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A rapid, standardized protein extraction method using adaptive focused acoustics for identification of mycobacteria by MALDI-ToF MS

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ABSTRACT

Mycobacterial identification using MALDI-ToF MS (MALDI) has been hindered by inadequate extraction methods. Adaptive Focused Acoustics™ uses concentrated ultrasonic energy to achieve cellular disruption. Using this technology, we developed a rapid mycobacterial inactivation/protein extraction method for MALDI-based identification. Agreement for identification to the species level versus conventional identification was stratified by log confidence cut-offs of ≥2.0, ≥ 1.8, or ≥1.7. A total of 182 mycobacterial isolates were tested. Complete inactivation of all species/strains was achieved after 2 min. Using a log confidence cut-off of ≥2.0, overall agreement for the commercial method (CM) was 41.7% versus 66.7% for the novel method (NM). For the CM, agreement increased to 66.7% and 83.3% using log confidence cut-offs of ≥1.8 and ≥1.7, respectively; for the NM, agreement was 100% for both cut-offs with all isolates. With no alteration to the existing database, overall agreement for the NM was 83.4%, largely due to low scores for clinical isolates of *M. chelonae* and *M. mucogenicum*. Addition of spectra from a single clinical strain of each species to the existing database increased overall agreement to 93.1%.

1. Introduction

Mycobacteria are ubiquitous in nature, being found in air, water, and soil. They are aerobic, non-motile rods which vary morphologically in growth on solid media (Pfyffer 2015). A large portion of the mycobacterial cell wall is composed of mycolic acids, complex glycolipids, glycopeptidolipids, waxes and other fatty acids providing a significant hydrophobic permeability barrier (Pfyffer 2015). Clinically, mycobacteria can cause devastating systemic and peripheral infections in both immunocompromised and immunocompetent individuals. However, appropriate treatment often varies between species making rapid identification essential for accurate diagnosis and effective antibiotic therapy.

Previous studies have established that matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF MS) can rapidly and accurately identify bacteria and yeasts from growth on solid media saving both time and expense (Croxatto et al. 2012; Fenselau and Demirev 2001; Mancini et al. 2013; Martiny et al. 2012; Tan et al. 2012). Also, MALDI-ToF MS has been used for mycobacterial identification to the species level (Lotz et al. 2010; Saleeb et al. 2011). However, unlike bacteria and yeast, routine clinical use of MALDI-ToF MS for mycobacterial identification has been hindered by difficulties related to peptide extraction due to the intrinsic characteristics of the cell wall (Pignone et al. 2006). To date, most published extraction protocols lack standardization, require multiple steps, including an initial 30 min heat inactivation step, and can take an hour to 2 hours for completion for a limited number of isolates at a time (Adams et al. 2015; Dunne et al. 2014; El Khéchine et al. 2011; Lin et al. 2015; Machen et al. 2013). A more rapid, standardized, extraction/inactivation method is needed to permit routine use of MALDI-ToF MS for mycobacterial identification in clinical laboratories.

Adaptive Focused Acoustics™ (AFA) uses concentrated bursts of ultrasonic energy at frequencies much higher than that can be achieved with an ordinary sonicator. These frequencies produce a wavelength of only a few millimeters which enables the ultrasonic acoustic energy to be focused into a discrete zone within a sample vessel immersed in a water bath (Li et al. 2015; Onigman et al. 2015; Quail et al. 2008). The variables which control the quality of energy utilized are peak incidence power (PIP, the amount of wattage applied), duty factor (DF, the percentage of time AFA is transmitted), the duration of time AFA is applied, and the temperature of the water bath. Small microTUBES™ containing glass beads provide for effective sample containment while ensuring exposure to AFA allowing for rapid disruption of the cell wall and concomitant peptide extraction into a mixture of acetonitrile/70% formic acid.

In the current study, we 1) evaluated the use of AFA and ultrasonication (Covaris®, Woburn, MA) as a means to rapidly extract mycobacterial peptides from growth on solid media with subsequent identification to the species level using MALDI-ToF MS and an existing commercial database, 2) investigated the use of AFA as a means to rapidly inactivate all species of mycobacteria tested versus conventional methods, 3) optimized the AFA-specific protocol for clinical use in

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identification of mycobacteria to the species level from growth on solid media, and 4) determined the time savings versus conventional extraction/inactivation methods.

