Evaluation of a semi-automated Seegene PCR workflow with MTB, MDR and NTM detection for rapid screening of tuberculosis in a low-prevalence setting.

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Running head: Evaluation of a TB-PCR workflow.
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**Evaluation of a semi-automated Seegene PCR workflow with MTB, MDR and NTM detection for rapid screening of tuberculosis in a low-prevalence setting.**

In areas of low tuberculosis (TB) prevalence, laboratory diagnosis of TB may essentially cover non-tuberculous mycobacteria (NTM) in addition to *Mycobacterium tuberculosis* (MTB). In this study, a semi-automated PCR workflow distinguishing MTB and NTM (Anyplex™ MTB/NTMe, Seegene) and subsequently detecting MTB isoniazid/rifampicin resistance (Allplex™ MTB/MDRe, Seegene) was evaluated for replacing smear microscopy of acid-fast bacilli as the rapid screening method for TB.

With 279 clinical samples, 47 cultures positive for MTB and 76 for NTM, the Anyplex™ MTB/NTMe assay and smear microscopy showed equal sensitivities (49.6% vs. 50.8%, respectively) but Anyplex™ MTB/NTMe was more sensitive for MTB (63.8% vs. 25.6%) than for NTM (40.8% vs. 64.5%). Allplex™ MTB/MDRe showed a slightly higher sensitivity of 68.1% for MTB (32/47 positive, n=222). Antibiotic resistance profiles were correctly identified for all MTB isolates (one MDR isolate). Specificity was 100% for both assays. Anyplex™ MTB/NTMe detected all the 18 NTM species present in the study.

The analytical performance of the evaluated high-throughput workflow was relatively weak compared to culture but potentially adequate as a rapid screening method analogous to smear microscopy with additional differentiation between TB, MDR-TB and NTM.

**Keywords:** Allplex; Anyplex; molecular diagnostics; non-tuberculous mycobacteria; PCR; tuberculosis

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Introduction

The detection of *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis (TB), is undeniably the number one priority when considering the laboratory diagnosis of mycobacteria. However, especially clinical settings of low TB prevalence, routine laboratory diagnosis may include non-tuberculous mycobacteria (NTM) in addition to MTB.

It is well known that the clinical significance of isolating environmental NTM in clinical samples is highly unknown compared to the obligatory parasitic MTB (1). Indeed, clinical symptoms and radiological findings are considered a prerequisite for the diagnosis of NTM pulmonary disease (NTMPD) (2). As NTMPD and TB may be clinically indistinguishable but differ greatly in antimicrobial therapy, a significant added value of detecting NTM lies in the exclusion of TB (3, 4). Several cases of MDR-TB misdiagnosis due to NTM infection have been described in areas where NTM are not routinely analyzed (5, 6). In countries where they are routinely analyzed, NTM make up a significant share of all mycobacterial laboratory findings and rates are systematically increasing (7, 8).

Smear microscopy is still a widely used initial screening method of TB (9). Although inexpensive, the method does not identify different acid-fast bacilli. Here, in a low TB prevalence setting in Finland, we evaluated the performance of two PCR assays, Anyplex™ MTB/NTMe (Seegene, Seoul, Korea) and Allplex™ MTB/MDRe (Seegene) using the automated NIMBUS IVD nucleic acid extraction and PCR setup system (Hamilton Company, Reno, NV, USA). The system was also surveyed as a combined workflow of rapid TB screening analogous to smear microscopy but with additional analysis of identification of TB, MDR-TB and NTM.

The detection of *Mycobacterium tuberculosis* complex (MTB), the causative agent of tuberculosis (TB), is undeniably the number one priority when considering the laboratory diagnosis of mycobacteria. Currently, this is still widely performed using traditional methods, which include rapid but insensitive smear microscopy of acid-fast bacilli and slow but sensitive mycobacterial culture. The main improvement to this process occurred in the mid-1990’s, with the introduction of molecular diagnostic tests to rapidly detect MTB (and later MTB multidrug resistance, MDR detection), bringing reliable MTB identification from weeks to potentially the same day of sampling (1,2). However, such assays may not be sufficient alone for replacing laborious smear microscopy in clinical settings, where routine analysis of TB culture samples includes non-tuberculous mycobacteria (NTM) in addition to MTB.
Materials and Methods

Clinical material

279 clinical NALC-NaOH sedimented samples (stored at -70 °C) and 35 culture-enriched (Löwenstein-Jensen agar or BD BACTEC™ MGIT™ growth indicator tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA)) mycobacterial isolates were collected for this study during the year 2018 with 230 of pulmonary origin (215 sputum or tracheal aspirate, 15 bronchoalveolar lavage) and 49 of extrapulmonary origin (23 soft tissue, 15 urine, 10 pus, and 1 bone). The samples were collected and the study was performed retrospectively in the Clinical Microbiology Laboratory of Fimlab Laboratories, Tampere, Finland.

