Comparison of two in-house real-time PCR assays with MTB Q-PCR Alert and GenoType MTBDRplus for the rapid detection of mycobacteria in clinical specimens

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An in-house IS\textsubscript{6110} real-time PCR (IH IS\textsubscript{6110}), MTB Q-PCR Alert (Q-PCR) and GenoType MTBDRplus (MTBDR; Hain Lifescience) were compared for the direct detection of \textit{Mycobacterium tuberculosis} complex (MTBC) in 87 specimens following automated NucliSENS easyMAG DNA extraction. This included 82 first smear-positive specimens and three smear-negative specimens. Another in-house real-time PCR with a \textit{Mycobacterium} genus-specific probe for the internal transcribed spacer (ITS) region (IH ITS) was used to allow a full comparison with culture results. The sensitivities of IH IS\textsubscript{6110}, Q-PCR, MTBDR and IH ITS for MTBC detection were 100, 92, 87 and 87\%, respectively, compared with culture. Both IS\textsubscript{6110}-based real-time PCRs (in-house and Q-PCR) were similar in performance, with 91.2\% concordant results for MTBC detection. Inhibition rates were low, with zero to three specimens producing uninterpretable results. However, the Q-PCR failed to detect MTBC in five samples that were smear negative or had few acid-fast bacilli (one to 10 bacilli in 10 microscopic fields) detected by IH IS\textsubscript{6110}. IH ITS was the least sensitive assay but may be useful when used in conjunction with IS\textsubscript{6110} PCR results to determine the presence of non-tuberculous mycobacteria in smear-negative specimens. None of the real-time PCR assays tested provided drug-resistance data. It was concluded that an IH IS\textsubscript{6110} assay could easily be incorporated into the workflow of a diagnostic laboratory for rapid and accurate identification of MTBC from clinical specimens. The inclusion of an internal control and amplification of an ITS target enhance the diagnostic utility of the test.

INTRODUCTION

Historically, laboratory diagnosis of tuberculosis has relied heavily on microscopy and the time-consuming process of culturing organisms from clinical specimens using solid or liquid media. Recently, real-time PCR methods have been proposed for the detection of \textit{Mycobacterium tuberculosis} complex (MTBC) or to differentiate between mycobacterial species (Flores et al., 2009; Miller et al., 2002; Richardson et al., 2009). This technique offers a fast and sensitive way to detect MTBC directly in smear-positive and smear-negative specimens, including non-respiratory samples (Armand et al., 2011).

The GenoType \textit{Mycobacterium tuberculosis} Direct Resistance plus assay (MTBDR; Hain Lifescience) has been used in our laboratory since 2008 for the simultaneous detection of MTBC and the majority of the gene mutations responsible for rifampicin and isoniazid resistance, directly from smear-positive decontaminated respiratory specimens. In our laboratory, MTBDR has also been used to attempt MTBC detection in a small number of smear-positive non-respiratory specimens, but the test remains fairly labour intensive compared with real-time PCR where no post-PCR processing is necessary. MTBC was detected in 12 smear-positive non-respiratory samples during the period of this study (unpublished data).

To determine whether real-time PCR would provide a significant improvement to our diagnostic laboratory service, we compared three real-time PCR assays and MTBDR for the detection of mycobacteria, including MTBC, in clinical specimens and compared the results obtained with culture. The real-time PCR probes detected either the MTBC-specific insertion element IS\textsubscript{6110} (Thierry et al., 1990) or
mycobacterial internal transcribed spacer (ITS) sequences (Park et al., 2000).

METHODS

Study design and specimen types. Routine specimens from patients strongly suspected to have tuberculosis (TB) were submitted for mycobacterial examination to the Scottish Mycobacteria Reference Laboratory over a 5-month period (April to September 2008). The cohort comprised 82 first smear-positive specimens, three smear-negative samples and two samples that had insufficient material for microscopy. A total of 78 respiratory samples (62 sputa, 15 bronchoalveolar lavages and one pleural fluid) and nine non-respiratory samples (four pus, two aspirates, one abscess fluid, one cerebrospinal fluid (CSF) and a tissue specimen) were included.

Microscopic examination. Following processing of respiratory samples with Sputasil (1:1, v/v; Oxoid), a loopful of each concentrated specimen was placed directly on a microscope slide and stained with auramine phenol using standard procedures. Slides were examined at ×400 magnification. The number of acid-fast bacilli (AFB) present was recorded as: + (one to 10 bacilli in 10 fields), + + (one to 10 bacilli per field) or + + + (10 or more bacilli per field).

