Comparison of real-time PCR and conventional PCR assay using IS6110 region of *Mycobacterium tuberculosis* for efficient diagnosis of tuberculous meningitis and pulmonary tuberculosis

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The present study was aimed at evaluating the role of a real-time polymerase chain reaction (RT-PCR) assay in the diagnosis of tuberculous meningitis (TBM) and pulmonary tuberculosis (PTB) and comparison of its performance with the in-house conventional PCR (C-PCR) assay. A RT-PCR assay using SYBR green methodology and C-PCR targeting a segment of the gene for mycobacterial IS6110 region were evaluated in 109 clinical samples consisting of 59 cerebrospinal fluid (CSF) samples and 50 sputum samples for diagnosis of tuberculosis (TB). When compared with the findings of clinical observations, the sensitivity and specificity of RT-PCR in detecting TBM was 87.5 and 88.5%, respectively and that of C-PCR assay was 79.1 and 88.5%, respectively in the same set of CSF samples. For detecting PTB, sensitivity and specificity of RT-PCR was 91.7 and 84.6% and C-PCR assays was 77 and 92.8%, respectively in sputum samples. The overall accuracy of the RT-PCR assay was higher compared with that of the C-PCR assay. Additionally, the RT-PCR has the advantage of a short experimental time, low risk of sample contamination, and offers the possibility to quantify bacterial load, making it a powerful tool for the rapid diagnosis of TBM and PTB.

**Keywords:** Conventional PCR, diagnosis, real-time PCR, tuberculosis

**Introduction**

Rapid diagnosis of tuberculous meningitis (TBM) is crucial in patients with impaired immune function, as early treatment may improve the clinical outcome. Considering the limitations of the conventional microbiological techniques during the last decade, a number of nucleic acid amplification methods have been developed for rapid detection and identification of *Mycobacterium tuberculosis* complex (*M. tuberculosis*) in clinical specimens¹-³. These techniques are attractive due to direct detection of few copies of genomic sequence of *M. tuberculosis* in clinical specimens. In our hospital, we are routinely using an endpoint in-house conventional polymerase chain reaction (C-PCR) for detection of *M. tuberculosis* in variety of clinical specimens, such as, cerebrospinal fluid (CSF), sputum and other body fluids for diagnosis of different forms of tuberculosis (TB)⁴-⁶. Although the PCR technique plays a substantial role in diagnosis but it possesses certain inherent technical limitations⁷. To overcome these limitations, in recent years, real-time methodology in PCR has been adopted. The real-time PCR (RT-PCR) assay allows for the rapid and sensitive detection of pathogens in a one-tube reaction without any further working steps. It has been reported that molecular diagnosis of pulmonary TB (PTB) can be improved and accelerated by performing RT-PCR protocols⁸⁹. However, there are few reports describing the utility of same in TBM¹⁰,¹¹. The objective of the present study was to evaluate the performance of a RT-PCR assay using a simple SYBR green based approach for the diagnosis of TBM. The performance of this RT-PCR assay was compared with the results of C-PCR assay in the same set of samples. A similar approach of comparative analysis between RT-PCR and C-PCR was performed for the analysis of sputum samples for detection of PTB to ascertain the usefulness of RT-PCR over end-point C-PCR assay for laboratory diagnosis of TBM and PTB.
Materials and Methods

Clinical Samples

A total of 109 specimens, including 59 cerebrospinal fluid (CSF) and 50 sputum specimens, were evaluated retrospectively for RT-PCR assay and C-PCR assay. CSF specimens were collected from patients admitted at Central India Institute of Medical Sciences (CIIMS), Nagpur and from patients attending TB ward of Government Medical College and Hospital, Nagpur between February 2009 and September 2010. Samples were collected before the initiation of treatment. The Institutional Ethics Committee of CIIMS, Nagpur approved the study and a written consent was obtained from all the patients.

Sample Grouping

CSF and sputum samples were categorized into TB and non-TB category based on their respective clinical criteria as follows:

**CSF Samples**

CSF (n=59) was obtained from 59 patients including 24 cases with TBM (24 clinically suspected with complete clinical findings) and 35 cases of non-TBM disorders. The age of the patients with TBM ranged from 6 to 73 yr, and there were 17 males and 7 females. CSF samples were obtained from almost all TBM patients before initiation of anti-TB treatment (ATT) and were stored at –20°C until they were tested. The Institutional Ethics Committee of CIIMS, Nagpur approved the study and a written consent was obtained from all the patients.