2. Materials and methods

2.1. Mycobacteria

All mycobacterial strains used in this study were cultured on Löwenstein–Jensen agar slants (LJ, Becton Dickinson, Sparks, MD) in an atmosphere of 5% CO_2 at 37 °C. A total of 182 isolates were tested representing 21 species (American Type Culture Collection, ATCC, Manassas, VA, the Johns Hopkins Hospital reference collection, and 160 unique clinical isolates) (Table 1). Conventional identification to the species level was performed using nucleic acid probes (Accuprobe, GenProbe, San Diego, CA) or 16S rDNA gene sequencing (Lin et al. 2015).

2.2. Peptide extraction and MALDI-ToF MS

Initial evaluation and optimization of the AFA-ultrasonic method (AFA) was performed using two species of non-tuberculous mycobacteria (NTM): *Mycobacterium abscessus* and the *M. avium* complex. Peptide extracts were prepared for AFA using an M220 ultrasonicator (Covaris) and microTUBES (Covaris) containing either glass beads (silica glass beads 0.5 mm, Covaris) or fibers (Covaris); and, 110 μL of water, acetonitrile (Sigma-Aldrich®, St. Louis, MO), and/or 70% formic acid (Sigma-Aldrich). For all assays, a 1 μL loopful of mycobacterial growth was collected from colonies grown on LJ agar slants and added to individual microTUBES. Instrument settings evaluated included PIP of 40 or 75 watts (W), DF of 20% or 50%, and duration ranging from 1 to 3 min at 18 °C with or without a final centrifugation at 13,000 rcf for 2 min. Following this, the supernatant from completed extracts was spotted (4 spots per sample) on polished steel plates (Bruker Daltonics, Billerica, MA) and identification to the species level achieved with MALDI-ToF MS using the Bruker MicroFlex LT (MicroFlex LT) mass spectrometer and Bruker Biotyper software and existing database (version 2.0, Bruker). All MALDI-ToF MS analysis included the use of 1 μL bacterial test calibration standard (BTS) and application of HCCA matrix (α-cyano-4-hydroxycinnamic acid) over each dried sample spot which was allowed to air dry (Bruker Daltonics 2015; Croxatto et al. 2012; Fenselau and Demirev 2001; Li et al. 2015). Once dried, steel plates were placed in the MicroFlex LT, and approximately 1000 laser shots over 20 sites applied to each sample generating spectra derived from calculation of the mass to charge ratio (m/z). All data was analyzed using log confidence score cut-offs of ≤1.7, ≥ 1.7, ≥ 1.8, and ≥2.0. All AFA results were compared to those obtained using a commercial extraction method (CM) for peptide extraction as previously described (Casanova et al. 2013). Briefly, for the CM, the biomass was initially placed in 75% ethanol and washed once with 500 μL of sterile water. Cell pellets were re-suspended in 50 μL of sterile water and heat inactivated at 95 °C for 30 min. Afterwards, 1.2 mL of 100% ethanol was added, followed by centrifugation, removal of the supernatant, and addition of 0.5 mm Zirconia/silica beads. Pellets were then re-suspended in acetonitrile, vortexed for 1 min, and 70% formic acid added. Finally, all samples were vortexed for 5 seconds, centrifuged, and 1 μL of the supernatant applied to the MALDI-ToF MS target for further analysis as described above (Casanova et al. 2013). All assays were performed in duplicate.

2.3. Mycobacterial inactivation using AFA and time studies

Determination of the effect of AFA on mycobacterial inactivation was done for all species of mycobacteria tested in this study. Initial viable counts used for AFA were estimated for all mycobacterial species by placing a 1 μL loopful of growth from an LJ agar slant into a microTUBE containing sterile water and glass beads, followed by vortexing to break up any clumps, and serially diluted to obtain countable colonies. This procedure was repeated after 2 min of incubation at 18 °C without AFA to demonstrate any temperature-mediated effects on viable counts. The effect of the optimized AFA protocol [40% watts (PIP)/50% DF] on

Table 1
Mycobacterium species and strains used in this study.

