



## Mycobacteriology

## Rapid one-step protein extraction method for the identification of mycobacteria using MALDI-TOF MS



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## ABSTRACT

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry is a quick and accurate method for mycobacterial identification from protein extracts. Our new one-step extraction method successfully reduced routine multistep extraction procedure time from over 60 min to under 10 min and used only 1  $\mu$ L loopful of mycobacteria while providing clinically acceptable identification scores ( $\geq 1.8$ ). Overall, 86.8% and 4.4% of mycobacteria isolates ( $n = 68$ ) were identified to the species/complex and genus levels, respectively, by one-step loop extraction method, comparable to the routine extraction method. Viability studies confirmed killing of mycobacterial isolates after 5 min in the extraction solution replacing lengthy heat killing step. Retrospective 7-month data analysis showed 100% of rapidly and slowly growing mycobacterial isolates were identified to the species/complex level by rapid extraction methods. Our rapid extraction methods substantially reduced processing time and microbial biomass required for testing without sacrificing quality and accuracy of mycobacterial identification.

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## 1. Introduction

Laboratory-based diagnosis of mycobacterial infections has evolved from conventional methods including smear microscopy, culture, and biochemical tests to molecular approaches (Forbes et al., 2018; Griffith et al., 2007; Lewinsohn et al., 2017). Unfortunately, molecular methods such as commercial probe assays or gene sequencing are laborious and time-consuming. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionized microbial identification in the clinical laboratory (Carbonnelle et al., 2011; Neville et al., 2011). Protein spectra obtained from isolates or prepared extracts are compared against a database providing reliable organism identifications within minutes. This technology has significantly reduced the overall time to identification, while also saving costs for both reagents and labor (Patel, 2015; Seng et al., 2010).

MALDI-TOF MS has been used for identification of both rapidly and slowly growing mycobacteria using commercially and laboratory-developed databases (Girard et al., 2016; Mather et al., 2014; Pignone et al., 2006; Rodríguez-Sánchez et al., 2016; Saleeb et al., 2011). Despite the advances of MALDI-TOF MS, sample preparation for hardy

organisms (e.g., mycobacteria and molds) is long and labor intensive (Lau et al., 2013; Saleeb et al., 2011). Due to safety concerns and their complex cell envelope, direct spotting techniques cannot be used for mycobacteria, and therefore, a protein extraction step is required prior to acquisition of spectra. Our first published mycobacterial extraction protocol required a 30-min incubation at 95 °C to heat-kill the organisms followed by multiple steps amounting to overall ~60 min of sample processing prior to MALDI-TOF MS analysis (Saleeb et al., 2011).

In order to decrease hands-on time and steps involved in sample extraction, we developed a rapid, one-step extraction method for the identification of mycobacteria by MALDI-TOF MS without the need of a heat inactivation step. This method was further optimized for slowly growing mycobacteria. Our new rapid extraction methods dramatically reduced hands-on extraction time and required less bacterial biomass compared to the standard method while yielding comparable and clinically acceptable identification scores.

## 2. Materials and methods

## 2.1. Clinical isolates and growth conditions

Mycobacterial isolates were derived from clinical specimens obtained from patients at the NIH Clinical Center. A total of 68 clinical mycobacterial isolates (mostly prospectively obtained) were tested in parallel with the routine and loop-rapid extraction methods. Of these,

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**Table 1**  
Identification of mycobacterial isolates by routine and loop-rapid extraction methods.

