Five-Minute Ultrasonic Inactivation and Protein Extraction of Mycobacteria for Identification by MALDI-ToF MS (Revised)

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Abstract (Revised)

Introduction MALDI-ToF MS (MALDI) has been used for identification of Mycobacteria. However, extraction methods are time consuming, lack standardization, and require pre-inactivation of the organism. Using Adaptive Focused Acoustics™ (AFA) technology with ultrasonication, we developed a rapid mycobacterial inactivation/ protein extraction method called the Ultrasonic Method (USM) and compared it to an existing commercial assay (CM) and database.

Methods A total of 184 mycobacterial isolates were used. Twenty-one species were represented including both type strains and clinical isolates. Optimization of the USM included an ultrasonicator (Covaris®, Woburn, MA), microTUBE™ with glass beads or fibers, water, acetonitrile, and/or formic acid. Instrument settings included: 40-75 watts (W) Peak Incident Power (PIP) and 20%-50% Duty Factor (DF) for 1-3 min with or without centrifugation. Completed extracts were spotted on polished steel plates and identification done by MALDI (Bruker MicroFlex LT) using Bruker Biotyper software and existing database. Viability assays and a time study were also performed.

Results The optimized USM was determined: microTUBES™ with glass beads, acetonitrile: formic acid, 40W PIP, 50%DF (2 min), with centrifugation. Complete inactivation of all species/strains was achieved with a 2 min exposure. Using a log confidence of ≥ 1.7, overall agreement for identification to the species level was 93% for the USM versus 83% for the CM. Higher log confidence scores of ≥ 1.8 or ≥ 2 resulted in higher agreement for the USM (90% and 71%) versus the CM (67% and 42%), respectively. One species, with only a single database entry, failed to identify by either method: *M. flavescens*. Optimal agreement was achieved with an average of 2 spots for the USM versus 5 spots for the CM. Assay completion for the USM was done in 5 min vs 90 min for the CM.

Conclusion These data illustrate the USM and use of focused ultrasonication provides for rapid inactivation/extraction of Mycobacteria for identification by MALDI. The USM requires significantly fewer steps, can be completed in 5 min and resulted in higher log confidence scores. In addition, optimum agreement can be achieved with 2 spots and use of an existing commercial database. Such considerations are important for high-volume laboratories using MALDI for mycobacterial identification.

Introduction

Previous studies have established that MALDI-ToF MS can rapidly and accurately identify bacteria and yeasts from growth in culture saving both time and expense. Unfortunately, Mycobacteria present a challenge for MALDI-ToF MS based identification because of inherent difficulties in disruption of the lipid-rich cell walls. In addition, Mycobacteria require longer incubation times for cultivation *in vitro* compared with other organisms extending up to 6 weeks for some species.

The existing literature proposes several different mycobacterial protein extraction methods for MALDI-ToF MS. However, these extraction methods are time consuming, lack standardization, and require pre-inactivation of the organism. Inactivation is necessary prior to conducting MALDI-ToF MS due to the risk of infectious aerosols with *M. tuberculosis* which could be generated during the assay. Using Adaptive Focused Acoustics™ (AFA) technology with ultrasonication, we developed a rapid method for extraction of mycobacterial peptides which simultaneously provides for complete inactivation of all species tested.

AFA™ uses focused bursts of ultrasonic energy at frequencies much higher than 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. The acoustic energy is applied to the sample to mechanically break the cell wall. When the acoustic burst is focally applied to a zone within the sample, numerous cavitation bubbles are generated. At the end of each acoustic burst, the microscopic bubbles collapse, creating intense localized jets of solute. When this process is repeated multiple times per second, rapid release of mycobacterial peptides occurs.

In this study, we compared the optimized AFA-ultrasonication method to a commercial extraction method and existing database/library for identification of both type and clinical strains of Mycobacteria. Clinical strains were obtained from the Johns Hopkins Hospital archive which represents isolates primarily from the mid-Atlantic region of the United States.

Table 1. Mycobacterial Reference and Clinical Isolates Used*

Slow Growers (n = 88)	ATCC	Clinical
M. asiaticum	1	0
M. avium complex	1	13
M. bohemicum	1	0
M. gordonae	2	23
M. hemophilum	1	0
M. kansasii	1	0
M. lentiflavum	1	0
M. marinum	0	11
M. scrofulaceum	1	0
M. simiae	1	0
M. szulgai	1	1
M. terrae	1	0
M. tuberculosis complex	3	24
M. xenopi	1	0