Slow growers* (n = 87)			Rapid growers* (n = 95)		
	ATCC and reference strains	Clinical		ATCC	Clinical
M. asiaticum ATCC25276	1	0	***M. abscessus*** ATCC19977	1	25
M. avium-intracellulare complex ATCC700898	1	13	***M. chelonae*** ATCC35752	1	16
M. bohemicum ATCC44277	1	0	***M. fortuitum* complex** ATCC6841	1	25
M. gordonae ATCC14470	1	24	***M. peregrinum*** ATCC700686	1	0
M. haemophilum JHH Reference Strain	1	0	***M. mageritense*** ATCC700351	1	1
M. kansasii ATCC12478	1	0	***M. mucogenicum*** ATCC49650	1	21
M. lentiflavum JHH Reference Strain	1	0	***M. phlei*** ATCC11758	1	0
M. marinum BAA535	1	10			
M. scrofulaceum JHH Reference Strain	1	0			
M. simiae ATCC25275	1	0			
M. szulgai ATCC35799	1	1			
M. terrae JHH Reference Strain	1	0			
M. tuberculosis complex ATCC25177 & ATCC27294	2	24			
M. xenopi JHH Reference Strain	1	0			

Abbreviations: ATCC = American Type Culture Collection, Manassas, VA.
* Mycobacteria are classified according to growth rates, rapid-growers exhibit growth in culture in ≤7 days; slow-growers require >7 days (Pfyffer 2015).

mycobacterial viability was determined by placing a 1 μL loopful of all samples into individual microTUBES containing 110 μL of acetonitrile: 70% formic acid, followed by ultrasonication for 2 min at 18 °C. At this point, in one series of experiments the entire contents of each microTUBE was completely transferred to a Mycobacterial Growth Indicator Tube (Becton Dickinson, MGIT 960 System) containing 0.8 mL of supplement (Becton Dickinson) and incubated on the MGIT 960 automated culture detection system until either a positive growth signal was obtained or for a total of 6 weeks. In a separate series of experiments, the entire contents of each microTUBE was transferred to a 1.5 mL Eppendorf tube (VWR, Radnor, PA) and the wash steps repeated as mentioned above. For all assays, a calibrated loop was used to inoculate (10 μL) of completely washed, re-suspended pellets onto Middlebrook 7H11 agar plates (Remel, Lenexa, KS). The remaining contents of the tube (~100 μL) were then transferred by pipetting to a separate plate. All plates were incubated for a total of 6 weeks for evidence of growth. All assays were performed in duplicate.

A separate study was conducted in which an observer monitored the time of two different technologists performing both AFA and the CM for all steps up to spotting of the MALDI plates. The average of 3 separate experiments for two different technologists was used to determine the final required time for completion of each assay (AFA versus CM).

3. Results

3.1. Optimization of AFA versus the CM and testing of mycobacterial type strains

Using type strains of *M. abscessus* and the *M. avium* complex, initial optimization of the AFA method revealed that a 1 μL loopful of bacterial biomass, 110 μL of acetonitrile: 70% formic acid and instrument settings of 75 watts PIP/20% DF versus 40 watts PIP/50% DF, resulted in similar average log confidence scores with the existing Bruker database of 1.84 and 1.87, respectively. However, there were differences and for this reason data was stratified by log confidence cut-offs of ≥2.0, ≥ 1.8, ≥ 1.7 for all subsequent assays. Instrument settings of 40 watts PIP/50% DF resulted in consistently higher ranges in log confidence scores (1.76–2.10) versus that observed for 75 watts PIP/20% DF (1.66–2.01). For the two species considered together, the use of beads in the microTUBES yielded higher log confidence scores (average 1.90, range 1.57–2.14) compared to fibers (average 1.80, range 1.62–2.02), regardless of instrument settings. Exposure time to AFA also affected log confidence scores. Two minutes was found to be optimal for both *M. abscessus* and the *M. avium* complex yielding an average log confidence score of 1.91, whereas for 1 and 3 min exposures, the log confidence average decreased to 1.84. Centrifugation of the microTUBES at 13,000 rcf for 2 min following AFA resulted in similar average log confidence scores (1.92) as those without centrifugation; however, the variability in the range was much narrower (± 0.15 points versus ±0.64 points). For this reason, a final centrifugation step was added to the protocol for subsequent testing. Additional ATCC strains of *M. tuberculosis* and other NTM were tested using the optimized parameters of 40 watts (PIP)/50% DF for 2 min, glass beads containing 110 μL of acetonitrile: 70% formic acid, and a final centrifugation step as shown in Table 2. These same strains were subjected to the standard CM for peptide extraction and compared to those obtained using the AFA method (Table 2). For all species considered together the CM resulted in an average log confidence score of 1.96 (range 1.52–2.20) versus 2.07 (range 1.90–2.26) for AFA. Differences were also noted between the two methods and rapid- and slow-growing mycobacteria. For the CM, log confidence scores were 1.85 (range 1.52–2.09) and 1.99 (range 1.73–2.20) for the rapid- and slow-growing species, respectively. For AFA, average log confidence scores for rapid-growing species was 2.06 (range 1.90–2.23) and 2.10 (range 1.90–2.26) for the slow-growing NTM. Data stratified by log confidence cut-offs of ≥2.0, ≥ 1.8, or ≥1.7, illustrate clear differences between the methods in percent agreement for identification to the species level. For instance, using a log confidence cut-off of ≥2.0, agreement for the CM for correct identification to the species level was 41.7% versus 66.7% for AFA. Log confidence scores did improve for the CM with cut-offs of ≥1.8 and ≥1.7 in which agreement versus conventional identification was 66.7%, and 83.3%, respectively. In comparison, the AFA method yielded 100% agreement for the ATCC strains versus conventional identification with log confidence scores of ≥1.8 or ≥1.7.

