Rapid Diagnosis of *Mycobacterium tuberculosis* with Truenat MTB: A Near-Care Approach

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**Abstract**

**Background:** Control of the global Tuberculosis (TB) burden is hindered by the lack of a simple and effective diagnostic test that can be utilized in resource-limited settings.

**Methods:** We evaluated the performance of Truenat MTB™, a chip-based nucleic acid amplification test in the detection of *Mycobacterium tuberculosis* (MTB) in clinical sputum specimens from 226 patients with suspected pulmonary tuberculosis (TB). The test involved sputum processing using Trueprep-MAG™ (nanoparticle-based protocol run on a battery-operated device) and real-time PCR performed on the Truelab Uno™ analyzer (handheld, battery-operated thermal cycler). Specimens were also examined for presence of MTB using smear microscopy, liquid culture and an in-house nested PCR protocol. Results were assessed in comparison to a composite reference standard (CRS) consisting of smear and culture results, clinical treatment and follow-up, and radiology findings.

**Results:** Based on the CRS, 191 patients had “Clinical-TB” (Definite and Probable-TB). Of which 154 patients are already on treatment, and 37 were treatment naive cases. Remaining 35 were confirmed “Non-TB” cases which are treatment naive cases. The Truenat MTB test was found to have sensitivity and specificity of 91.1% (CI: 86.1–94.7) and 100% (CI: 90.0–100) respectively, in comparison to 90.58% (CI: 85.5–94.3) and 91.43% (CI: 76.9–98.2) respectively for the in-house nested PCR protocol.

**Conclusion:** This preliminary study shows that the Truenat MTB test allows detection of TB in approximately one hour and can be utilized in near-care settings to provide quick and accurate diagnosis.


**Editor:** Olivier Neyrolles, Institut de Pharmacologie et de Biologie Structurale, France

**Received** May 28, 2012; **Accepted** October 29, 2012; **Published** January 21, 2013

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**Funding:** Bigtec Laboratories, Bangalore, India, has funded the study but the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** Manjula Jagannath, Manoj Mulakkapurath Narayanan and Vinaya Ramanabhiraman are employees of bigtec Labs, the funder of this study. The Truenat MTB test is based on a proprietary primer set/TaqMan probe designed by bigtec Labs. Bigtec holds patents in various countries for the Truelab handheld PCR device (PCT Pub. No WO/2009/047804) and and microchip (PCT Pub. No WO/2009/047805). Authors Manjula Jagannath, Manoj Mulakkapurath Narayanan, Vinaya Ramanabhiraman, are involved in training and use of bigtec devices. There are no further patents, products in development or marketed products to declare. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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**Introduction**

Tuberculosis (TB) causes the highest number of deaths globally, attributable to a curable infectious agent [1], despite the availability of potent anti-TB medication. Reduction in TB-related morbidity and mortality is impeded by the lack of rapid and cost-effective diagnostic tests that are implementable in resource-limited settings. Over 95% of new TB cases and TB deaths occur in developing countries [2], where smear microscopy, which detects only 45% of TB infections [3], remains the most practical and often only test available.

Even where infrastructure exists, more sensitive tests are currently time- and cost-prohibitive. Performing a culture can take weeks because of the slow growth rate of TB bacilli. Molecular tests such as polymerase chain reaction (PCR), which are considerably faster than culture, often have a high turnaround time as specimens are often sent to distant laboratories. The expense involved in PCR testing makes it out of reach of most patients in TB-endemic countries. High risk of transmission of TB makes cost-effective and rapid detection crucial to control the spread of infection.

There has been considerable interest in the miniaturization of the PCR platform as this would confer advantages such as reduction in cost of instruments and tests, faster turnaround times and enhancement in the availability and accessibility of PCR tests in resource-poor geographies. With the combined advantages of affordability, simplicity in operations, diagnostic sensitivity and portability, micro-PCR devices are strong candidates for wide-scale use among the peripheral laboratories of India and other countries of South-East Asia which account for 50% of the global burden of MTB [4].