**Sputum Samples**

Sputum samples (n=50) were obtained from 50 patients, which included 31 cases of confirmed TB cases, 5 clinically suspected TB cases with complete clinical findings and 14 other non-TB cases. The age of the patients categorized under TB group ranged from 16 to 58 yr and there were 22 males and 14 females. Sputum samples were obtained from all the patients before initiation of ATT treatment and stored at –20°C until used. Samples were collected from all study groups for which patient’s consent was obtained. The selection of patients under PTB and non-PTB category was based on the criteria described below.

**TBM Group (=24)**

TBM diagnosis was based on clinical features including sub-acute or chronic fever and signs of meningeal irritation with or without other features of central nervous system (CNS) abnormality. CSF findings in these patients included increased protein levels, decreased glucose levels (CSF/blood glucose ratio, ≤0.5) and pleocytosis with lymphocyte predominance. Patients where an acid-fast bacillus (AFB) was demonstrated by smear and/or cultures were considered “confirmed” cases of TBM. In the remaining cases, evidence of TBM from either computed tomography or magnetic resonance imaging; high adenosine deaminase activity (ADA) values and response to ATT were used as the criteria for the “suspected/probable” cases of TBM diagnosis.

**Non-TBM Group (=35)**

**Non-TBM Infectious Group (=12):** These were determined by the presence of pathogenic organisms other than mycobacteria AFB in CSF through staining and/or culture and/or with the following observations: a) fever and/or signs of meningeal irritation in patients who have undergone cranial surgery to treat tumor(s), stroke or head injury, and who have already received antibiotics in pre- and post-operative regime, or high fever and/or signs of meningeal irritation with or without CNS manifestations, but who have not received broad-spectrum antibiotics; b) CSF findings showing increased proteins, decreased glucose (CSF to blood glucose ratio <0.2), and/or pleocytosis with a predominance of polymorphonuclear cells (in patients who have received broad spectrum antibiotics, CSF may resemble that of chronic meningitis patients); and c) good clinical response to broad-spectrum antibiotics.

**Non-TBM Non-Infectious Group (=23):** All other patients that lacked evidence of CNS bacterial or viral infections were grouped into the non-TBM infectious group.
Non-PTB Infectious Group (≈10): The diseases included bacterial pneumonia, chronic obstructive pulmonary disease, bronchial asthma, bronchiectasis, pneumoconiosis, and lung cancer.

Non-PTB Non-Infectious Group (≈4): This group included patients with normal chest radiographs, no history of ATT and absence of infection.

Microbiological Investigations
CSF sample of approx 3-4 mL was initially available. A total of 2 mL of CSF sample was used for routine biochemical, pathological and microbiological tests. Briefly, 2 mL of CSF was centrifuged, and a portion of the pellet was examined by Gram, India ink and Ziehl-Neelsen stains. The remaining portion of the pellet was cultured (on blood and chocolate agar) for bacteria and fungi on Löwenstein-Jensen medium (Becton Dickinson) and in liquid 7H9 media (Mycobacterium Growth Indicator Tubes, Becton Dickinson) for mycobacteria. CSF cultures were incubated at 37°C for 6 wk and examined weekly for growth. The supernatant was used in routine biochemical tests. As for sputum samples, these were first digested and decontaminated with N-acetyl cysteine-2.5% NaOH, concentrated by centrifugation and finally suspended in 2 mL of phosphate buffered saline. These were then processed similar to the protocol for CSF samples.

Phenol Chloroform Based DNA Extraction
Approx 1-1.5 mL of CSF or sputum sample was used to extract DNA. The DNA extracted was further used in the C-PCR and RT-PCR assays as previously described\(^6\). Briefly, 100 µL of pellet suspensions of CSF/sputum samples were subjected to cell lysis using detergents and then purified by phenol chloroform extraction. The resulting DNA was ethanol precipitated and dissolved in 50 µL of TE buffer.