Table 2
Log confidence scores for AFA method versus the CM for ATCC and reference strains of mycobacteria used for protocol optimization.*

Mycobacterial species	CM	AFA
M. abscessus	1.95	1.90
M. asiaticum	1.73	1.99
M. avium complex	2.12	1.96
M. fortuitum	1.52	2.03
M. gordonae	1.78	1.90
M. kansasii	2.11	2.08
M. lentiflavum	1.97	2.23
M. phlei	2.09	2.25
M. simiae	1.92	2.09
M. szulgai	2.20	2.26
M. tuberculosis complex	2.13	2.11

Abbreviations: CM = commercial method; AFA = adaptive, focused, acoustics-ultrasonication method.
* Numbers indicate log confidence scores averaged for 4 replicates for each species.

To reiterate, optimization of the AFA-based protocol was done using a select number of type strains of NTM species commonly encountered in our laboratory. The number of type and reference strains did not include all isolates in our archive nor were clinical isolates utilized for initial optimization since prior investigation using the standard CM had shown the latter to be more variable. Thus, in order to optimize the AFA-based protocol, a more limited approach was taken in which the number of type- and reference-strains was restricted to those viewed in Table 2.

Determination of the optimal number of spots to be tested for the CM versus the optimized AFA method was done using all ATCC species/strains as listed in Table 2 with the number of spots ranging from 2 to 4. As shown in Table 3, for the AFA method, agreement for all species considered together was 100% regardless of the number of spots tested using a log confidence score of ≥1.7 and the existing Bruker database. In contrast, for the CM, overall agreement using the same log confidence score required 4 spots to achieve 77.5% agreement which then decreased to 38.3% when using only 2 spots. No significant differences in agreement were observed between rapid- and slow-growing species.

3.2. AFA testing of clinical isolates and addition of spectra to the existing database

Testing of clinical isolates with the optimized AFA method revealed differences versus those observed with type and reference strains. As shown in Table 4, overall agreement varied by the cut-off used for log

Table 3
Effect of the number of spots between methods on % agreement for ATCC and reference strains of mycobacteria.

	% Agreement (log confidence score ≥ 1.7)*					
	2 replicates		3 replicates		4 replicates	
	AFA	CM	AFA	CM	AFA	CM
Overall	100	38.3	100	57.9	100	77.5
Slow growers	100	37.5	100	56.7	100	75.8
Rapid growers	100	39.2	100	59.2	100	79.2

Abbreviations: CM, commercial method; AFA, adaptive, focused, acoustics-ultrasonication method.
* A log confidence cut-off of ≥1.7 was used for assessment of the optimal number of spots to be used for each method.

Table 4
Differences in % agreement for the AFA method before and after the addition of spectra from 2 JHH clinical isolates to the existing commercial library.