In this study, we evaluate a novel TB test, Truenat MTB (bigtec Labs, India). The test requires the user to add 5 µl of extracted
DNA to a pre-loaded microchip [5] containing room temperature stabilized reagents and start the PCR run on a handheld battery-operated device, Truelab Uno™, which is a fully portable stand-alone thermal cycler [6]. Briefly, the Truelab platform consists of a PDA (personal digital assistant) running the software application, a handheld unit housing the control electronics and optical detection system for real-time monitoring and a microchip with integrated temperature control elements. The Truenat MTB test involves sputum processing using a battery-operated sample preparation device, Trueprep-MAG™, which extracts nucleic acids by a simple menu driven process using a nanoparticle-based protocol optimized for sputum. The device integrates all operations (heating, fluid mixing, magnet control, step timing) using on a programmed micro-controller, and easy to follow screen instructions, thereby enabling nucleic acid isolation without the need for any additional equipment. The chip-based test has been designed to simplify the process of real-time PCR from ‘sample to result’ so that laboratories with minimal infrastructure can easily perform these tests routinely in their facilities and report PCR results in less than an hour.

Materials and Methods

Ethics

This study was approved by the Institutional Review Board of Hinduja hospital. Waiver of consent was obtained by Institutional Review Board, PD Hinduja Hospital and MRC., Mumbai, India. Waiver of consent was obtained as the study was carried out on left-over banked sediments identified by a laboratory generated number with no traceability to the patients. All patients’ details were thus kept confidential. The Truenat MTB results were not used in clinical decision making.

Settings

Sample collection, Smear Microscopy, MGIT culture and nested PCR was performed at Hinduja Hospital and Medical Research Centre, Mumbai. The Truenat MTB tests were performed by Hinduja staff at bigtec Laboratories, Bangalore.

Study population and specimens

This was a single site, blinded, cross-sectional study to determine the performance of the Truenat MTB in patients with symptoms of pulmonary TB in comparison to conventional methodologies. Sputum specimens were taken from patients presenting routinely to our hospital with suspected pulmonary TB. Standard diagnostic follow-up (smear, culture, and in-house nested PCR) was performed on all patients. Where available, left-over sputum specimens were tested using Truenat MTB. This study was approved by the Institutional Review Board of Hinduja hospital. (Fig. 1)

Methods

As described previously [7], direct and concentrated acid-fast bacillus (AFB) microscopy (Ziehl-Neelsen [ZN] staining) was performed, followed by sputum processing with 2% N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) and centrifugation. The re-suspended pellet was subjected to cultivation on liquid medium [MGIT [mycobacteria growth indicator tube]].

Digested and decontaminated (2% NALC-NaOH) sputum specimens that were culture negative for mycobacterium and confirmed “Non-TB cases” were pooled for use as a negative control. A suspension of *M. tuberculosis* H37RV was prepared in sterile saline and adjusted to the density of a 1.0 McFarland standard. The suspension was diluted 1:10 in saline and used to spike the pooled above mentioned negative control and used as a positive control. Spiked specimens were stored at -70°C until further processing.
Patient categories

A composite reference standard (CRS) was used to categorise patients. Patients were allocated into the following groups based on a combination of smear status, culture results, clinical treatment and follow-up, and radiology findings. As per routine hospital protocol, follow-up was performed on all patients based on their culture results which were available 4–6 weeks after inoculation.

**Definite-TB.** Smear positive and Culture positive (S+C+) or smear negative, culture positive (S−C+) or smear positive, culture negative (S+C−).

**Probable-TB.** Smear positive and Culture positive (S+C+) or smear negative, culture negative (S−C−) but clinical-radiological picture highly indicative of TB. Patients showing a response to anti-TB treatment at follow-up were assigned to this group.

**Non-TB.** Smear negative culture negative (S−C−). Patient was assessed as ‘clinically negative’ when the patient had no previous history of TB infection and no microbiological evidence indicating current infection. Patients were symptomatic (weight loss and prolonged cough) but showed improvement without anti-TB treatment.

**Nested PCR**

1. **Nucleic acid extraction.** Untreated sputum specimens were processed using the QIAamp DNA mini kit (Qiagen) with the following modifications: 1) 30 mg/ml lysozyme (Amresco) was added to the sputum specimen which was then kept at 37°C overnight to ensure enzymatic digestion. 2) This was followed by addition of 250 µl lysis buffer (AL buffer from the QIAamp kit) and 25 µl Proteinase K and incubation at 56°C for 2 hours. DNA was then extracted as per manufacturer’s recommendations in a total volume of 100 µl.