Decontamination and culture. For all specimens except those from sterile sites, decontamination was performed using 2% N-acetyl-L-cysteine sodium hydroxide (Health Protection Agency, 2006) prior to extraction and culture. The resulting pellet was resuspended in 1.5 ml phosphate buffer. One aliquot of 750 µl was used for routine mycobacterial culture using solid egg medium (two modified Löwenstein–Jensen slopes, one containing glycerol and one containing pyruvate as growth supplements) and rapid liquid culture (Mycobacterial Growth Indicator Tube; Becton Dickinson). The remaining 750 µl was stored at 4°C until extracted for molecular testing. Liquid and solid cultures were considered negative if no growth was obtained after 6 or 12 weeks of incubation, respectively.

MTBDR. This test was performed following the manufacturer’s instructions for simultaneous detection of MTBC (using a 235 rRNA probe) and its resistance to rifampicin (using rpoB probes) and/or isoniazid (using katG and inhA probes) from cultured isolates or smear-positive respiratory pulmonary specimens. Briefly, 500 µl decontaminated specimen was heat-inactivated and lysed by boiling and sonication. The resulting crude extract was then used for PCR and the products detected by reverse hybridization to immobilized probes. An internal control was included in every reaction to check for inhibitory substances.

Automated DNA extraction. Heat inactivation was performed by immersion in a water bath set at 95°C for 20 min, rendering the samples safe. A NucliSSENS easyMAG platform (bioMérieux) was used to isolate nucleic acids from 200 µl decontaminated specimen using silica extraction technology (Boom et al., 1990). The DNA was extracted on the easyMAG machine using the Generic 2.0.1 program. The on-board lysis protocol incorporates the non-human seal herpes virus type 1 (PhHV) in the lysis buffer. This is co-extracted and co-amplified in the in-house PCRs to aid validation of the assay performance (Niesters, 2002). Elution was performed in 50 µl NucliSens Extraction Buffer 3.

In-house real-time PCR detecting the IS6110 element (IH IS6110). The IS6110 primers and minor groove-binding probe from Savelkoul et al. (2006) and the PhHV primers and TaqMan probe from Niesters (2002) were used. Each 30 µl reaction contained 15 µl TaqMan Universal PCR master mix (Applied Biosystems), 0.3 µM each of IS6110 and PhHV primer, 0.2 µM FAM-labelled MTBC probe, 0.05 µM Cy5-labelled PhHV probe and 10 µl DNA. PCR amplification was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using the following amplification profile: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 50 cycles of 15 s at 90°C and 1 min at 60°C.

In-house real-time PCR detecting the ITS (IH ITS). The primers and TaqMan probe from Bruijnestein van Coppenraet et al. (2004) and the PhHV primers and TaqMan probe from Niesters (2002) were used. Each 30 µl reaction contained 15 µl TaqMan Universal PCR master mix, 0.3 µM each of ITS and PhHV primer, 0.25 µM FAM-labelled MTBC probe, 0.05 µM Cy5-labelled PhHV probe and 10 µl DNA. PCR amplification was performed on an ABI Prism 7500 using the following amplification profile: one cycle at 95°C for 3 min, followed by 50 cycles of 30 s at 95°C, 40 s at 55°C and 30 s at 72°C.

MTB Q-PCR Alert (Q-PCR). All primers and probes for this assay were provided in the kit (Nanogen Advanced Diagnostics). The target was the IS6110 element with an internal control specific for the human tumour necrosis factor-α gene. The same extractions used for the in-house real-time PCR assays were used, so the human tumour necrosis factor-α plasmid DNA was not co-extracted with the sample as per the manufacturer’s recommendations. Instead, 1 µl internal control DNA and 4 µl extract were added to each 25 µl PCR. The ABI Prism 7500 was used as defined by the manufacturer’s instructions (‘9600 emulation’ mode).

Identification of cultured isolates. Culture isolates were identified using GenoType Mycobacterium CM and AS assays (Hain Life-science). These, in combination, allow identification of 31 different non-tuberculous mycobacteria (NTM). Both tests were performed following the manufacturer’s instructions using 300 µl culture suspension.

Statistical analysis. The results from each PCR test were compared with culture of the same specimen, here considered the ‘gold standard’ of mycobacterial diagnosis. Because the analysis was carried out using specimens that were known to contain mycobacteria or not, they could all be considered as true positives, for both MTBC and NTM, and true negatives, respectively. The PCR results were classified as true positive, true negative, false positive or false negative. Specimens that were contaminated or contained inhibitors to PCR were excluded from the statistical analysis.

RESULTS

Microscopy was performed on 85 samples using auramine phenol staining (Table 1), whilst two had insufficient volume for microscopy. Forty-five (60%) smear-positive respiratory samples contained MTBC organisms by culture. Of the remaining 30 samples, 18 grew NTM and 12 were culture negative, reflecting prior anti-mycobacterial treatment, small numbers of mycobacteria or fastidious organisms.