Mycobacterial isolate identification (n)	Routine extraction method				Loop-rapid extraction method			
	No. (%) of isolates with the following highest scores				No. (%) of isolates with the following highest scores			
	≥2	1.8–1.99	1.7–1.79	≤1.69	≥2	1.8–1.99	1.7–1.79	≤1.69
<b>Rapidly growing mycobacteria</b>								
<i>M. abscessus</i> complex (16)	15	1	0	0	14	2	0	0
<i>M. chelonae</i> (3)	3	0	0	0	1	0	2	0
<i>M. fortuitum</i> (9)	9	0	0	0	8	1	0	0
<i>M. immunogenum</i> (1)	1	0	0	0	1	0	0	0
<i>M. mucogenicum</i> group (6)	4	2	0	0	3	3	0	0
<i>M. smegmatis</i> (2)	2	0	0	0	2	0	0	0
<i>M. wolinskyi</i> (2)	0	1	0	1	0	1	0	1
<b>Total (39)</b>	<b>34 (87.2)</b>	<b>4 (10.3)</b>	<b>0</b>	<b>1 (2.6)</b>	<b>29 (74.4)</b>	<b>7 (17.9)</b>	<b>2 (5.1)</b>	<b>1 (2.6)</b>
<b>Slowly growing mycobacteria</b>								
<i>M. avium</i> (11)	7	2	0	2	4	5	1	1
<i>M. colombiense</i> (1)	0	0	0	1	0	0	0	1
<i>M. gordonae</i> (3)	2	1	0	0	1	0	0	2
<i>M. intracellulare/M. chimaera</i> (6)	5	1	0	0	4	2	0	0
<i>M. kansasii</i> (3)	3	0	0	0	2	1	0	0
<i>M. lentiflavum</i> (1)	1	0	0	0	0	0	0	1
<i>M. tuberculosis</i> complex (4)	3	1	0	0	2	2	0	0
<b>Total (29)</b>	<b>21 (72.4)</b>	<b>5 (17.2)</b>	<b>0</b>	<b>3 (10.3)</b>	<b>13 (44.8)</b>	<b>10 (34.5)</b>	<b>1 (3.4)</b>	<b>5 (17.2)</b>
<b>Grand Total (68)</b>	<b>55 (80.9)</b>	<b>9 (13.2)</b>	<b>0</b>	<b>4 (5.9)</b>	<b>42 (61.8)</b>	<b>17 (25.0)</b>	<b>3 (4.4)</b>	<b>6 (8.8)</b>

Identification scores: ≥2 and 1.8–1.99 = species-/complex-level identification, 1.7–1.79 = genus-level identification, ≤1.69 = no identification.

39 isolates (57.4%) were rapidly growing mycobacteria, and 29 isolates (42.6%) were slowly growing mycobacteria (Table 1). In addition, with the goal to improve MALDI-TOF MS performance with slowly growing mycobacteria, a comparison between 2 rapid extraction methods (loop and swab methods) was carried out with 15 isolates of slowly growing mycobacteria including *M. avium* (n = 1), *M. gordonae* (n = 2), *M. intracellulare/M. chimaera* (n = 3), *M. marinum* (n = 1), *M. simiae* (n = 1), *M. triplex* (n = 1), and *M. tuberculosis* complex (n = 6). Mycobacterial strains were grown at 35 °C, 8% CO<sub>2</sub> on Middlebrook 7H11 agar (Remel™, ThermoFisher Scientific) until visible colonies were present.

## 2.2. Protein extraction

Protein extraction by the standard routine and rapid methods was performed in parallel from the same mycobacterial cultures. Routine extraction method was performed as previously described (Saleeb et al., 2011). Briefly, a disposable 10-μL inoculating loop was used to obtain organisms grown on Middlebrook 7H11 agar [approximately 10<sup>9</sup> CFU (Dunne et al., 2014)] which were then inoculated in 500 μL of distilled water in a 1.5-mL screw-cap tube and heat-killed at 95 °C for 30 min followed by organism resuspension using a plastic micropestle. The pellets were washed with distilled water and then 70% ethanol. The proteins were extracted from washed pellet in 70% formic acid by vortexing with 0.1-mm-diameter zirconia/silica beads for 10 min (BioSpec Products) followed by addition of 100% acetonitrile with additional 10 min vortexing step. Then, the supernatant was utilized for analysis by MALDI-TOF MS (Fig. S1).

For the loop-rapid extraction method, a disposable 1-μL inoculating loop was used to obtain approximately 10<sup>7</sup>–10<sup>8</sup> CFU mycobacteria (Dunne et al., 2014) grown on Middlebrook 7H11 agar. A loopful of organisms was inoculated into 200 μL of extraction solution containing 70% formic acid and 100% acetonitrile (1:1) along with 50 mg of 0.1-mm-diameter zirconia/silica beads in a 1.5-mL screw-cap tube. The samples were incubated at room temperature for 5 min to kill the mycobacteria. Proteins were extracted by disruption of the organisms in a PowerLyzer 24 high power bead-based homogenizer (Mo Bio Laboratories, Inc.) with 2 cycles of 45 s at 4,000 rpm with a 30-s rest interval in between. Samples were centrifuged at 13,000 rpm for 1 min. Then, 1 μL of extract was directly spotted onto a target plate for MALDI-TOF MS analysis. For

