Comparison of an in-house multiplex PCR with two commercial immuno-chromatographic tests for rapid identification and differentiation of MTB from NTM isolates

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ABSTRACT

Background: Species specific diagnosis of mycobacterial infection is crucial because treatment of infections caused by Mycobacterium tuberculosis (MTB) differs from that of non-tuberculous mycobacterial (NTM) species. The species identification used to be cumbersome and non-reproducible a decade ago.

Objectives: Recently, some commercial tests have been made available to differentiate the MTB and NTM growths in culture media. Sensitivity and specificity of these tests was evaluated.

Materials and methods: In this double blind study 572 clinical samples were cultured in an automated BACTEC-MGIT-960 system. A total of 147 (25.7%) samples were MGIT culture positive. These cultures were subjected to an in-house m-PCR (which amplifies hsp-65, esat-6 and ITS region for MAC), two commercial immune-chromatographic tests (ICTs) and phenotypic tests.

Results: Of the 147 MGIT positive cultures, m-PCR was able to correctly identify MTB in 123 cultures and NTM in 24 which included 3 MAC isolates. m-PCR showed 100% agreement with two gold standard methods—the nitrate reductase assay and PNB tests—in correctly identifying MTB. Commercial strips were able to correctly identify MTB in 120 (97.5%) of 123 cultures, while 3 (2.5%) isolates were falsely identified as NTM. However, none of the growth negative spent medium gave false positive results in any of the tests. None of the commercial strips misidentified any of the 24 NTM as MTB; hence, specificity of these strips was 100%. Of the 2 IC test systems, both SD Bioline and BD TBc strip tests missed 2.5% of MTB isolates and misidentified these as NTM.

Conclusion: The in-house m-PCR was found to be the most accurate and efficient tool for identifying the MTB, MAC and other NTMs.
Introduction

Early detection of tuberculosis (TB) is one of the key recommendations of the World Health Organisation Stop-TB strategy [1]. TB is mainly caused by Mycobacterium tuberculosis (MTB), but non-tuberculous Mycobacteria (NTM) can also cause similar manifestations, under certain conditions, such as if the host is immuno-compromised or if exposed to a very high dose of NTM species. More than 45 mycobacterial species are reported to be pathogenic or opportunistic pathogens to humans [2]. Despite similar clinical manifestations, the therapeutic regimen and course of treatment differ significantly for MTB and NTM. Therefore, accurate and timely species-specific diagnosis of mycobacterial infections becomes of paramount importance.

However, before identification of the species, it is important that it grows in vitro for further phenotypic tests. The conventional Lowenstein-Jensen (L-J) medium takes up to 60 days to grow MTB to a detectable level. Hence, in 2007, the World Health Organisation (WHO) recommended the use of an automated liquid culture system, i.e., BACTEC® MGIT-960 (Mycobacterium Growth Indicator Tube 960 system) for rapid detection and drug susceptibility tests as a standard method for TB diagnosis [3]. However, the MGIT system does not provide information about the species grown. Hence, MGIT flashed positive cultures need to be further confirmed by Ziehl-Neelsen (Z-N) stained smear examination, whether the growth is mycobacterium or some contaminating bacterial growth [4], and then subculture is required on solid medium for colony characteristics. Mycobacterial species of the growth is confirmed by biochemical or molecular tests [5]. The differentiation of MTB from NTM can be achieved by conventional methods (growth rate, pigmentation of colonies and biochemical features), selective inhibition tests, such as para-nitrobenzoic acid (PNA)/p-nitro-a-acetylaminobeta-hydroxypropion-phenone (NAP) and other commercial systems, recently introduced in the market. Although conventional biochemical methods are time-consuming, often requiring the use of hazardous/carcinogenic chemicals (e.g. niacin test), and are prone to subjective errors in the interpretation of results, these are considered the standard methods. Currently, rapid immunochromatographic tests (ICTs) have been developed that are based on a predominant protein MPT-64 secreted by M. tuberculosis, Mycobacterium africanum, and some strains of Mycobacterium bovis [6–9]. ICTs provide quick results and hence WHO recommended use of ICTs for species identification in positive cultures [3].