2. **PCR.** The multi-copy IS6110 sequence was selected as the target region for the nested PCR test (Table 1). The outer primers, INSF and INSR, were used to amplify a 245 bp fragment. IS1 and IS2 were used as inner primers for the nested PCR, yielding a 123 bp fragment. Primers were purchased from Sigma. The in-house nested PCR was performed as described previously [8]. Briefly, 20 µl of extracted DNA was added to 80 µl of mastermix to bring the total reaction volume to a 100 µl. PCR was run on the Eppendorf Mastercycler gradient. Amplicons was detected by gel electrophoresis using a 3% agarose gel. An internal amplification control, β-globin, was used to detect PCR inhibition in extracts.

### Table 1. Primers used for nested PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Region</th>
<th>Product size</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward:5’CGTGAGGCCATCGAGGTGGC3’</td>
<td>INS</td>
<td>245 bp</td>
<td>631–650</td>
</tr>
<tr>
<td>Reverse:5’GCATCGAGGTGGC3’</td>
<td></td>
<td>856–875</td>
<td></td>
</tr>
<tr>
<td>Forward:5’CTCGTCCAGCCGCTGGAGG3’</td>
<td>IS</td>
<td>123 bp</td>
<td>762–781</td>
</tr>
<tr>
<td>Reverse:5’CTCGTCCAGCCGCTGGAGG3’</td>
<td></td>
<td>865–884</td>
<td></td>
</tr>
<tr>
<td>Forward:5’TGAACGTGGATGAGTGTTGGTG3’</td>
<td>β-globin</td>
<td>291 bp</td>
<td>-</td>
</tr>
<tr>
<td>Reverse:5’ACGTCTGCCATGAGCCCTCCT3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Sample loading on Trueprep-MAG device. doi:10.1371/journal.pone.0051121.g002

Figure 3. Addition of 5 µl of DNA to Truenat MTB chip. doi:10.1371/journal.pone.0051121.g003
case of inhibition the PCR, was repeated after dilution of DNA in a 1:1 ratio with sterile water.

**Truenat MTB test**

1. **DNA extraction using Trueprep-MAG protocol.** Untreated sputum specimens were liquefied with 500 µl of liquefaction buffer. The liquefied sputum was centrifuged at 14000 rpm for 5 minutes. After discarding supernatant, sputum pellets were re-suspended with 500 µl of lysis buffer and the suspension was incubated at 90°C for 10 minutes in the extraction tube (Fig. 2).

After the lysis step, 500 µl of Binding Reagent A and 100 µl of Binding Reagent B were added to the extraction tube. The bound DNA was washed once with 1 ml of Wash Buffer A and 5 times with 1 ml of Wash Buffer B. 100 µl of elution buffer was added to the tube followed by incubation at 90°C for 5 minutes. Elute was used directly in PCR reactions.

2. **Real-time PCR on ABI 7500.** PCR reactions were run using the DNA extracted using the Trueprep-MAG protocol. 4 µl of extracted DNA was mixed with 6 µl of the Truenat MTB mastermix and real-time PCR was performed on ABI 7500 (Applied Biosystems) under the following cycling conditions: 1 min at 95°C and 45 cycles of 10 s at 95°C and 34 s at 58°C.

3. **Real-time PCR on chip.** 5 µl of DNA extracted added to the Truenat MTB microchip (Fig. 3) and the real-time PCR was done using a pre-programmed profile on the device. Results were observed on the screen and compared to the results obtained on the ABI 7500 using the same mastermix.

**Buffers, reagents and mastermixes.** All buffers and reagents used for nucleic acid extraction and all mastermixes used for PCR are proprietary components of the Truenat MTB kit.

**Table 2. Performance of PCR tests in various patient groups.**

<table>
<thead>
<tr>
<th></th>
<th>Smear</th>
<th>Culture</th>
<th>$S+C+$ (n = 112)</th>
<th>$S−C−$ (n = 77)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Truenat MTB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>119</td>
<td>55</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td>−</td>
<td>1</td>
<td>51</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td><strong>In-house nested PCR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>117</td>
<td>59</td>
<td>41</td>
<td>35</td>
</tr>
<tr>
<td>−</td>
<td>3</td>
<td>47</td>
<td>44</td>
<td>42</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0051121.t002
**Table 3.** Comparison of Truenat MTB results with in-house nested PCR results.