the swab-rapid extraction method developed for limited biomass of the slowly growing mycobacteria, a swab moistened in sterile water was used to obtain mycobacteria from the Middlebrook 7H11 agar into 500 μL of sterile water in a 1.5-mL screw-cap tube. The samples were centrifuged at 13,000 rpm for 2 min and the supernatant discarded. The pellets were then resuspended in the extraction solution with zirconia/silica beads and vortexed briefly, and then protein was extracted as a loop-rapid extraction method (Fig. S1).

## 2.3. Viability study

Determination of mycobacterial viability was assessed after 5-min exposure to extraction solution. Ten mycobacterial isolates of rapidly and slowly growing mycobacteria including *M. abscessus* complex (n = 2), *M. avium* (n = 2), *M. fortuitum* (n = 2), *M. bovis* BCG (n = 1), *M. smegmatis* (n = 1), *M. gordonae* (n = 1), and *M. chelonae* (n = 1) were tested. A 1-μL loopful of organisms on Middlebrook 7H11 agar was resuspended in 100 μL of either phosphate buffer (M/15, AlphaTec) or 70% formic acid and 100% acetonitrile mix (1:1) and then incubated for 5 min at room temperature. A volume of 45 μL was then inoculated into a mycobacteria growth indicator tube (MGIT) (Becton Dickinson) supplemented with 0.8 mL of PANTA and supplement solution as per manufacturer's procedures. The MGITs were incubated at 37 °C for 6 weeks in BD BACTEC MGIT 960 automated mycobacterial detection system at which time to positivity (TTP) was recorded for all tubes that flagged positive. Positive MGIT tubes were evaluated by auramine-rhodamine (AR) and Gram staining. Negative MGIT tubes at 6 weeks were reported as no growth.

## 2.4. MALDI-TOF MS for mycobacterial identification

One microliter of extracted supernatant was spotted in duplicate on MSP BigAnchor 96 BC target plates (Bruker Daltonics, Inc.) and dried on a slide warmer at 42 °C. Additionally, 1 μL of bacterial test standard and extraction solution was spotted onto the plate as a calibration standard and negative control, respectively. Dried sample and control spots were overlaid with 1 μL of matrix solution (50% acetonitrile and 2.5% trifluoroacetic acid solution with α-cyano-4-hydroxycinnamic acid) and allowed to dry on a slide warmer. Sample spots were then analyzed by the MALDI-TOF Microflex LT mass spectrometer (Bruker Daltonics, Inc.) as described previously (Luethy and Zelazny, 2018). Spectra were

analyzed by the Biotyper program utilizing both the Bruker database (version 3.1) and a laboratory-developed NIH database previously described (Saleeb et al., 2011). The reference intervals for MALDI-TOF MS identification for reporting mycobacteria are: scores  $\geq 1.8$  for species, group- or complex-level identification, scores 1.70–1.79 for genus-level identification, and score  $\leq 1.69$  for no identification (Rodriguez-Sanchez et al., 2016; Saleeb et al., 2011). From 2 analyzed spots of each isolate, the highest scoring spot was taken for analysis. *M. smegmatis* ATCC 19420 (CCUG 21002T) was used as positive quality control in all experiments. Identification results were reported at genus, species, or group/complex following laboratory protocols.

### 2.5. Targeted genomic sequencing for mycobacterial identification

Isolates that had scores  $\leq 1.69$  on repeat testing with MALDI-TOF MS were identified by sequencing targeting *secA1* (Zelazny et al., 2005) and/or 1500-bp 16S rRNA as per standard laboratory protocols. Briefly, primers complementary to target gene were used to amplify DNA, and the amplicon was sequenced with a Big Dye Terminator X kit (Life Technologies). The ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) was utilized for sequencing. The sequencing results were analyzed for quality and compared against GenBank and our laboratory-created database.