C-PCR
Amplification of PCR products was carried out by using in-house protocol using a specific pair of primers designed to amplify an insertion sequence IS\(6110\) in the \textit{M. tuberculosis} complex having expected band size of 123-bp\(^7\). The sequences of forward and reverse primers were 5'-CCT GCG AGC GTA GGC GTC GG-3' and 5'-CTC GTC CAG CGC CGC TTC GG-3', respectively. A 50 µL reaction was set up containing 10× assay buffer (Applied Biosystem, Calif., USA), 10 mM dNTP's (Bangalore Genei, Bangalore, India), 12.5 pmole of each primer (Sigma-Genosys, USA), 1.25 units Amplitaq DNA polymerase (Applied Biosystem, Calif., USA) and 5 µL of extracted DNA from sputum or CSF samples. Amplification was carried out in a thermal cycler (Applied Biosystems, Foster City, Calif., USA), involving 40 cycles of denaturation at 94°C for 1 min, annealing of primers at 68°C for 1 min, and primer extension at 72°C for 10 min. The positive control included the DNA of \textit{H}\textsubscript{17}R\textsubscript{v} strain provided by Colorado State University, Fort Collins, USA, (Contract No 1-A1-40091). Amplified products were detected by electrophoresis on 2% agarose gel and visualized on a UV gel documentation system (Bio-Rad Laboratories, CA, USA).

RT-PCR
Amplification in RT-PCR assay was carried out by using the same primers as in C-PCR. RT-PCR was carried out in 10 µL reaction mixture consisting of 1 µM primers, 5 µL of SYBR green dye mix (Applied Biosystem, Foster City, Calif., USA) containing buffer, dNTPs, MgCl\(_2\) and hot start Taq polymerase. Reaction was performed in a StepOne RT-PCR system (Applied Biosystem, Foster city, Calif., USA) using an amplification programme of initial denaturation at 95°C and 40 PCR cycles of 95°C for 1 min and 68°C for 1 min, followed by melt curve stage of 95°C and 60°C. The results were analyzed and displayed by the software in terms of melt curve graphs of each sample. Positive control DNA was included in each run, which gave melt curve at 84°C. Similarly melt curve for each unknown samples were observed and samples showing peak at 84°C were considered as positive for presence of IS\(6110\) gene target. The PCR results obtained by both the assays were analyzed for assessing the advantage of RT-PCR over end-point C-PCR as a routine diagnostic test for sensitive, specific and rapid detection of \textit{M. tuberculosis} DNA in clinical samples. The preparation of master mix was carried out in a separate PCR/UV workstation. To prevent cross-contamination, different sets of pipettes and distinct work areas were used for DNA template preparation, PCR mixture preparation, DNA amplification and gel analysis. Moreover, one positive and one negative control were included with every set of samples, used during DNA extraction, RT-PCR and C-PCR.

Statistical Analysis
A concordance test was applied between the RT-PCR and C-PCR using kappa statistics and was expressed as a \(\kappa\)-value.
Results

The results depicting sensitivity and specificity of C-PCR and RT-PCR assay in TBM and non-TBM cases are shown in Fig. 1. RT-PCR was highly sensitive in detecting TBM cases (87.50%) and the sensitivity was comparatively higher compared to C-PCR (79.1%). However, specificity of the two tests was same (88.5%).

The level of concordance calculated between RT-PCR assay and C-PCR for TBM diagnosis by using kappa statistics is shown in Table 1. In TBM cases, RT-PCR assay was positive in 21 out of 24 cases. C-PCR was positive in 19 of those 21 cases that were positive for RT-PCR and thus overall missed the diagnosis for 2 cases. The agreement thus was found to be 91% (κ=0.703). In non-TBM group, RT-PCR assay was positive in 4 cases and all the four cases were positive in C-PCR assay. Out of 4 positive cases, 2 were in non-TBM infectious group and 2 in non-TBM non-infectious group. The agreement between two test for all these categories was found to be 100% (κ=1).

The evaluation of RT-PCR was also done in extra-TBM category and compared with the findings of C-PCR. Fig. 2 in this respect depicts the sensitivity and specificity of C-PCR and RT-PCR in PTB and non-PTB cases. RT-PCR was considerably more sensitive (91.6%) compared to C-PCR (77.7%). However, C-PCR was more specific (92.80%) compared to RT-PCR (84.61%).