The results obtained by culture, IH IS6110, Q-PCR, IH ITS and MTBDR are shown in Tables 2 and 3. These five methods detected MTBC or MTBC DNA in 62, 66, 60, 50 and 58% of specimens, respectively. The contamination or inhibition rate was comparable for all of the tests, with zero to three specimens producing uninterpretable results.
Q-PCR was the only real-time PCR assay to show no lack of signal due to PCR inhibitors.

Both IS6110-based real-time PCRs (in-house and Q-PCR) were similar in performance, with 91.2% of results concordant with culture for MTBC. When clinical specimens were stratified by their acid-fast smear status and the anatomical site from which they were obtained, IH IS6110 exhibited a sensitivity of 100% for the detection of MTBC compared with culture (the ‘gold standard’ for TB diagnosis), independent of the smear result or sample type (Table 4). Three of four false-positive IH IS6110 results were obtained from patients who were treated for TB infection. We were unable to confirm whether the other patient was being treated for TB infection. Despite detecting the same multi-copy target sequence as IH IS6110, the Q-PCR test sensitivity for smear-positive respiratory specimens was 94% (95% CI 88–100%), but this decreased to 50% for smear-negative specimens. Q-PCR failed to detect MTBC in five samples that were smear negative or had few AFB (+) detected by IH IS6110. To allow the kit internal control to be added to the MTB Q-PCR, 4 µl DNA extract was used instead of 5 µl. The reduced template volume may account for the reduction in sensitivity found using Q-PCR.

MTBDR was less sensitive than both IH IS6110 and Q-PCR for MTBC detection in smear-positive samples. Six of seven specimens found not to be positive for MTBC using MTBDR, but which later grew MTBC, were smear negative or had few AFB (+) seen on microscopy. With regard to specificity, all the uninhibited respiratory specimens that were culture negative for MTBC were negative using IH IS6110, Q-PCR and MTBDR.

Table 1. Summary of the microscopy and culture results for the study specimens

<table>
<thead>
<tr>
<th>Microscopy result</th>
<th>All specimen types</th>
<th>Respiratory (MTBC culture positive)</th>
<th>Non-respiratory (MTBC culture positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Many AFB (+ + +)</td>
<td>30 (24 SPT, 4 BAL, 2 PUS)</td>
<td>28 (23)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Moderate AFB (+ +)</td>
<td>21 (18 SPT, 3 BAL)</td>
<td>21 (9)</td>
<td>0</td>
</tr>
<tr>
<td>Few AFB (+)</td>
<td>31 (19 SPT, 7 BAL, 2 PUS, 1 Fluid, 1 ASP, 1 Tiss)</td>
<td>26 (13)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>No AFB*</td>
<td>3 (1 CSF, 1 BAL, 1 PF)</td>
<td>2 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>ISM†</td>
<td>2 (1 SPT, 1 ASP)</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
<td>78 (47)</td>
<td>9 (7)</td>
</tr>
</tbody>
</table>

*Two microscopy-negative specimens grew MTBC. One of these was from a CSF specimen.
†Insufficient specimen for microscopy.

Table 2. Summary of culture and PCR results for all assays

<table>
<thead>
<tr>
<th>Result</th>
<th>Culture</th>
<th>IH IS6110</th>
<th>Q-PCR</th>
<th>IH ITS</th>
<th>MTBDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive MTBC</td>
<td>54 (62.1)</td>
<td>57 (65.5)</td>
<td>52 (59.8)</td>
<td>44 (50.1)</td>
<td>50 (57.5)</td>
</tr>
<tr>
<td>Positive NTM</td>
<td>18 (20.7)*</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>22 (25.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Negative</td>
<td>13 (14.9)</td>
<td>27 (31.0)</td>
<td>35 (40.2)</td>
<td>18 (20.7)</td>
<td>36 (41.4)</td>
</tr>
<tr>
<td>Cont./Inhib.</td>
<td>2 (2.3)</td>
<td>3 (3.5)</td>
<td>0 (0)</td>
<td>3 (3.4)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
<td>87</td>
<td>87</td>
<td>87</td>
<td>87</td>
</tr>
</tbody>
</table>

*Identified using the GenoType Mycobacterium CM/AS assays:
- Mycobacterium avium (n=6), Mycobacterium intracellulare (n=4),
- Mycobacterium chelonei (n=2), Mycobacterium malmoense (n=1),
- Mycobacterium simiae (n=1), Mycobacterium fortuitum (n=1),
- Mycobacterium xenopi (n=1) and Mycobacterium kansasii (n=1).