### 2.6. Retrospective comparison study

The MALDI-TOF MS identification scores from mycobacterial isolates from patients at the NIH Clinical Center during 7-month periods in 2 consecutive years were compared retrospectively to assess the performance of 2 rapid extraction methods using loop or swab methods compared to the routine extraction method. The loop-rapid extraction method was used for rapidly growing mycobacteria, while the swab-rapid extraction method was used for slowly growing mycobacteria. The highest scores from 2 spots from a total of 195 isolates including 97 rapidly growing and 98 slowly growing mycobacteria extracted by the routine method during June 2016 to December 2016 and total of 176 isolates including 80 rapidly growing and 96 slowly growing mycobacteria extracted by loop- and swab-rapid extraction methods, respectively, during June 2017 to December 2017 were collected for analysis (Tables 2 and 3).

### 2.7. Data and statistical analysis

McNemar's test using GraphPad Prism was used to analyze scores generated by the routine extraction method and the loop-rapid extraction method as well as those from the comparison study between loop- and swab-rapid extraction methods on slowly growing mycobacteria. A *P* value less than 0.05 was considered as a statistically significant difference.

## 3. Results

### 3.1. Performance of one-step rapid protein extraction procedure and identification analysis

A comparison of routine and loop-rapid extraction methods was performed on mycobacterial isolates from patients at the NIH Clinical Center. In total, 68 mycobacterial isolates, mostly prospectively collected (39 rapidly growing mycobacteria and 29 slowly growing mycobacteria), were tested (Table 1). Using the routine extraction method, 64/68 (94.1%) of mycobacterial isolates were identified to the species/complex level (score  $\geq 1.8$ ). However, 4/68 (5.9%) of isolates comprising *M. wolinskyi* ( $n = 1$ ), *M. avium* ( $n = 2$ ), and *M. colombiense* ( $n = 1$ ) failed species- or genus-level identification (Table 1). MALDI-TOF MS scores range using the routine extraction method was 1.39 to 2.45 with a median score of 2.25. In comparison, using the loop-rapid extraction method, the Biotyper identified 59/68 (86.8%) isolates to the species/complex level; 3/68 (4.4%) isolates to the *Mycobacterium* genus level; and 6/68 (8.8%) isolates including *M. wolinskyi* ( $n = 1$ ), *M. avium* ( $n = 1$ ), *M. colombiense* ( $n = 1$ ), *M. gordonae* ( $n = 2$ ), and *M. lentiflavum* ( $n = 1$ ) with no genus- or species-level identification (Table 1). The range of scores was 1.37 to 2.37 with a median score of 2.03. Overall reference intervals scores obtained with the routine extraction method versus the loop-rapid extraction method were not significantly different ( $P = 0.0736$ ). Duplicate spots from each isolate generated scores that fell within the same reporting interval ( $\geq 1.8$ , 1.7–1.79, and  $\leq 1.69$ ) for 61/68 (89.7%) isolates by the routine method and 63/68 (92.6%) isolates by loop-rapid extraction method (data not shown).

For rapidly growing mycobacteria, the Biotyper could identify 38/39 (97.4%) isolates to the species/complex level, while 1/39 (2.6%) isolate failed genus and species identification using the routine extraction method. The range of identification scores for rapidly growing mycobacteria by routine extraction method was 1.53 to 2.45 with a median score of 2.3. Using loop-rapid extraction method, 36/39 (92.3%) isolates, 2/39 (5.1%) isolates, and 1/39 (2.6%) were identified to species-/complex-level, genus-level, and no identification, respectively (Table 1). The range of identification scores for rapidly growing mycobacteria by loop-rapid extraction method was 1.37 to 2.37 with a median score of 2.1. The routine extraction method and loop-rapid extraction method were not significantly different at overall reference intervals scores ( $P = 0.4795$ ) for the rapidly growing mycobacteria identification.

For the slowly growing mycobacteria, the routine extraction method produced reporting scores to species/complex level for 26/29 (89.7%) isolates and no identification for 3/29 (10.3%) isolates. The range of identification scores for slowly growing mycobacteria with the routine extraction method was 1.39 to 2.38 with a median score of 2.21. By using the loop-rapid extraction method, there were 23/29 (79.3%)

**Table 2**

Identification of rapidly growing mycobacterial isolates by routine and loop-rapid extraction methods (retrospective comparison study).