Similar to the CNS category, concordance was also calculated between RT-PCR and C-PCR performances in PTB and non-PTB group. Out of 36 PTB cases, 33 were positive by RT-PCR assay (Table 2). C-PCR was overall positive in 29 cases and all these were positive by RT-PCR. The agreement between the two tests was found to be 88% (κ=0.547). RT-PCR thus detected 4 additional cases, 3 in confirmed PTB category and 1 in suspected PTB category. The concordance between the two test in confirmed PTB category and suspected PTB category was thus 90% (κ=0.527) and 80% (κ=0.41), respectively. In the non-PTB group, RT-PCR was positive in 3 out of 14 cases, while C-PCR was only positive in 1 case, yielding an concordance of

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>RT-PCR results</th>
<th>C-PCR positive</th>
<th>C-PCR negative</th>
<th>Concordance (κ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBM group (=24)</td>
<td>RT-PCR positive=21</td>
<td>19</td>
<td>2</td>
<td>91% (0.703)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=3</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Non-TBM group (=35)</td>
<td>RT-PCR positive=4</td>
<td>4</td>
<td>0</td>
<td>100% (1)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=31</td>
<td>0</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Non-TBM infectious group (=12)</td>
<td>RT-PCR positive=2</td>
<td>2</td>
<td>0</td>
<td>100% (1)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=10</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Non-TBM non-infectious group (=23)</td>
<td>RT-PCR positive=2</td>
<td>2</td>
<td>0</td>
<td>100% (1)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=21</td>
<td>0</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>
Table 2—Concordance between RT-PCR and C-PCR results for all categories of PTB and non-PTB patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>RT-PCR results</th>
<th>C-PCR positive</th>
<th>C-PCR negative</th>
<th>Concordance (κ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTB (=36)</td>
<td>RT-PCR positive=33</td>
<td>29</td>
<td>4</td>
<td>88% (0.547)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=3</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Confirmed PTB (=31)</td>
<td>RT-PCR positive=29</td>
<td>26</td>
<td>3</td>
<td>90% (0.527)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=2</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Suspected PTB (=05)</td>
<td>RT-PCR positive=4</td>
<td>3</td>
<td>1</td>
<td>80% (0.411)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Non-PTB (=14)</td>
<td>RT-PCR positive=3</td>
<td>1</td>
<td>2</td>
<td>85% (0.44)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=11</td>
<td>0</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Non-PTB infectious group (=10)</td>
<td>RT-PCR positive=3</td>
<td>1</td>
<td>2</td>
<td>80% (0.411)</td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=7</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Non-PTB non-infectious group (=04)</td>
<td>RT-PCR positive=0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RT-PCR negative=4</td>
<td>0</td>
<td>4</td>
<td>100% (1)</td>
</tr>
</tbody>
</table>

85% between the specificity of two tests (κ=0.44). The concordance was 100% in the non-PTB non-infectious group as there was no positive result with both the tests in this category (κ=0.1). However, in non-PTB infectious group, 3 cases were positive by RT-PCR assay, while only 1 was positive by C-PCR assay, yielding a concordance of 80% (κ=0.41).

Discussion

The biggest challenge in TBM diagnosis is lack of evidence of *M. tuberculosis* infection in CSF by microscopic examination of culture. Therapy is usually initiated based on clinical features of TBM\(^12-15\). Therefore, newer diagnostic techniques are needed that can rapidly aid in confirming the clinical suspicion of TBM. Over the years, several studies have evaluated PCR for the detection of *M. tuberculosis* DNA in CSF samples and have found it to be more sensitive compared to AFB smears and culture for the diagnosis of TBM\(^16,17\). However, these methods require many steps and take several hours to complete. To overcome these limitations, RT-PCR assays have given a significant breakthrough in PCR amplification and amplicon detection compared to conventional detection methods. The benefits of the RT-PCR assays in detecting PTB have also been reported earlier\(^18,19\). However, the evaluation of RT-PCR in diagnosis of TBM is limited. In the present study, a RT-PCR assay using SYBR green methodology has been evaluated for the specific identification of *M. tuberculosis* from CSF samples for TBM diagnosis.