Data for smear microscopy (auramine staining) and culture (Löwenstein-Jensen agar and BD BACTEC™ MGIT™ growth indicator tubes) were available for all but 15 urine samples with culture results only. Additionally, mycobacterial species identification (GenoType® Mycobacterium CM VER 2.0 assay, Hain Lifescience GmbH, Nehren, Germany) and antimicrobial susceptibility results (determined by National Institute for Health and Welfare, Finland) were available for cultured mycobacterial isolates.

Sample pretreatment

To ensure safe working outside a biosafety cabinet, and to reduce sample viscosity, a separate protocol inactivation was employed, previously described by Qi et al. for the RealTime MTB assay (Abbott Molecular, Des Plaines, IL, USA) (10). Briefly, samples were mixed 1:3 with inactivation reagent (0.6% sodium hydroxide [wt/vol], 60% isopropanol [vol/vol], and 1.8% Tween 20 [vol/vol]) and vortexed twice during a 1 to 24 h incubation at room temperature. To assess if the inactivation protocol had any adverse effects to the workflow, a subset of samples was analyzed without the inactivation pretreatment (non-paired comparison to inactivated samples) and another smaller subset with and without the inactivation pretreatment (paired comparison). Samples for which no inactivation pretreatment was employed were known to be negative for MTB.

DNA extraction, amplification and result analysis

Two different PCR assays were used, Anyplex™ MTB/NTMe (Seegene) and Allplex™ MTB/MDRe (Seegene). DNA was extracted with NIMBUS IVD (Hamilton Company, Reno, NV, USA) system using the STARMag 96x4 Universal Cartridge Kit (Seegene, Seoul, Korea) and amplified with CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Extraction and PCR setup was controlled with Seegene Launcher IVD (Seegene) and result analysis with Seegene Viewer IVD software (Seegene). All protocols were preset by the
assay manufacturer. DNA concentrations were measured using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Workflow analysis
To assess the turnaround time of the workflow, including Anyplex™ MTB/NTMe and subsequent testing of MTB-positive samples with Allplex™ MTB/MDRe, durations of different steps with realized sample batches were timed. To assess the range of assay durations, the instrument software was tested for analysis with a minimal and maximal number of samples, while still allowing for single batch analysis.

Statistical Analysis
Statistical tests were employed, when appropriate, using the Prism software (GraphPad Software, San Diego, CA, USA). Mann–Whitney U test and Wilcoxon signed-ranked test were used to compare non-paired and paired data of DNA concentrations, respectively. Fisher’s exact test was used for comparing sample clotting during DNA extraction and PCR results with different sample types. Sensitivity, specificity, and positive (PPV) and negative predictive values (NPV) with 95% confidence intervals (CI) and medians with interquartile range were used to describe qualitative and quantitative results, respectively.

Ethics declaration
Leftover samples were analyzed retrospectively in this study and no patient data was collected. The study design required no ethical committee approval. Informed consent was not required for this study.

Results
Clinical material
123 of the 279 analyzed clinical samples were culture-positive for mycobacteria, 47 with MTB (including 3 MDR and 2 M. africanum isolates) and 76 with NTM (consisting of 9 different NTM species). With the culture-enriched isolates included, a total of 18 NTM species were analyzed in the study.

Performance of the Anyplex™ MTB/NTMe assay
A total of 279 clinical samples were analyzed with the Anyplex™ MTB/NTMe assay. The assay showed overall sensitivity of 49.6% (61/123) with 67.2% for smear-positive (SP) and 30.5% for
smear-negative (SN) samples. Separately, sensitivity was 63.8% (30/47) for MTB detection (SP: 91.7%, 11/12; SN: 53.1%, 17/32) and 40.8% (31/76) for NTM detection (SP: 61.2%, 30/49; SN: 3.7%, 1/27) (Table 1–2). The overall sensitivity was equal to that of smear microscopy (50.8%, 61/120) with reciprocal figures for smear microscopy’s theoretical sensitivities for MTB (25.6%, 12/47) and NTM (64.5%, 49/76) detection whatsoever. True assay specificity and PPV were 100%, as the three observed culture-negative, PCR-positive cases were all recently culture-positive for MTB. NPV for MTB/NTM, MTB and NTM detection were 71.2%, 93.1% (229/246) and 81.9% (203/248), respectively.