Mycobacterial isolate identification	Routine extraction method (2016)				Loop-rapid extraction method (2017)					
	No. of isolates	No. (%) of isolates with the following highest scores				No. of isolates	No. (%) of isolates with the following highest scores			
		$\geq 2$	1.8–1.99	1.7–1.79	$\leq 1.69$		$\geq 2$	1.8–1.99	1.7–1.79	$\leq 1.69$
<i>M. abscessus</i> complex	50	49	1	0	0	47	40	7	0	0
<i>M. chelonae</i>	1	1	0	0	0	1	0	1	0	0
<i>M. fortuitum</i> group	6	6	0	0	0	3	3	0	0	0
<i>M. mucogenicum</i> group	11	8	3	0	0	2	2	0	0	0
<i>M. immunogenum</i>	1	1	0	0	0	0	0	0	0	0
<i>M. neoaurum</i>	0	0	0	0	0	1	1	0	0	0
<i>M. obuense</i>	1	1	0	0	0	0	0	0	0	0
<i>M. smegmatis</i>	27	25	2	0	0	26	25	1	0	0
<b>Total</b>	<b>97</b>	<b>91 (93.8)</b>	<b>6 (6.2)</b>	<b>0</b>	<b>0</b>	<b>80</b>	<b>71 (88.8)</b>	<b>9 (11.2)</b>	<b>0</b>	<b>0</b>

Identification scores:  $\geq 2$  and 1.8–1.99 = species-/complex-level identification, 1.7–1.79 = genus-level identification,  $\leq 1.69$  = no identification.

**Table 3**  
Identification of slowly growing mycobacterial isolates by routine and swab-rapid extraction methods (retrospective comparison study).

Mycobacterial isolate identification	Routine extraction method (2016)				Swab-rapid extraction method (2017)					
	No. of isolates	No. of isolates with the following highest scores				No. of isolates	No. of isolates with the following highest scores			
		≥2	1.8–1.99	1.7–1.79	≤1.69		≥2	1.8–1.99	1.7–1.79	≤1.69
<i>M. avium</i>	52	52	0	0	0	37	36	1	0	0
<i>M. europaeum</i>	0	0	0	0	0	1	0	1	0	0
<i>M. intracellulare/M. chimaera</i>	35	35	0	0	0	44	44	0	0	0
<i>M. kansasii</i>	0	0	0	0	0	3	3	0	0	0
<i>M. goodii</i>	4	2	2	0	0	4	4	0	0	0
<i>M. heckeshornense</i>	1	1	0	0	0	0	0	0	0	0
<i>M. lentiflavum</i>	0	0	0	0	0	1	0	1	0	0
<i>M. paragordoniae</i>	0	0	0	0	0	3	1	2	0	0
<i>M. simiae</i>	1	1	0	0	0	1	1	0	0	0
<i>M. tuberculosis</i> complex	4	4	0	0	0	2	2	0	0	0
<i>M. xenopi</i>	1	1	0	0	0	0	0	0	0	0
<b>Total</b>	<b>98</b>	<b>96 (98.0)</b>	<b>2 (2.0)</b>	<b>0</b>	<b>0</b>	<b>96</b>	<b>91 (94.8)</b>	<b>5 (5.2)</b>	<b>0</b>	<b>0</b>

Identification scores: ≥2 and 1.8–1.99 = species-/complex-level identification, 1.7–1.79 = genus-level identification, ≤1.69 = no identification.

isolates, 1/29 (3.4%) isolate, and 5/29 (17.2%) isolates identified to species/complex, genus, and no identification, respectively (Table 1). The range of scores produced by loop-extraction method for slowly growing mycobacteria was 1.40 to 2.20 with a median score of 1.95. No statistically significant difference of overall reference intervals scores between the 2 methods was observed for slowly growing mycobacteria ( $p = 0.2482$ ).