In recent years, molecular techniques have made deep inroads in TB diagnosis as they are highly sensitive, specific and can be applied directly on different types of samples as well as on grown cultures. Polymerase chain reaction (PCR)-based methods have also been used for rapid detection and differentiation of MTB and NTM [10]. However, multiplex PCRs are bound to miss the co-infections and solo infections with NTM. Therefore, in this study we used the standardized in-house multiplex PCR (m-PCR) for the rapid detection and differentiation of the M. tuberculosis, Mycobacterium avium complex (MAC) and other non-tubercular mycobacterial species in a single reaction [2,11,12].

In the present study, the efficacy of in-house m-PCR was evaluated and compared with two commercial ICTs: SD Bio-line TB Ag MPT64 Rapid™ and BD MGIT Tbc™ identification kits for their capabilities in differentiating MTB from NTM in MGIT cultures.

Materials and methods

Data collected between January 2010 and December 2011 were analyzed retrospectively. All the samples which were submitted to the laboratory for mycobacterial culture and drug susceptibility testing as TB routine patient care service were included in the study. Hence, no ethical clearance was required for this study. However, all the study participants gave informed consent, as a standard diagnostic procedure of the laboratory, which is accredited by the central TB division of the Government of India and certified by the Stop-TB/WHO for non-commercial rapid culture and drug susceptibility tests [13].

Clinical specimens

In this study, a total of 572 clinical samples received from patients with suspected TB were cultured in an automated MGIT960 culture system. Of these, 125 were pulmonary specimens and 447 were extra-pulmonary specimens, which comprised of 123 CSF, 113 gastric aspirates, 49 pus, 55 pleural fluids, 30 peritoneal fluids, 27 tissue, 14 lymph node aspirates, 18 synovial fluids, 4 pericardial fluids and 14 ascitic fluids.

MGIT culture

Pulmonary and contaminated clinical specimens were decontaminated by using NALC–NaOH method in a biosafety cabinet, as described earlier [11]. The decontaminated specimens (no decontamination of clean specimens) were inoculated in the MGIT culture containing 800 µl of OADC and PANTA supplement as per the manufacturer’s instructions (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA). The inoculated MGI tube was loaded in the automated BACTEC™ MGIT-960 system and the growth was continuously monitored by BD Epi-center®. From the flashed positive culture tubes, Z-N smear examination and ICTs were performed on the next working day. For this, 200 µl of suspension from MGIT culture was inoculated onto L-J medium for phenotypic testing, and a 500 µl suspension was aliquoted out for DNA extraction. All procedures were carried in a biosafety-level 2B cabinet.

DNA extraction from MGIT cultures

DNA isolation from the MGIT cultures was done as described elsewhere [11,12,14]. Briefly, the culture pellets from the 0.5 ml culture suspensions were lysed with lysozyme followed by proteinase-K-SDS treatment. Proteins and macromolecules were precipitated using NaCl and hexadecyltrimethylammonium bromide solutions. Nucleic acids were recovered from the aqueous phase after extraction with chloroform and isomyl alcohol followed by precipitation with 70% ice cold ethanol. The resulting DNA pellets were solubilised in 1X Tris-EDTA buffer and 5 µl were used for the multiplex PCR.
Multiplex PCR (m-PCR)

The in-house m-PCR was performed according to the patented protocol [15] as published previously [11,12]. This m-PCR targets hsp-65 (genus specific), esat-6 (MTB specific) and internal transcribed spacer (ITS) MAC region (MAC specific). The sequences of primers were:

- hsp-65 F- ACCAAGCAGTGGTGTTCCCAT and hsp-65 R- CTTTGCAGGCGCATCCCT;
- esat-6 F- GCGGATTCCATGACAGGAGCAGTTGGA and esat-6 R- CCAAGCTCTTATGCGACATCCGACTGAGC;
- ITS F- CCGTGA GACAACACTCGGTC and ITS R- ATTACA CATTTCGATGAACGC.