Of the nine NTM species present in clinical samples, _M. avium_, _M. chelonae_, and _M. simiae_ were detected by Anyplex™ MTB/NTMe (Table 3). _M. xenopi_ was the only NTM species not identified in any smear-positive clinical sample. Examining PCR data of culture-enriched isolates indeed showed that the detection of _M. xenopi_ was relatively weak, showing late PCR cycles of 35.19 and 36.06 compared to the median of the whole culture-enriched sample set (23.30, 21.62–26.21). All the 18 NTM species present in the study were detectable in culture-enriched samples (Table 4).

Performance of the Allplex™ MTB/MDRe assay

222 of the available 279 clinical samples were analyzed with the Allplex™ MTB/MDRe assay. The Allplex™ MTB/MDRe assay showed sensitivity of 68.1% (32/47) (SP: 91.7%, 11/12; SN: 59.4%, 19/32) and NPV of 91.9% (171/186) for MTB detection (Table 1–2). As with Anyplex™ MTB/NTMe, true specificity and PPV were 100%. 29/30 (96.7%) of the samples analyzed as MTB-positive with Anyplex™ MTB/NTMe were also analyzed as positive with Allplex™ MTB/MDRe.

There were three MDR-MTB positive samples in the study, all culture-positive, smear-negative, none detected by the Allplex™ MTB/MDRe assay (MTB negative). However, both MTB and MDR were detected from a culture-enriched sample. No false positive MDR results were detected.

Effect of the inactivation protocol in DNA extraction and PCR results

The inactivation protocol had a significant effect on DNA extraction yields (P=0.003) when compared between a subset of samples with the inactivation protocol employed (11.8 ng/µl, 9.5–16 ng/µl; n=96) and a different subset of samples without the inactivation protocol employed (13.9 ng/µl, 10–32 ng/µl; n=96) (non-paired subsets). The observed difference was strengthened by a smaller subset of samples analyzed both with and without inactivation pretreatment (P=0.0001) (paired subset). (Figure 1A). However, the analysis of paired samples showed a mean 2.2-fold
(1.6–2.6, n=14) shift in DNA concentration, whereas the inactivation protocol diluted the sample 4-fold. A similar comparison of the same subset of paired samples was done with the Anyplex™ MTB/NTMe assay. Here, only a slight shift in PCR cycle levels was seen with no significant difference (P=0.14) (Figure 1B). No PCR abortion occurred in the study. However, sample clotting events in the automated DNA extraction were significantly more frequent with the inactivated samples (13.6%, 25/184) compared to the non-inactivated samples (3.2%, 3/95) (P=0.006). This increased the need for manual pipetting during the DNA extraction protocol.

Semi-automated workflow

The workflow of rapid TB screening with Anyplex™ MTB/NTMe and subsequent testing of MTB-positive samples with Allplex™ MTB/MDRe is illustrated in Figure 2. Assuming a 96 well-plate format, the workflow allowed analysis of 72 samples with 45 MTB-positive samples in one cycle. The highest walkaway time was achieved with batches of no more than 40 clinical samples (not to mix with analysis of already extracted DNA). More samples resulted in two separate interruptions of the DNA extraction protocol, as the instrument required more pipette tips and an empty waste bag. Occasional sample clots further reduced walkaway time and increased extraction time. There was a separate interrupt in the extraction protocol for manually transferring samples with clots. Without these interruptions, the turnaround time of a 40-sample batch was approximately 6 h from decontaminated samples to MTB/NTM (inactivation: 1.5 h, DNA extraction and PCR setup: 2.25 h, amplification and result analysis: 2.25 h, hands-on time included). The turnaround time for subsequent MDR-analysis of five MTB-positive samples took approximately 3.5 h (PCR setup: 0.25 h, amplification: 3 h, result analysis and hands-on time: 0.25 h), making up a total turnaround time of 9.5 h.