Additionally, we performed a pilot study to evaluate 2 rapid extraction methods on slowly growing mycobacteria using a loop or a swab for colony collection from Middlebrook 7H11 agar. We hypothesize that the swab collection method can improve the scores from MALDI-TOF MS analysis for lower biomass samples as seen with slowly growing mycobacteria. Using the loop-rapid extraction method resulted in identification to species level (score ≥ 1.8) for 15/15 (100%) isolates (Table S1) with a range of scores of 1.80 to 2.32 and a median score of 2.06. The use of swab-rapid extraction method demonstrated 14/15 (93.3%) isolates with scores identified to species level and 1/15 (6.7%) isolate with genus-level identification (Table S1) with a range of scores of 1.78 to 2.45 and a median score of 2.20. The result between 2 rapid extraction methods for slowly growing mycobacteria identification was not significantly different for overall reference intervals scores ( $P = 1$ ). However, the swab-rapid extraction method generally yielded improved scores compared to the loop-rapid extraction method (Table S2).

### 3.2. Viability study

Loss of viability was determined using MGIT cultures on 10 mycobacterial isolates immersed in the extraction mixture of the rapid-extraction method. As expected, all 10 mycobacterial isolates exposed to phosphate buffer (positive control) showed positive growth with TTP of MGIT tubes ranging between 9 and 102 h. All positive MGIT cultures were confirmed for growth of acid-fast bacilli by AR and Gram staining. No growth was observed for all 10 mycobacterial isolates placed in the 70% formic acid and 100% acetonitrile solution (1:1) for 5 min with or without bead-beating step (data not shown). Four of the strains (*M. abscessus*, *M. avium*, *M. fortuitum*, and *M. bovis* BCG) placed in 70% formic acid and 100% acetonitrile solution were also inoculated (45 µL) onto Middlebrook 7H11 agar plates; no growth was observed for any of them (data not shown). Overall, these data demonstrate that 5-min exposure to a 1:1 solution of 70% formic acid and 100% acetonitrile is sufficient for killing of mycobacteria.

### 3.3. Retrospective analysis of mycobacterial identification with routine and rapid extraction methods

We analyzed two 7-month intervals of reported scores of mycobacterial isolates before and after implementation of rapid extraction methods

(which occurred in 2017). Data from 2016 showed that the routine extraction method identified 97/97 (100%) of rapidly growing mycobacteria to species or group/complex level (Table 2) with a score range of 1.87 to 2.52 and a median score of 2.32 and a total of 98/98 (100%) slowly growing mycobacteria to species and complex level (Table 3) with a score range of 1.84 to 2.46 and a median score of 2.24. The same 7-month period in 2017 showed 80/80 (100%) of rapidly growing mycobacteria identified to species using the loop-rapid extraction method (Table 2) with a score range of 1.82 to 2.46 and a median score of 2.19. For the slowly growing mycobacteria, the swab-rapid extraction method yielded 96/96 (100%) identification to the species or group/complex level (Table 3), with a score range of 1.89 to 2.44, and a median score of 2.2.

Our retrospective data comparison revealed equivalent performance of MALDI-TOF MS identification using routine extraction method compared to rapid extraction methods for the 2 most common rapidly and slowly growing nontuberculous mycobacteria (NTM) in our institution, *M. abscessus* complex and *M. avium* complex which includes *M. avium* and *M. intracellulare/M. chimaera*. *M. abscessus* complex showed 51.5% (50/97) prevalence within the rapidly growing mycobacteria between June and December 2016 with 100% (50/50) identified with routine extraction method versus 58.8% (47/80) prevalence between June and December 2017 with 100% (47/47) identified using the loop-rapid extraction method (Table 2). For *M. avium* complex, the prevalence was 88.8% (87/98) within slowly growing mycobacteria between June and December 2016 with 100% (87/87) identified by routine extraction method versus 84.4% (81/96) prevalence between June and December 2017 with 100% (81/81) identified using the swab-rapid extraction method (Table 3).

## 4. Discussion

MALDI-TOF MS has emerged as a reliable, fast, and cost-effective system for microbial identification (Carbonnelle et al., 2011; Neville et al., 2011) for a variety of microorganisms (Chen et al., 2013; Lau et al., 2013; Luethy and Zelazny, 2018; Neville et al., 2011; Totty et al., 2016). Commercial MALDI-TOF MS databases have been expanded with additional mycobacteria (Mather et al., 2014; Rodriguez-Sanchez et al., 2016). Protein extraction procedures for the bacterial identification by MALDI-TOF MS are simple and can be done with one extraction step (Alatoom et al., 2011; Neville et al., 2011). In contrast, mycobacterial protein extraction procedures require additional steps because of their unique waxy cell envelope containing mycolic acids. A recent meta-analysis review of published data concluded that the diagnostic performance of MALDI-TOF MS for mycobacterial identification could not fully meet the need of clinical microbiology laboratory with only 85% and 71% of the isolates identified to genus and species level, respectively. Differences in databases and MALDI-TOF MS system, sample preparation,

and the species included in each study were mentioned as possible factors to explain the varying overall identification rates among the studies (Cao et al., 2018). Interestingly, in our institution, we have experienced high identification success rates using a combined in-house and commercial database as shown in this and a previous study (Saleeb et al., 2011).