	Before addition			After addition		
Log confidence score cut-off	Overall	RG	SG	Overall	RG	SG
≥1.70	83.4	78.1	90.6	93.1	95.3	90.6
≥1.80	79.3	73.1	86.2	90.1	93.7	86.2
≥2.00	57.0	49.1	65.9	71.4	76.3	65.9
<1.70	16.0	22.0	9.4	7.0	4.8	9.4

confidence. With no alteration to the existing database, 83.4% of tested strains considered together were identified to the correct species when using a log confidence score of ≥1.7. Percent agreement decreased with increasing cut-offs for log confidence of ≥1.8 (79.3%) and ≥2.0 (57.0%). However, when stratified by intrinsic growth rate, differences emerged which highlighted that agreement was lower for the rapid-growing species ranging from a high of 78.1% to a low of 49.1% for log confidence cut-offs of ≥1.7 and ≥2.0, respectively. In contrast, using a log confidence cut-off of ≥1.7, the slow-growing species resulted in agreement of 90.6% which decreased to 65.9% when the cut-off was raised to ≥2.0. Further investigation revealed that two clinical strains representing two species of rapid-growing mycobacteria, *M. chelonae* and *M. mucogenicum,* were primarily responsible for the observed lower agreement. Using the existing database, clinical isolates of these two species rarely resulted in log confidence scores above ≥1.70. The average log confidence score for clinical strains (n = 16) of *M. chelonae* was 1.64 whereas for *M. mucogenicum* (n = 21) it was higher (1.91). However, with respect to the latter, log confidence scores demonstrated greater variability ranging from 1.46 to 2.28. Closer examination of the obtained spectra for these isolates, indicated the identification obtained was correct although below the minimum log confidence cut-off of ≥1.7. The addition of spectra from a single clinical strain of *M. chelonae* (strain #5) and *M. mucogenicum* (strain #3) increased log confidence scores and agreement for all clinical strains of the same species (Table 4). Following the addition of spectra to the database, average log confidence scores increased to 2.19 (range 2.35–2.44) for *M. chelonae* and 2.12 (range 2.11–2.24) for *M. mucogenicum*. Augmentation of the existing commercial database was also reflected in the change in agreement as shown in Table 4. Prior to augmentation, the highest overall agreement (log confidence cut-off ≥1.7) was 83.4%. This agreement increased to 93.1% following the addition of spectra. This increase in agreement was also noted with higher log confidence scores of ≥1.8 or ≥2.0 (Table 4). The greatest change in agreement was noted for the rapid-growing species which increased 17.2, 21.0, and 27.2 percentage points for log confidence scores of ≥1.7, ≥1.8, ≥2.0, respectively. Moreover, results with log confidence scores below 1.7 decreased 17.2 percentage points for the rapid-growing species resulting in overall agreement of 93.1%. Augmentation of the existing database resulted in decreasing the number of spots needed for identification to the species level for clinical isolates. As shown in Table 5, overall agreement with two spots increased from 69.1% prior to the addition to the database; whereas afterwards overall agreement was 97.6%.

Table 5
Effect of the number of spots on % agreement for the AFA method before and after addition of spectra to the existing database.

	% Agreement (log confidence score ≥ 1.7)*					
	2 replicates		3 replicates		4 replicates	
	Before	After	Before	After	Before	After
Overall	69.1	**97.6**	71.4	**98.4**	71.4	**97.6**
Slow growers	96.4	**96.4**	97.6	**97.6**	96.4	**96.4**
Rapid growers	85.7	**100**	85.7	**100**	85.7	**100**

Note: Numbers bolded are based on data after to the addition of two clinical strains to the existing library.
Abbreviations: AFA = adaptive, focused, acoustic-ultrasonication method.
* A log confidence cut-off of ≥1.7 was used for assessment of the optimal number of spots to be used for analysis before and after the addition of spectra to the existing database.

3.3. The effect of AFA on inactivation and time studies

The optimized AFA method, consisting of 40 watts PIP, 50% DF for 2 min at 18 °C resulted in complete inactivation of all species of mycobacteria tested from an estimated starting inoculum of ~10^8 CFU/mL. No growth was demonstrated in any MGIT tubes inoculated with the entire contents of individual microTUBES following AFA, even when incubated for up to 6 weeks. Likewise, no growth was observed when either 1 μL or 100 μL of washed pellets following AFA were plated onto Middlebrook 7H11 agar and incubated for a total of 6 weeks. Additionally, no samples demonstrated detectable peaks without exposure to AFA.

In a separate time study, the optimized AFA method required 4.5 min per sample for inactivation and peptide extraction versus 95.5 min for the CM.