Discussion

Rapid screening of TB is still widely based on smear microscopy of acid-fast bacilli – a method that could be replaced by more automated and objective technology such as PCR. It is clear that a PCR-based method is more expensive per test than a nonspecific staining method. Additionally, a multi-stage PCR workflow such as the one evaluated in this study requires a high level of infrastructure and therefore does not suit small laboratories or low-income settings. Compared to smear microscopy, however, the system greatly increases the sensitivity of rapid screening of TB as was shown in this study. It also reduces the need for a high-expertise-demanding method, and increases assay reproducibility with respect to workflow and result interpretation. With MTB, smear microscopy has the status as a determinant of TB infectivity, although there are also studies where smear-negative cases have been a significant source of infection (11, 12). No
similar practice has been described for NTM (2). We previously showed that in this specific clinical setting of low TB-prevalence and low population, an average of one sample was positive for acid-fast bacilli per day (13). In such a clinical setting, it would be easy to perform a subsequent smear microscopy analysis for PCR-positive samples. Thus, the status as an infectivity determinant does not essentially impair the applicability of PCR-screening.

The evaluated semi-automated system is not exclusive for TB. The proposed TB screening workflow can be employed to complement a uniform and comprehensive molecular diagnostic system. This also allows the possibility of assigning automated work steps for other laboratory personnel not working in a TB laboratory. Here, the evaluated workflow also enabled a cost-efficient reflex strategy of antibiotic resistance testing where MTB-positive samples could be specifically selected for subsequent antibiotic resistance analysis without the need for overlapping analysis of all samples (13). As a follow-up test for MTB-positive samples with Anyplex™ MTB/NTMe assay, the Allplex™ MTB/MDRe assay provided reliable results, although more resistant MTB isolates would have been needed to fully evaluate the assay’s MDR feature with 7 isoniazid and 18 rifampicin resistance determining mutation targets.

Moreover, the evaluated semi-automated system is not exclusive for TB. As Seegene holds an array of different molecular diagnostic assays available for its automated systems, the proposed TB screening workflow can be employed to complement a uniform and comprehensive molecular diagnostic system. This also allows the possibility of assigning automated work steps for other laboratory personnel not working in a TB laboratory. Here, the evaluated workflow enabled a cost-efficient reflex strategy of antibiotic resistance testing where MTB-positive samples could be specifically selected for subsequent antibiotic resistance analysis without the need for overlapping analysis of all samples. The system is analogous to the RealTime MTB system employed by Abbott, but, as previously shown, the Seegene TB assay selection may currently better suit the needs of low TB-prevalence, low population settings (16). The use of the Seegene Launcher software allowed easy and fluent control of samples, as a single sample worklist could be used from extraction to result analysis and follow-up testing of selected samples. As a follow-up test for MTB-positive samples with Anyplex™ MTB/NTMe assay, the Allplex™ MTB/MDRe assay provided reliable results, although more resistant MTB isolates would have been needed to fully evaluate the assay’s MDR feature with 7 isoniazid and 18 rifampicin resistance determining mutation targets.

Considering analytical performance, the two evaluated PCR assays showed overall sensitivities close to smear microscopy. While such figures are far from definitive laboratory diagnosis and therefore do not compete with culture, the relatively low sensitivity may be adequate for replacing
smear microscopy as the initial screening method. As studies even for similar PCR assays have been shown to have higher performance figures (14, 15) one must consider the possible adverse effects of the inactivation protocol or sample storage on the quality of the results. In addition, performance evaluation studies of Seegene TB assays are generally performed using a manual extraction protocol and, to our knowledge, there are no previous studies assessing the performance of the NIMBUS IVD in molecular diagnosis of TB. The inactivation protocol used in this study seemed, in fact, not to have any apparent effect on assay results despite sample dilution. However, as sample clotting occurred significantly more often with inactivated samples, this step seemed to be redundant in terms of reducing sample viscosity. In terms of laboratory safety, though, the process requires a separate MTB killing step as a sample-handling instrument may not prevent pathogen exposure if not situated inside a biosafety cabinet or a fume hood with an accessory HEPA filter. This is exceptionally important when handling MTB as laboratory transmission due to prolonged exposure may be very difficult to identify due to the slow nature of the MTB course of infection (16).