RT-PCR assay provided sensitivity and specificity of 87.5 and 88.5%, respectively in providing clinical confirmation of TBM cases. These results when compared to those of C-PCR assay showed high concordance, which shows that molecular tests are well suited for clinical confirmation of TBM cases. RT-PCR assay also provided correct diagnosis for two additional cases of TBM, which were missed by C-PCR; although specificity was similar in either case. The results obtained in the present study are in good agreement with the previous studies performed in detecting *M. tuberculosis* in CSF samples. In one such study, Bhigjee *et al*\(^20\) observed ~16% improvement in sensitivity with RT-PCR compared to C-PCR assays. The reasons for better sensitivity of RT-PCR assay is that the fluorescent dyes used in the system are much more sensitive and can detect as little as a 2-fold change in DNA load, while agarose gel based system of C-PCR stained with EtBr or SYBR-Green dye can detect only 10-fold differences in DNA load\(^21\). It is thus very much possible that samples having less quantity of mycobacterial DNA may get detected by RT-PCR assay but could be missed by C-PCR assay. This aspect was very well addressed in one study performed by Haldar *et al* where a ~ 22% increase in sensitivity with RT-PCR vs C-PCR was found in ‘sediments’ where bacterial load was estimated to be low. However, their gel based assay and RT-PCR was equally sensitive in ‘filtrates’ where bacterial load was high\(^22\). This indicates that RT-PCR are more efficient in samples where bacterial load is low.

In the next step, RT-PCR assay as well as C-PCR assay was also performed in sputum samples to
confirm earlier findings of performance of RT-PCR assay in detecting PTB cases. As in CSF samples, both the test were sensitive and were in good agreement to each other. However, RT-PCR detected 4 additional cases in the PTB category (sensitivity 91.7%) than that of C-PCR assay (sensitivity 77%). These results thus ascertain that both RT-PCR and C-PCR assay can be efficient in both CSF and sputum samples for diagnosis of TBM and PTB but RT-PCR has an advantage of detecting cases that are missed by C-PCR. These results are thus contradictory to the earlier studies, where it is mentioned that RT-PCR performs better in respiratory specimens compared to in non-respiratory specimens. However, we obtained appreciable results of RT-PCR assay with CSF and sera samples of TBM and PTB cases, respectively.

The results in the present study show marginal increase in sensitivity of RT-PCR assay over C-PCR. However, the differences would be more appreciable if large number of samples were studied. It should also be noted that RT-PCR assay has additional advantage over C-PCR in terms of time duration, as it was able to provide results in less than 1 h (excluding time for DNA extraction) and additional steps, such as, gel analysis has not be done in RT-PCR assay. Further, an important area of concern is the specificity of the assay. Since the RT-PCR assay is a closed system in which the reaction tube is never opened after amplification, chances of false positives should be minimal. However, in our study, both RT-PCR and C-PCR assay were positive in 4 out of 35 CSF samples of non-TBM patients. In sputum samples, 3 out of 14 samples in the non-TBM infectious category were detected by RT-PCR assay, while only 1 was detected by C-PCR assay in the same category. Although to reduce the chances of contamination, we carried out necessary precautions for avoiding any discrepancies in the results, but technical limitations can be possible for erroneous results.

RT-PCR has distinct advantage of being a non-gel-based technique having automated data interpretation, which saves considerable time and labour. However, a major limitation of the RT-PCR is the initial capital investment for equipment, as well as the investment required for staff training and expertise, which may be beyond the means of many laboratories. Besides, there could be technical limitations when using non-specific dyes, such as, SYBR green. The sensitivity of detection may be compromised as SYBR green dye is a non-specific dye and can incorporate into any double stranded structures either it is primer dimmers or secondary structures. This factor could register a fluorescent signal and may lead to false positivity.

To overcome the limitations, other RT-PCR based methods, such as, TaqMan probe and Beacon detection based system offer additional level of specificity due to the hybridization of a PCR product to a specific probe. However, these assays are costly since real-time probes are expensive as compared with conventional oligonucleotides. It was also mentioned by Ponchel et al that even the 5'-nuclease assay using TaqMan probes can also be compromised by primer dimers and although not detected, but alter the amplification efficiency of the specific product.

In conclusion, the results of the present study demonstrate that the use of the SYBR green based RT-PCR assay for the detection of M. tuberculosis in CSF samples could detect additional cases, which are missed by C-PCR assay and is very important for management of patients.

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References