<table>
<thead>
<tr>
<th>Truenat MTB</th>
<th>Nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>160</td>
</tr>
<tr>
<td>−</td>
<td>16</td>
</tr>
</tbody>
</table>

Sensitivity: 99.12% (111/112), Specificity: 100% (110/110), Positive Predictive Value: 100%, Negative Predictive Value: 100%

**Statistical Analysis**

Evaluation of the Truenat MTB test was performed in comparison to the other molecular methods for detection of *Mycobacterium tuberculosis* DNA from sputum, following the STARD recommendations [9]. Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value, Positive Likelihood Ratio, Negative Likelihood Ratio were calculated by using Bayesian sensitivity/specificity calculator and ROC curve and forest plot were calculated using Meta disc (version 1.4).

**Results**

As shown in fig. 4, outcome of study out of total 230 specimens screened, 4 were detected as nontuberculous mycobacteria (NTM) by phenotypic MGIT and hence were excluded from this study. Of the remaining 226 sputum specimens, 141 were MTB culture positive (+) and 85 were culture negative (−). Out of 141 (+), 104 patients were on antitubercle treatment and 37 were treatment naive, out of 85 (−), 50 patients were on treatment and 35 were treatment naive. A total of 112 specimens were smear positive (+).

Among the (+) specimens and 104 (93.33%) were (+) and 8 (+) specimens failed to grow in culture medium. Of the 8 (+), 3 had a smear status of 3+, 2 were 2+, 2 were 1+ and 1 had occasional AFB (acid fast bacilli). These (+) specimens were CRS+ and therefore the inhibition rate for liquid culture among (+) specimens was 6.67% (8/120). As seen in Table 2, of the 120 (+), 117 (97.50%) were detected as positive by the IS6110 nested PCR, and 119 (99.16%) were detected as positive by the Truenat MTB test. One sample with smear status 3+ showed inhibition and was MTB negative by all molecular methods. Of 141 (+), specimens, 135 (95.74%) were detected as positive by the IS6110 nested PCR, and 132 (93.62%) were detected as positive by the Truenat MTB test.

Among the (+) specimens, both the in-house nested PCR and Truenat MTB detected 99.12% (111/112) of specimens accurately.

Among the S− specimens, 75.86% (22/29) were Truenat MTB positive and 82.76% (24/29) were positive by the IS6110 nested PCR protocol.

The Truenat MTB results were largely concordant with the in-house nested PCR results, 196 of 226 specimens showed the same result by either PCR test (Table 3). Of the 30 discordant results, 16 specimens were MTB positive by nested PCR but not by Truenat. Of this group, 3 specimens were CRS+ and treatment naive but consequently false positives. On the other hand, 14 of the 30 were MTB positive by Truenat but not nested by PCR. Of this group, all 14 were CRS+ and on antitubercle treatment indicating no false positives.

Performance estimates of all tests using the CRS as a reference standard are presented in Table 4. As can be seen, the PCR tests have higher sensitivity than smear and culture tests. The IS6110 nested PCR protocol had a PCR inhibition rate of 8.4% (19/226) whereas the PCR reaction had to be repeated after the DNA was diluted as 1:1 with sterile water.

Liquid culture had an average time to positivity (TTP) of 25 days, in-house nested PCR had a TTP of 7 days (additional 7 days if PCR was inhibited) and the Truenat MTB test had a TTP of approximately 1 hour.

**Discussion**

Appliance of molecular methods in routine for diagnosing in developing countries like India depends on diverse factors like high cost, rapid and accessibility of skilled personnel to perform the test. Although economical and extensively available, microscopy was non-specific in our study population and less sensitive than Nucleic Acid Amplification Test (NAAT) and thus not satisfactory for rapid diagnosis.

In present study, the Truenat MTB test was found to have sensitivity of 91.1% (CI: 91.1–94.7) and in-house nested PCR have sensitivity of 90.5% (CI: 85.5–94.3). NAAT demonstrated excellent specificities in our study as shown in forest plot and ROC curve (Fig. 5 and Fig. 6) against smear, culture.