PCR reaction was set up for 25 μl final volume and was run in a PTC-100 thermal cycler (MJ Research, USA) at the amplifying conditions of initial denaturation at 95 °C for 10 min and 30 cycles of 95 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min and a final extension of 72 °C for 10 min. Amplified products were resolved through 2% agarose gel in Tris–acetate buffer. Target bands of 441 bp for the genus specific, 320 bp for the MTB specific and 144 bp for the genus specific target are amplified but not the MTB or MAC specific, it indicates other NTM than MAC (Fig. 1). The m-PCR was performed according to the patented protocol [15] as published previously [11,12]. This m-PCR target is amplified but not the MTB or MAC specific, it indicates other NTM than MAC (MAC specific). The sequences of primers were:

- hsp-65 F- ACCAAGCAGTGGTGTTCCCAT and hsp-65 R- CTTTGCAGGCGCATCCCT;
- esat-6 F- GCGGATTCCATGACAGGAGCAGTTGGA and esat-6 R- CCAAGCTCTTATGCGACATCCGACTGAGC;
- ITS F- CCGTGA GACAACACTCGGTC and ITS R- ATTACA CATTTCGATGAACGC.

Nitratereductase assay

Nitratereductase assay (NRA) was performed as a reference test to confirm if the growth was MTB or not [19]. Briefly, 2 loopful colonies of 21–28 days old culture were emulsified in 0.2 ml of distilled water in the screw-cap tube. Two milliliters of 0.01 M sodium nitrate substrate buffer was added into the tube and placed in the water-bath at 37 °C for 2 h. After that, the tubes were removed from the water bath and one drop of 50% hydrochloric acid solution, two drops of 0.2% sulfanilamide solution and two drops of 0.1% N-naphthyl ethylenediamine solution were added. The results were examined immediately by reading the color visually by comparing with the prescribed WHO color standards [20]. Only dark pink color readings between 3+ and 5+ were considered positive results for M. tuberculosis. In order to confirm the negative results, a small amount of zinc powder was added to each negative tube that should not develop any color to confirm the test result as being truly negative.

Para-nitrobenzoic acid test (PNB)

All the culture isolates that showed negative results by both ICTs and identified as Mycobacterium sp by PCR were further subjected to the PNB test. The PNB was incorporated into the L-J medium at a final concentration of 500 μg/ml which inhibits the growth of MTB [21]. Medium without any inhibitory substance was considered as growth control. Five microliters of McFarland 1 inoculum suspension was inoculated onto the PNB and plain media and incubated at 37 °C for 28 days.

Statistical analysis

The m-PCR results were analyzed and compared with ICT, NRA and PNB assay as standard. The data were statically analyzed to calculate the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV).

![Fig. 1 – Multiplex PCR amplifications of genus-specific 441 bp (hsp-65), MTB-specific 320 bp (esat-6) and M. avium complex-specific (MAQ) 144 bp (ITS region) targets. Lane, M-100 bp marker; Lanes, 1 &2-M. tuberculosis; Lane, 3-NTM; Lane, 4-M. avium complex; Lanes, 5,6-negative control; Lane 7- M. tuberculosis control; Lane, 8- M. avium control and Lane, 9-negative control.](image-url)
Results

Out of 572 specimens, 147 were MGIT cultures flashed positive and 425 were MGIT culture negative for *Mycobacterium* sp (Fig. 2). All flashed positive and negative cultures were checked for acid fast bacilli by Z-N stained smear examination (X1000) as per standard criteria. Of the 147 MGIT culture positive growths, 52 mycobacterial cultures were from pulmonary specimens and 95 from extra-pulmonary specimens as shown in Table 1. From 425 flashed negative (spent) cultures (after 42 days of incubation), at least 5 samples were also selected randomly on the day of experimentation to check the specificity of each test.