Table 3. Summary of PCR results for all assays compared with culture as the ‘gold standard’ for TB diagnosis

<table>
<thead>
<tr>
<th>Culture</th>
<th>Result</th>
<th>IH IS6110</th>
<th>Q-PCR</th>
<th>IH ITS</th>
<th>MTBDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive MTBC</td>
<td>PCR positive</td>
<td>56</td>
<td>51</td>
<td>62</td>
<td>49</td>
</tr>
<tr>
<td>PCR negative</td>
<td>0</td>
<td>6</td>
<td>11</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>PCR inhibited</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>PCR positive</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>PCR negative</td>
<td>25</td>
<td>27</td>
<td>5</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>PCR inhibited</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cont./Inhib.</td>
<td>PCR positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCR negative</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PCR inhibited</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
<td>87</td>
<td>87</td>
<td>87</td>
<td>87</td>
</tr>
</tbody>
</table>

http://jmm.sgmjournals.org
IH ITS was the least sensitive assay for MTBC detection as it failed to detect MTBC in five culture-positive specimens and one culture-negative specimen that were all PCR positive using IH IS6110. The major advantage of IH ITS, however, is the potential to determine whether other NTM species are present when used in conjunction with IS6110 PCR results. When our PCR data were analysed in this way, 15 smear-positive specimens gave IS6110-negative and ITS-positive results. Eleven NTM were confirmed by culture. Of the remaining four samples that failed to grow despite moderate or many AFB being seen on microscopy, two were from patients with known M. malmoense infection. The other two were single patient samples submitted to our laboratory with no others to confirm NTM infection.

**DISCUSSION**

Real-time PCR offers improved sensitivity compared with MTBDR for the detection of MTBC directly from specimens. Such assays are easy to perform and allow faster sample turnaround times than MTBDR, which is currently used in our laboratory. IH IS6110, Q-PCR and MTBDR were all highly specific and exhibited ≥90% sensitivity for MTBC detection in smear-positive respiratory samples.

IH IS6110 in particular showed excellent sensitivity for smear-positive respiratory specimens. Whilst the level of sensitivity using IH IS6110 remained stable for non-respiratory and smear-negative specimens, Q-PCR and MTBDR showed a decrease in sensitivity for these specimen types.

The high sensitivity (100%) and NPV (100%) of IH IS6110 using 75 smear-positive respiratory specimens means it is unlikely that false-negative MTBC results will occur using this method. Whilst some M. tuberculosis strains that lack IS6110 have been reported (Chauhan et al., 2007; Das et al., 1995; van Soolingen et al., 1993), our laboratory previously examined 2526 referred isolates from Scottish laboratories and found no strains without this element (unpublished data). Three of four false-positive IH IS6110 results were obtained from patients with TB infection. As these patients were on anti-tuberculous therapy, it is not surprising that the culture result was negative; the amplification of mycobacterial DNA would therefore indicate the presence of dead mycobacteria and is not a true false-positive result. It has recently been shown that real-time PCR can be a useful adjunct for rapid diagnosis of smear-negative TB (Min et al., 2010), but further studies using smear-negative samples are required to assess the suitability of IH IS6110.
to detect MTBC infection in smear-negative patients, as only five smear-negative samples were included in this study.

In our laboratory, MTBDR showed lower sensitivity than has been reported previously using smear-positive specimens (Barnard et al., 2008; Hillemann et al., 2007; Ling et al., 2008; Nikolayevskyy et al., 2009). As 13% of MTBC-positive specimens were missed, we confirmed that this assay is not suitable for testing smear-negative specimens. Whilst IH ITS had a lower sensitivity than IH IS6110, Q-PCR and MTBDR, it was useful to confirm that other mycobacterial species may be present. None of the real-time PCR assays tested provided drug-resistance data.

Our analysis focused on smear-positive specimens because our laboratory is not funded to perform direct testing on smear-negative specimens. We appreciate that including a higher number of smear positives rather than selection of all samples sent for laboratory investigation influences the PPV of the assay. However, our results suggest that our routine laboratory service could be improved by replacing MTBDR with automated specimen extraction and an in-house real-time PCR as described here. Following a positive IH IS6110 result, a resistance detection assay (such as MTBDR) could then be performed, providing both MTBC identification and drug susceptibility within 1–2 days of the specimen arriving at the laboratory. This would reduce laboratory hands-on time and improve sample turnaround time, providing a better service for clinicians and patients alike.

Ideally, further optimization of the NucliSENS easyMAG extraction protocol is required for non-respiratory specimen types, such as CSF and tissues, to improve DNA extraction efficiency, which in turn may increase the accuracy of non-pulmonary TB diagnosis. Primers and probes for amplification of an internal control and an ITS target should be incorporated, ideally as a multiplex PCR, to enhance the diagnostic utility of IH IS6110 and to limit the number of PCRs performed. Oligonucleotides to detect mutations that confer drug resistance, particularly to rifampicin and isoniazid, could also be included.

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REFERENCES


