture +ve (N=19)</td>
<td>10 (52.3%)</td>
</tr>
<tr>
<td>Smear -ve &amp; Culture -ve (N=37)</td>
<td>8 (21.6%)</td>
</tr>
<tr>
<td>Controls (Non-TB patients N=72)</td>
<td>3 (2.7%)</td>
</tr>
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</table>
illustrated good sensitivities and specificities. Our target also showed a very high sensitivity and specificity proving its usefulness. The elimination of inhibitors is one of the most difficult challenges in the diagnostic PCR test. Thus, DNA extraction procedure yielding pure and clean DNA from clinical specimens is the key to the success of PCR. Use of silica membrane in the kit was observed to have great efficacy for removing Taq polymerase inhibitors from blood, sputum and other biological material and hence, no inhibition of PCR and no false negativity was seen, Our studies also emphasized the fact that we may need to use appropriate extraction and amplicon detection methods for different clinical samples.

**Clinical evaluation**

**Pulmonary tuberculosis:** As per the recent meta-analysis by Greco et al. the diagnostic sensitivity and specificity of different PCR tests for smear positive respiratory samples varied among different laboratories and was in the range of 80-95%. Our prototype kit could detect 84% smear and culture positive cases. Additionally, our test could also detect 52% of smear negative but culture positive TB cases and 22% of smear and culture negative TB cases, which is considered to be a challenge in TB diagnosis.

**Tuberculous meningitis:** TBM is one of a common clinical manifestation of EPTB which can be fatal. Definitive diagnosis is not possible as smear and culture are rarely positive and in the absence of gold standards, a PCR test like ours with 90% sensitivity will be very useful in the early diagnosis of TBM and this will further help in decreasing the rate of mortality.

**Abdominal tuberculosis:** It is an important EPTB which is difficult to diagnose due to the diagnostic dilemma in histopathology. When AFB and culture are negative, PCR is the only reliable technique that can confirm the presence of *MTB* in the affected site and further help in ruling out malignancy. Ours is one of the very few studies that were done on fresh laparoscopic abdominal biopsies which showed reasonably good sensitivity and specificity.

RMC, BARC and JONAKI, BRIT have successfully developed an inhouse TB-PCR test for both pulmonary and extra-pulmonary tuberculosis and have made it commercially available after different levels of quality evaluation and validation.

**References**