Multiplex-PCR was tested on 238 MGIT cultures, of which 147 were MGIT culture positive. Out of 147 positive cultures, m-PCR detected all (100%) as mycobacterial growth: 123 (83.67%) cultures as MTB, 21 (14.28%) other NTM, and 3 (2%) as MAC. All 91 MGIT negative cultures were negative by m-PCR (Fig. 1, Table 1). In the strip test, all results were valid as indicated by the presence of a control band (C band). No significant difference was observed in band intensity in two strip tests. Out of 147 MGIT positive cultures, 120

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**Table 1 – Evaluation of in-house multiplex PCR and two strip test assays on *Mycobacterium* isolates isolated from pulmonary and extra-pulmonary samples.**

<table>
<thead>
<tr>
<th>MGIT culture positive</th>
<th>m-PCR (147)</th>
<th>Strip test (147)</th>
<th>NRA (147)</th>
<th>PNB test (27)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTB NTM MAC</td>
<td>SD Bioline BD</td>
<td>Pos Neg</td>
<td>Pos Neg</td>
</tr>
<tr>
<td>Sputum (52/125)</td>
<td>45 6 1</td>
<td>45 45 7</td>
<td>45 7</td>
<td>7 0</td>
</tr>
<tr>
<td>CSF (34/123)</td>
<td>33 1 0</td>
<td>33 33 1</td>
<td>33 1</td>
<td>1 0</td>
</tr>
<tr>
<td>GA (18/113)</td>
<td>12 6 0</td>
<td>11 11 7</td>
<td>12 6 1</td>
<td>6 1</td>
</tr>
<tr>
<td>Pus (17/49)</td>
<td>14 3 0</td>
<td>14 14 3</td>
<td>14 3 3</td>
<td>0 0</td>
</tr>
<tr>
<td>Tissue (4/27)</td>
<td>2 1 1</td>
<td>1 1 3</td>
<td>2 2 1</td>
<td>2 1</td>
</tr>
<tr>
<td>Pleural fluid (10/55)</td>
<td>7 3 0</td>
<td>7 7 3</td>
<td>7 3 3</td>
<td>0 0</td>
</tr>
<tr>
<td>Peritoneal fluid (5/30)</td>
<td>0 0 5</td>
<td>5 5 0</td>
<td>5 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Lymph node (4/14)</td>
<td>3 0 1</td>
<td>2 2 2</td>
<td>3 1 1</td>
<td>1 1</td>
</tr>
<tr>
<td>Pericardial fluid</td>
<td>(1/4)</td>
<td>0 1 0</td>
<td>0 1 1</td>
<td>0 0</td>
</tr>
<tr>
<td>Synovial fluid (1/18)</td>
<td>1 0 0</td>
<td>1 1 0</td>
<td>1 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Ascitic fluid (1/14)</td>
<td>1 0 0</td>
<td>1 1 0</td>
<td>1 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Total (147/572)</td>
<td>123 21 3</td>
<td>120 120 27</td>
<td>123 24 24</td>
<td>24 3</td>
</tr>
</tbody>
</table>

MTB: *M. Tuberculosis*; NTM: non-tuberculous mycobacteria; MAC: *M. avium* complex.
SD Bioline: SD BIOLINE TB Ag MPT 64 Rapid test; BD: BD MGIT TBc Identification Test; PNB: para-nitro benzoic acid; Pos: positive; Neg: Negative.

* Both ICT strips misidentified 3 MTB isolates.
cultures were positive for MTB and 27 (18.45%) negative by both SD Bioline ICT and BD ICT strips (Tables 1 and 2). Hence both strips had the same sensitivity and specificity.

Most notable performance of m-PCR was on 27 strip negative but MGIT and smear positive cultures. Of these, 3 (10.1%) were detected as MTB, 21 (77.7%) as other NTM and 3 (10.1%) as MAC. Of the 91 MGIT negative cultures, all were found negative by both ICT strip tests as well. Hence, the sensitivity and specificity rates of ICT kits were 97.5% and 100% respectively, as compared with m-PCR (Table 3).

NRA test and PNB test were used as standard methods. All 123 cultures that identified MTB in m-PCR showed positive nitrate reduction test and identified these cultures as true MTB. All 24 mycobacterial isolates that were identified as NTM (21) and MAC (3) by m-PCR showed negative reaction in nitrate tests (Tables 1 and 2).

