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Two novel protocols for inactivation and extraction were developed and used to identify 107 Mycobacterium clinical isolates, including Mycobacterium tuberculosis complex, from solid cultures using Vitek matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. The protocol using heat inactivation with sonication and cell disruption with glass beads resulted in 82.2% and 88.8% species and genus level identifications, respectively.

The Mycobacterium genus consists of over 100 species of rapidly growing and slow-growing acid–fast bacilli (AFB) (1–4). Rapid and accurate diagnosis of mycobacteria infections is important to patient care and public health (4). Inappropriate treatment may lead to unnecessary exposure to toxic drugs or drug resistance (5). Rapid identification (ID) of mycobacteria has proven difficult due in part to their fastidious growth requirements and low growth rate (3, 6). Molecular probes and DNA hybridization are relatively fast and simple but are available only for a limited number of clinically common species (2, 3, 5, 7, 8). High-performance liquid chromatography (HPLC) (2, 3, 9) and electrospray ionization–tandem mass spectrometry analysis (2) have recently been used to analyze mycolic acid but are labor-intensive and require technical expertise (1, 2).

Recent studies have shown that matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is an accurate and rapid method for identifying mycobacteria and yeast from solid culture media (6, 10–13). MALDI-TOF MS has also recently been adapted for the identification of mycobacteria (1–5, 8, 9, 14), mostly using a Bruker Daltonics Flex system (1, 2, 4, 5, 8). We previously evaluated one inactivation procedure, described in a 2010 training manual from bioMérieux, that suspended mycobacteria in trifluoroacetic acid (TFA) for 30 min (15) and found that both the procedure and the database were ineffective (data not shown). There is no standard inactivation procedure currently available for identifying mycobacteria by using MALDI-TOF MS for either the Bruker or bioMérieux system.

This study evaluates two novel inactivation and extraction protocols used to identify clinical mycobacterial isolates, including Mycobacterium tuberculosis complex (MTC), from solid culture media. In addition, this study evaluates an improved database for mycobacteria by using Vitek MALDI-TOF MS RUO (Vitek MS) (bioMérieux, Durham, NC) with a reference database developed in-house.

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A 1-µL loop was used to transfer a colony of mycobacteria from 7H11 solid medium into a microcentrifuge tube containing 500 ml of 70% ethanol and 200 ml of 0.5-mm glass beads (Sartorius Stedim; catalog no. 14-559-084). The microcentrifuge tube was vortexed for 15 min using a vortex adaptor (MoBio; catalog no. BMX13000-V1-24) and allowed to incubate at room temperature for another 10 min. The contents of the tube were vortexed for 5 to 10 s to suspend the mycobacteria. The suspension was transferred to an empty microcentrifuge tube with care to avoid the transfer of any bead. The tube was centrifuged at 10,000 × g for 2 min to create a sufficient pellet. The pellet was resuspended in 10 µL of formic acid and allowed to incubate for 2 to 5 min at room temperature. Ten microliters of acetonitrile was added to the suspension. The tube was then centrifuged at 10,000 × g for 2 min. One microliter of supernatant was added to a spot on a disposable target plate. The spot was allowed to dry completely and covered with 1 µL of CHCA matrix.

The remaining supernatant of each run was inoculated onto 7H11 solid medium to ensure successful inactivation. No mycobacterial growth was seen after 6 weeks of incubation at 37°C in 4.0 to 8.0% CO₂. This is particularly important for MTC inactivation and identification. Thus, the inactivated isolates can be used for MALDI-TOF MS in the clinical setting.

One hundred seven clinically relevant isolates, consisting of 14 species of mycobacteria, were included in the study (Table 1). After inactivation by protocol A, 88/107 (82.2%) mycobacterial isolates were correctly identified to the species or genus level. After inactivation by protocol B, 95/107 (88.8%) mycobacterial isolates were correctly identified to the species or genus level. Inactivation by protocol A resulted in a higher percentage of correct identifications at species level for *M. avium* complex and *M. kansasii* isolates. It could be argued that protocol A had an unfair advantage in identifying species of the *M. avium* complex.

### Table 1: Results of *Mycobacterium* ID by Vitek MS after inactivation by either protocol A or protocol B

<table>
<thead>
<tr>
<th>Organism</th>
<th>Total no. of isolates</th>
<th>A with:</th>
<th></th>
<th>B with:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Species level ID</td>
<td>Genus level ID</td>
<td>No ID</td>
<td>Incorrect ID</td>
</tr>
<tr>
<td><em>M. avium complex</em></td>
<td>64</td>
<td>42</td>
<td>10</td>
<td>10</td>
<td>2*</td>
</tr>
<tr>
<td><em>M. tuberculosis complex</em></td>
<td>18</td>
<td>16</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>9</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><em>M. fortuitum</em></td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. abscessus</em></td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>M. gordonae</em></td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. kubicae</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. lentiflavum</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. mucogenicum</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. scrofulaceum</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. simiae</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. szulgai</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>M. triplex</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>74</td>
<td>14</td>
<td>17</td>
<td>2*</td>
</tr>
</tbody>
</table>

* One *M. avium* complex isolate was incorrectly identified as a *Microbacterium* species and the other as *Trichophyton violaceum*.
advantage, because the database used to identify isolates was built using spectral fingerprints of isolates inactivated with the same protocol. Inactivation by protocol B resulted in a higher percentage of correct identifications at species level. Protocol B requires less processing time and fewer steps than protocol A. The higher percentage of correct identifications at species level may be a result of fewer protocol steps, resulting in fewer chances to decrease the sample recovery yield. One more MTC isolate was correctly identified by using protocol B than by using protocol A.

Failure to obtain an ID can most likely be attributed to an insufficient protein signal or an absence of an adequate reference spectrum in the database (16–18). Protocol A yielded an average of 119 mass spectrum peaks. This was not statistically different from protocol B, which yielded an average of 128 peaks ($P = 0.1426$). Spectral fingerprints of representative MAC and MTC isolates can be found in Fig. 2. The median percent match for protocol A was 61% with a range of 40 to 88%. The median percent match for protocol B was 50% with a range of 42 to 76%.

Protocols A and B are significantly simpler and safer than previously published protocols (1, 2, 5, 8, 9). Protocol A does not require washes or centrifugation before heating. Protocol B does not require any washes; processing time is under 1 h.

One limitation of this study is the relatively small database, which resulted in lower percent matches and a lack of identifications below the complex level for MTC and MAC. Though it seems that there is a lack of diversity of mycobacterial isolates in this study, MAC isolates are the most common mycobacteria isolated in our clinical setting, partially due to the high number of HIV-positive patients. Though it is important to study the clean, pure colony from solid media, future studies should adapt these protocols for liquid culture.

In summary, this study demonstrates the effectiveness of a novel heat inactivation protocol as well as a novel cell disruption inactivation protocol used for identification of clinically relevant mycobacterial isolates, including M. tuberculosis complex, from solid culture media by using Vitek MALDI-TOF (RUO) MS.

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