The Anyplex™ MTB/NTMe assay showed poorest performance on smear-negative NTM-positive samples but also specifically on *M. xenopi*, even with smear-positive samples. Depending on geographical location, *M. xenopi* is one of the species most often reported to cause NTM pulmonary disease, in addition to *M. abscessus*, *M. avium* complex, *M. fortuitum*, *M. kansasii*, and *M. malmoense* (4). Even though the regional incidence of NTM has a great diversity, *M. xenopi* is globally the third most common NTM identified (8%) after the *M. avium* complex (47%), and *M. gordonae* (11%) (17-19). Closer inspection of the test insert revealed that the manufacturer had in fact not tested specificity to *M. xenopi*. Other relevant species assayed in this study, but not by the manufacturer, were *M. celatum*, *M. interjectum*, *M. lentiflavum*, *M. malmoense*, *M. marinum*, *M. simiae*, and *M. schimoidei*. Except for *M. marinum*, which is the causative agent of a superficial infection known as fish tank granuloma, all of the mentioned NTM species have been reported to cause pulmonary disease (20). Within tuberculous mycobacteria, information on specificity to *M. africanum* also appeared to be lacking. Regionally important in West Africa, testing assay specificity for *M. africanum* was highly relevant even though different MTB complex species have been recently proposed to comprise a single species of MTB (21). Although only with individual isolates, specificity of the Anyplex™ MTB/NTMe assay for these eight NTM species and one MTB variant was shown in this study with, as far as we are aware, no previous demonstration elsewhere.

Most importantly, the Anyplex™ MTB/NTMe assay showed reliable resolution between tuberculous and non-tuberculous mycobacteria with no observed false-positive results. This was
Based on the relatively higher threshold for the generic mycobacterial target (i.e. lower sensitivity for NTM) than for the MTB specific target, so that MTB could not be falsely reported as NTM. However, this configuration did not compromise the sensitivity of MTB detection, which was superior to that of smear microscopy. The assays amplify the known gene targets IS6110 and Mpb64 for MTB detection, and a panmycobacterial 16S rRNA gene target for NTM detection.

The Anyplex™ MTB/NTMe and Allplex™ MTB/MDRe assays, accompanied with the NIMBUS IVD system showed relatively low but adequate overall sensitivity to be used as an initial screening method for mycobacteria analogous to smear microscopy. However, future research assessing the cost-efficiency of PCR compared to smear microscopy is vital.

Acknowledgments

We thank Seegene Inc. and Triolab Ltd. for providing the NIMBUS IVD instrument and accompanying labware, Allplex™ MTB/MDRe kits, and product support by Aliisa Mäkinen. The study was funded by Tampere Tuberculosis Foundation and Finnish Anti-Tuberculosis Foundation.

Conflicts of interest

None to declare.
References


Table 1. Agreement of Anyplex™ MTB/NTMe and Allplex™ MTB/MDRe with mycobacterial culture and smear microscopy results.

<table>
<thead>
<tr>
<th>Culture (n=279)</th>
<th>Smear microscopy</th>
<th>MTB Detection</th>
<th>Anyplex MTB/NTMe</th>
<th>MTB/NTM Detection</th>
<th>Allplex MTB/MDRe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFB detection</td>
<td>MTB Detection</td>
<td>NTM Detection</td>
<td>MTB/NTM Detection</td>
<td>MTB Detection</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>61 59</td>
<td>30 17</td>
<td>31 45</td>
<td>61 62</td>
<td>32 15</td>
</tr>
<tr>
<td>Negative</td>
<td>5 139</td>
<td>3* 229</td>
<td>0 203</td>
<td>3* 153</td>
<td>4* 171</td>
</tr>
<tr>
<td>Smear Microscopy</td>
<td>Positive</td>
<td>11 1</td>
<td>30 19</td>
<td>41 20</td>
<td>11 1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>17 15</td>
<td>1 26</td>
<td>18 41</td>
<td>19 13</td>
</tr>
</tbody>
</table>

*All cases recently culture-positive for MTB
**All culture-positive
AFB: acid-fast bacilli

Table 2. Performance data of Anyplex™ MTB/NTMe and Allplex™ MTB/MDRe assays.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (CI), %</th>
<th>Specificity (CI), %</th>
<th>PPV (CI), %</th>
<th>NPV (CI), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear microscopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFB Detection</td>
<td>50.8 (42.0–59.6)</td>
<td>96.5 (91.9–98.7)</td>
<td>92.4 (83.1–97.1)</td>
<td>70.2 (63.5–76.2)</td>
</tr>
<tr>
<td>Anyplex MTB/NTMe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTB Detection</td>
<td>91.7 (62.5–100)</td>
<td>98.7* (96.1–99.7)</td>
<td>90.9* (75.7–97.6)</td>
<td>93.1 (89.2–95.7)</td>
</tr>
<tr>
<td>NTM Detection</td>
<td>61.2 (47.2–73.6)</td>
<td>100 (97.8–100)</td>
<td>100 (86.9–100)</td>
<td>81.9 (76.6–86.2)</td>
</tr>
<tr>
<td>MTB/NTM Detection</td>
<td>67.2 (54.7–77.7)</td>
<td>98.1* (94.2–99.6)</td>
<td>95.3* (86.6–98.9)</td>
<td>71.2 (64.8–76.8)</td>
</tr>
<tr>
<td>Allplex MTB/MDRe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTB Detection</td>
<td>91.7 (62.5–100)</td>
<td>97.7* (94.1–99.3)</td>
<td>88.9* (74.1–96.2)</td>
<td>91.9 (87.0–95.1)</td>
</tr>
</tbody>
</table>