Culture isolates that showed negative results by both the ICTs and were identified as *Mycobacterium* by PCR were further evaluated by a PNB test. Out of 27 ICTs negative, 24 isolates were resistant and 3 isolates were sensitive to PNB. Thus, these 3 isolates were truly detected as MTB by m-PCR, while misidentified by both the ICTs (Tables 1 and 2). Hence, the sensitivity and specificity of the m-PCR was 100% when compared with the nitrate and PNB tests (Table 3). An excellent agreement (Cohen’s kappa $\kappa = 1$) was observed among m-PCR, nitrate and PNB test for MTB detection.

**Discussion**

Recently, WHO estimated 8.7 million incident cases of TB and 1.4 million deaths occurred due to TB worldwide in 2011 [22]. Beside MTB infections, a significant percentage of patients are infected by NTM, especially those co-infected with HIV [2,23]. Clinically, the symptoms of disease caused by NTM are often similar to MTB disease. These patients are empirically prescribed with standard anti-TB drugs which are inherently ineffective against these NTM and most often these cases are labeled MDR on the basis of smear examination. It is mainly because in most of the resource-limited countries where TB is endemic, facilities for species-specific identification of the causative agent are lacking [24]. The treatment of infections caused by NTM species and MTB is entirely different. Though some NTM can be treated with simple antibiotics in a few days, but for that correct species has to be identified in order to initiate the appropriate treatment [25].

Mycobacterial growth on conventional L-J medium is fairly identifiable if the growth is MTB. Rapid growers, photochromogenic and scotochromogenic mycobacteria can also be identified without much difficulty [19]. However, liquid cultures have this limitation, though these provide a faster diagnosis of TB. Hence after inclusion of liquid cultures in most of the national TB control programmes, a number of ICT assays have been developed to resolve this limitation. Most of these are based on the presence of MPT-64 and/or MPT-63 antigens in MTB, but not in NTM. The widely used commercial tests for this purpose are Capilia TB assay (Tauns Laboratories, Inc., Numazu, Japan), the Tibilia rapid test (Hangzhou, China), the SD Bioline TB Ag MPT64 strip test (Standard Diagnostics, South Korea), and the BD MGIT Tbc identification test. MPT-64 protein was first used for rapid detection of MTB by the researchers in Japan which showed excellent specificity and sensitivity [4,5,10,26–30].

However, these tests can be applied only after a significant growth has taken place in the medium which takes several days to weeks. To minimize this duration, several nucleic acid amplification assays (NAAT) have been developed, which are already commercialized or undergoing clinical evaluation. Centers for Disease Control and Prevention (CDC) and the American Thoracic Society (ATS) recommend

<table>
<thead>
<tr>
<th>m-PCR (238)</th>
<th>SD Bioline strip (238)</th>
<th>BD Tbc strip (238)</th>
<th>NRA (147)</th>
<th>PNB test (27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>MTBC (123)</td>
<td>120</td>
<td>3</td>
<td>120</td>
<td>3</td>
</tr>
<tr>
<td>NTM (21)</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>MAC (3)</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Negative (90)</td>
<td>0</td>
<td>91</td>
<td>0</td>
<td>91</td>
</tr>
<tr>
<td>Total</td>
<td>238</td>
<td>238</td>
<td>147</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 2 – Comparison of in-house multiplex PCR results with BD Tbc identification strip, SD Bioline TB Ag MPT64 strip test, nitrate reductase and PNB tests.

<table>
<thead>
<tr>
<th>Comparison with m-PCR</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (PPV) (%)</th>
<th>Negative predictive value (NPV) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD Bioline strip</td>
<td>100</td>
<td>97.5**</td>
<td>97.5</td>
<td>100</td>
</tr>
<tr>
<td>BD Tbc Strip</td>
<td>100</td>
<td>97.5**</td>
<td>97.5</td>
<td>100</td>
</tr>
<tr>
<td>Nitrate test</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PNB test</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Considered as confirmatory test for identification of MTB complex.
** Due to 3 false negative results obtained with strip tests.

| Table 3 – Sensitivity, specificity, PPV and NPV of in-house multiplex PCR as compared with strip tests and nitrate reduction and PNB tests. |
The authors declare that they have no competing interests.

PK carried out the experiment and drafted the manuscript; PB & MJ carried out the experiments; SS conceptualized the study and arranged reagents and funds for carrying out the study.

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