4. Discussion

The overall objective of the current study was to evaluate and optimize the use of AFA as a means to provide a more rapid and consistent method of mycobacterial peptide extraction for subsequent identification to the species level using MALDI-ToF MS and an existing commercial database. Use of an existing commercial database required that all assay parameters be optimized with no additions to the library unless identification with a log confidence score of ≥1.7 had failed. This strategy was designed with clinical laboratories in mind where a more rapid, standardized extraction protocol in tandem with an existing available database would provide for a more feasible avenue to utilize MALDI-ToF MS for mycobacterial identification. In many clinical laboratory settings, lengthy protocols, including a 30 min inactivation step, and generation of a unique library, is not a practical undertaking. In this study, the optimized AFA method inactivated all 21 mycobacterial species tested including the *M. tuberculosis* complex negating the requirement for a separate 30 min inactivation step while simultaneously providing for more efficient peptide extraction in ≤5 min per sample. In addition, use of the AFA method resulted in a decrease in the optimum number of spots required for identification to the species level using a log confidence cut-off of ≥1.7. Taken together, this represents a significant time savings versus conventional peptide extraction methods which are time consuming, lack standardization, and require pre-inactivation of the organism.

It is important to note that using the AFA method, all of the type and reference strains of mycobacteria tested resulted in identification to the species level with log confidence scores ≥1.7. Identification of these isolates to the species level did not require alteration of the existing database. This was also the case for all clinical isolates tested with the exception of strains of *M. chelonae* and *M. mucogenicum*. Closer examination of individual spectra obtained for clinical isolates of these 2 species revealed a pattern consistent with the correct species, although the log confidence scores were <1.7. Importantly, initial identification of these clinical strains was performed using conventional test methods such as 16S rDNA sequencing and ancillary biochemical tests as in the case of *M. chelonae*. As a result, one could postulate that intrinsic differences may exist between the strains used to construct the commercial database and the clinical isolates used in this study. Such intrinsic differences may reflect strain-to-strain variation based on the geographical location from which they were derived. The clinical strains tested in this study were primarily from the mid-Atlantic region of the United States. Addition of a single strain of each species to the existing database resulted in the positive identification of all other remaining clinical strains tested with no further modifications required to the database. This suggests it may be possible for clinical laboratories to utilize the existing commercial database in conjunction with the optimized AFA protocol and only modify the library when clinical strains for which log confidence scores of <1.7 are obtained so long as the spectra

generated are consistent with the correct identification. Such strain-to-strain variation based on the geographical location from which they were derived has been previously documented for *M. tuberculosis* (Ahmed et al. 2004; Rindi et al. 2014).

This study does have some important limitations. The type strains used in this study represent species typically encountered in our laboratory. However, it is by no means a comprehensive list of mycobacteria. It is not known if other species, not tested in this study, would result in similar log confidence scores, especially with regard to rarely encountered species or those for which a single library entry exists. Additionally, we did not obtain clinical collections from other regions of the Unites States or abroad to determine the extent to which geographic differences might contribute to differences in identification. In our hands, only the clinical strains of *M. chelonae* and *M. mucogenicum* required additions to the database. However, it is possible that in other settings, completely different clinical species may present similar problems. Additionally, for this study, all isolates tested were grown on solid media and not in broth. Thus, additional testing is needed to determine if the optimized AFA protocol can be used for mycobacteria grown in Middlebrook 7H9 broth or in the MGIT 960 system.

Lastly, although most of the widely used methods of mycobacterial identification require significant investment in instrumentation (e.g. DNA sequencer for 16S rDNA sequencing; luminometer for nucleic acid probes; MALDI-ToF MS and ultrasonicator for the AFA method) the novel approach for rapid inactivation and extraction described in this work provides a significant cost savings in consumables and labor versus the other methods: identification by sequencing costs $110.00 per isolate; by nucleic acid probe $60; and by MALDI-ToF $12.

In conclusion, to the best of our knowledge, this work represents the first use of AFA for both inactivation and peptide extraction from mycobacteria in ≤5 min with subsequent identification to the species level using MALDI-ToF MS and a log confidence cut-off of ≥1.7. The optimized AFA method does not require extensive library construction, but rather can be utilized for the most part with the existing commercial database. The time savings and decreased cost of AFA-based inactivation and peptide extraction for MALDI-ToF MS identification provides the means for a rapid, standardized protocol which can be utilized easily by clinical laboratories.

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