*True specificity and PPV was 100% as all culture negative, PCR-positive cases had been recently culture-positive for MTBC
AFB: acid-fast bacilli
Table 3. Array of non-tuberculous mycobacteria present in the study: clinical samples. PCR performance is shown as positive agreement with culture.

<table>
<thead>
<tr>
<th>Species</th>
<th>Smear+ Culture+</th>
<th>Smear- Culture+</th>
<th>Total Culture+</th>
<th>PCR+ %</th>
<th>PCR+</th>
<th>PCR+ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. avium</td>
<td>40</td>
<td>25</td>
<td>62.5</td>
<td>15</td>
<td>1</td>
<td>6.7</td>
</tr>
<tr>
<td>M. intracellular</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M. xenopi</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M. chelonae</td>
<td>3</td>
<td>3</td>
<td>100</td>
<td>3</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>M. simiae</td>
<td>3</td>
<td>2</td>
<td>66.7</td>
<td>3</td>
<td>2</td>
<td>66.7</td>
</tr>
<tr>
<td>M. gordonae</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M. szulgai</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M. mucogenicum</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>30</td>
<td>61.2</td>
<td>27</td>
<td>1</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Table 4. Array of mycobacteria present in the study: culture-enriched isolates. Additional species tested for positive NTM identification by manufacturer but not in this study are shown in a separate column.

<table>
<thead>
<tr>
<th>Cultured isolates</th>
<th>n</th>
<th>MTB</th>
<th>NTM</th>
<th>Other species tested by manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>M. bovis BCG</td>
</tr>
<tr>
<td>M. avium</td>
<td>3</td>
<td>3</td>
<td></td>
<td>M. asiaticum</td>
</tr>
<tr>
<td>M. abscessus</td>
<td>2</td>
<td>2</td>
<td></td>
<td>M. fallax</td>
</tr>
<tr>
<td>M. chelonae</td>
<td>2</td>
<td>2</td>
<td></td>
<td>M. massiliense</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>2</td>
<td>2</td>
<td></td>
<td>M. neoaurum</td>
</tr>
<tr>
<td>M. gordonae</td>
<td>2</td>
<td>2</td>
<td></td>
<td>M. paraseoulense</td>
</tr>
<tr>
<td>M. interjectum*</td>
<td>2</td>
<td>2</td>
<td></td>
<td>M. phlei</td>
</tr>
<tr>
<td>M. intracellulare</td>
<td>2</td>
<td>2</td>
<td></td>
<td>M. seoulense</td>
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<td>M. smegmatis</td>
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<tr>
<td>M. lentiflavum*</td>
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<td>M. vaccae</td>
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<tr>
<td>M. szulgai</td>
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<tr>
<td>M. xenopi*</td>
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<tr>
<td>M. celatum*</td>
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<tr>
<td>M. malmoense*</td>
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<td>M. marium*</td>
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<tr>
<td>M. schimoidei*</td>
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<tr>
<td>NTM total</td>
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<tr>
<td>Other AFB</td>
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<td>1</td>
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</tr>
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</table>

AFB: acid-fast bacilli (non-mycobacterial)

*Assay specificity not tested by manufacturer
Figure 1. Effect of the inactivation protocol on sample quality and results on DNA extraction yield (A) and PCR cycles (B).
*Statistically significant difference

Figure 2. Illustration of the suggested semi-automated PCR workflow. Vertical arrows show workflow-to-result with live (dark) and dead (light) mycobacteria. Hands-on time excluded.
Primary sample(s)
(e.g. sputum)

Decontamination → (Smear microscopy)

Inactivation
1–1.75 h (n=1–72)

DNA extraction + PCR setup
1–3 h (n=1–72)

Amplification
2 h (n=1–94)

Result analysis:
MTB pos/neg
NTM pos/neg

PCR setup of MTB pos
10–30 min (n=1–45)

Amplification
3 h (n=1–45)

Result analysis:
(MTB pos/neg)
MDR pos/neg

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