Our initial mycobacterial extraction protocol required heat, ethanol, and beads to disrupt and kill the mycobacteria. Although this routine extraction method has proven effective in our laboratory for species-/complex-level identification by MALDI-TOF MS, it is extremely time consuming, taking more than 60 min, with extra time added for each additional sample (Saleeb et al., 2011). Due to safety concerns, various mycobacterial killing approaches have been evaluated along extraction protocols including mechanical disruption (Totty et al., 2016) or heat inactivation followed by cell wall disruption (Adams et al., 2015; Machen et al., 2013; Mather et al., 2014) or incubation in 70% ethanol for 10 min (Lotz et al., 2010). Several studies have shown the role of bead-beater homogenizers in mycobacterial inactivation (Dunne et al., 2014; Machen et al., 2013; Totty et al., 2016). Recently, a mycobacterial protein extraction protocol using Adaptive Focused Acoustics (AFA) technology (Adams et al., 2016) was evaluated. The optimized AFA method provided both a reliable mycobacterial inactivation and a rapid and efficient protein extraction. However, AFA technology requires the purchase of a focused ultrasonicator and manufacturer-provided consumables, making it more costly than our laboratory-developed method.

The recommended extraction method for mycobacterial identification with the Bruker Biotyper instrument includes heat inactivation (BRUKER, 2014; O'Connor et al., 2016). However, our rapid extraction methods provided reliable results without the need for a heat inactivation step. In addition, our viability study showed exposure to 70% formic acid and 100% acetonitrile extraction solution for 5 min even without bead-beating procedure could kill mycobacteria. Therefore, 5 min post inoculation in the extraction mixture allows the rapid extraction protocol to be performed outside the BSL-3.

Although the median scores from the loop-rapid extraction method from the prospective study were slightly lower than those from routine extraction method (2.03 vs 2.25), both methods were not significantly different for mycobacterial identification to species and complex/group level for both rapidly and slowly growing mycobacteria with the cutoff score  $\geq 1.8$  used in our institution and published studies (Rodriguez-Sanchez et al., 2016; Saleeb et al., 2011). The possible reason for the lower identification score of loop-rapid extraction method could be the lower biomass from utilizing only 1  $\mu\text{L}$  loopful of organisms compared to the 10  $\mu\text{L}$  loopful of organisms used for the routine method and recommended by Bruker (BRUKER, 2014). The size of loop was shown to impact the quantity of cell density of inoculum with different degrees based on the mycobacterial species analyzed (Dunne et al., 2014). The inconsistencies in mycobacterial number could affect the efficacy of mycobacterial identification; in particular, low number of mycobacteria can decrease the identification score. Therefore, we have modified the loop rapid extraction method by using the swab to obtain more biomass of organisms with a focus on the slowly growing mycobacteria. More mycobacteria can bind to a moistened swab compared to using a loop prior to elution into the water. However, the higher number of organisms collected using a swab may also increase the potential for interferences from the solid media. The brief resuspension and washing step with water not only helps to remove mycobacterial cells from the swab but also reduces possible interferences from the solid media. Based on this and our previous study (Saleeb et al., 2011), spotting mycobacterial extracts in duplicates is recommended for mycobacterial identification by MALDI-TOF MS.

## 5. Conclusions

The new rapid mycobacterial extraction methods for MALDI-TOF MS described in this work demonstrated an equivalent performance at

species-/complex- or group-level identification (score  $\geq 1.8$ ) to our routine extraction method for both rapidly and slowly growing mycobacteria. In addition, the rapid extraction method provides several advantages: low cost, shorter extraction time, fewer number of steps, lower number of organisms required, and high reproducibility as compared to the routine extraction method.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.diagmicrobio.2019.03.004>.

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