**Positivity of culture vs PCR**

Pulmonary TB constitutes the major form of TB in clinical practice. In this study, we evaluate the Truenat MTB test for detection of pulmonary TB in near-care settings. As can be seen in Table 3, based on CRS criteria, PCR tests had higher sensitivity compared to smear microscopy and culture. In this study, 50 CRS positive TB cases were culture negative. This could be attributed to unequal distribution of mycobacteria in paucibacillary respiratory specimens. Clumping of micro-organisms is a common

**Table 4.** Comparison of all methods against CRS as reference standard.

<table>
<thead>
<tr>
<th>(N = 226)</th>
<th>Smear (%)</th>
<th>Culture (%)</th>
<th>Nested (%)</th>
<th>Truenat (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pos</strong></td>
<td><strong>Neg</strong></td>
<td><strong>Pos</strong></td>
<td><strong>Neg</strong></td>
<td><strong>Pos</strong></td>
</tr>
<tr>
<td>Test Pos</td>
<td>120</td>
<td>71</td>
<td>141</td>
<td>50</td>
</tr>
<tr>
<td>Test Neg</td>
<td>0</td>
<td>35</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>63</td>
<td>74</td>
<td>91</td>
<td>91</td>
</tr>
<tr>
<td>Specificity</td>
<td>100</td>
<td>100</td>
<td>91</td>
<td>100</td>
</tr>
<tr>
<td>PPV</td>
<td>100</td>
<td>98</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NPV</td>
<td>33</td>
<td>64</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** Comparison of Truenat MTB results with in-house nested PCR results.

<table>
<thead>
<tr>
<th>Truenat MTB</th>
<th>+</th>
<th>−</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>160</td>
<td>14</td>
</tr>
<tr>
<td>−</td>
<td>16</td>
<td>36</td>
</tr>
</tbody>
</table>
problem with mycobacteria, therefore, uniform dispersion in clinical specimens is difficult to obtain [10,11]. Additionally, during the decontamination procedure, errors such as (i) inappropriate specimen dilution, (ii) accidental aspiration of the pellet when removing supernatant, and (iii) cross-contamination could have resulted in false negative results. Furthermore, the decontamination procedure could have caused a large reduction (80%) of colony forming units (CFU) recovered in cultures [12]. This may have increased the likelihood of culture-negative results among CRS positive specimens.

Many of the culture-negative CRS positive, and on effective treatment cases were accurately identified by the PCR techniques. Discrepancies between conventional mycobacterial culture and PCR-based detection have been previously associated with TB patients who undergo anti-tubercular chemotherapies [13,14]. PCR can detect DNA from non-viable MTB as a result of anti-tubercular treatment and also viable MTB present in paucibacillary specimens, whereas culture detects viable bacilli.

The load of bacilli require to obtain a positive culture is $10^5$ viable bacilli, and lower detection limit of conventional PCR is 10 copies and for real-time PCR it is 6 copies. However as our laboratory is a tertiary care laboratory with a referral bias towards non-responders, most of the patients, 154 out of 226 patients were on anti tubercle treatment. Therefore in some instances the bacilli may not be viable. Hence these bacilli may not grow in culture but their DNA could be detected using PCR.

Various nucleic acid amplification tests (NAAT) have been developed for detection of MTB in sputum [15]. In general, different NAAT tests have been found to have positivity in 95–100% of smear and culture positive specimens where as the positivity ranges from 40–60% in smear negative paucibacillary pulmonary disease [15–25]. The positivity of the PCR methods evaluated in this study (~99% positivity in S+C+ specimens and >75% in S−C+ specimens for the in-house nested PCR and Truenat MTB) was in accordance with previously observed values. All of the NAAT studied here have analytical sensitivities for
nucleic acid detection quantitatively equivalent to 1–10 mycobacteria [26–31].

In terms of sensitivity, assays that detect insertion sequence IS6110 for molecular diagnosis of MTB, such as the one described here, benefit from the fact that IS6110 often presents in high copy numbers.

**Time to positivity (TTP) and ease-of-use**

Though culture is inexpensive, the high TTP is an important barrier to rapid detection. In-house PCR protocols such as the IS6110 nested PCR protocol described here, though sensitive, suffer from false positive results due to inherent pitfalls associated with PCR techniques that require post-amplification analysis (carryover contamination between specimens, reagent contamination due to aerosol-based transmission of amplicon). The TTP can be quite long as specimens need to be batched to increase cost-effectiveness. Additionally, PCR inhibition rates can be high (here 8.4%), increasing the TTP by a few days, at the same time increasing the overall cost of PCR-based diagnosis. The Truenat MTB test evaluated in this study had a TTP of approximately one hour, enabling rapid detection of MTB DNA. The optimized spumt processing protocol ensured that PCR inhibitors were removed from the isolated DNA.

**References**