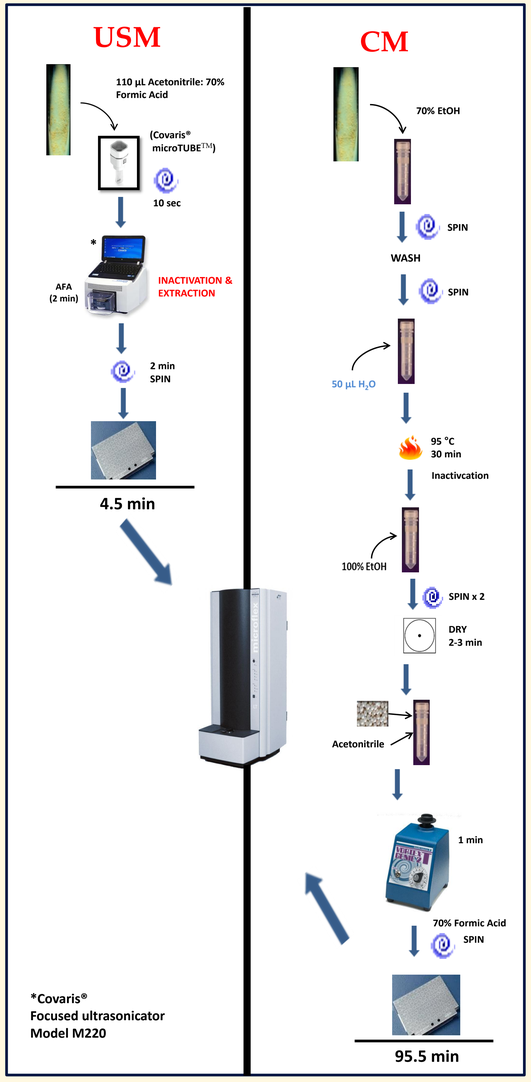
Rapid Growers (n = 96)	ATCC	Clinical
M. abscessus	1	25
M. chelonae	1	16
M. fortuitum complex	1	25
M. peregrinum	1	0
M. magaritense	1	1
M. mucogenicum complex	1	21
M. phlei	2	0

*Isolates previously identified by HPLC,16S rRNA gene sequencing, or AccuProbe.

AFA Method Conditions Evaluated

- **Peak Incidence Power (40 – 75 watts PIP)**
- **Duty Factor (20 – 50 % DF)**
- **Time of Adaptive Focused Acoustics (AFA) (1 – 3 min)**
- **With and without centrifugation after AFA**
- **Glass beads vs. Fibers**
- **Protein Inactivation**
- **Formic acid only vs. formic acid/acetonitrile**

Methods



Results

Table 2. Comparison of the USM versus the CM for protein extraction and identification by MALDI-ToF MS

	CM	USM*
Score ≥ 1.7	94.4%	93.1%
P value		NS
Score ≥ 1.8	80.6 %	90.1%
P value		NS
Score ≥ 2.0	55.6%	71.4%
P value		NS

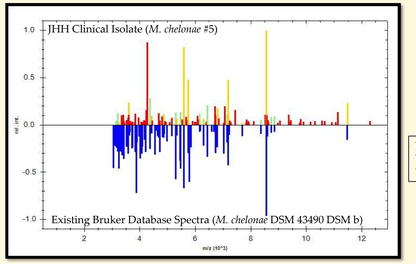
*Both methods compared to HPLC/16S rRNA gene sequencing used as the reference method. *NS, not statistically different from the CM ($P > 0.5$).

Table 3. Effect of increased number of spots on % agreement

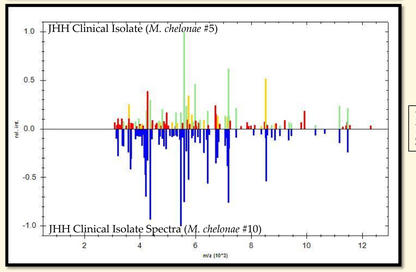
Mycobacterium spp.	Identification (score ≥ 1.7)					
	2 replicates		3 replicates		4 replicates	
	USM	CM	USM	CM	USM	CM
Overall	88.6 (93.2)	38.3	89.4 (93.4)	57.9	88.6 (93.2)	77.5
Slow growers	96.4	37.5	97.6	56.7	96.4	75.8
Rapid growers	75.0 (100)	39.2	75.0 (100)	59.2	75.0 (100)	79.2

NOTE:
- Numbers in black are based on data prior to the addition of 2 JHH-RG strains to the existing library.
- Numbers in red are based on data after the addition.

Figure 1. Effects of augmentation of the existing commercial database with 2 JHH clinical entries and changes in log confidence scores for other strains.



Best Match
Log Confidence Score
1.6



Best Match
Log Confidence Score
2.1

Table 5. Differences in % agreement for the USM before and after the addition of spectra from 2 JHH clinical isolates to the existing commercial library*

Log Confidence Score	Before Addition			After Addition		
	Overall	RG	SG	Overall	RG	SG
≥ 1.70	83.4	78.1	90.6	93.1	95.3	90.6
≥ 1.80	79.3	73.11	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

*Additional spectra added for a single JHH derived clinical strain of *M. chelonae* and *M. mucogenicum*.

Summary

- The optimized protocol required for inactivation and protein extraction consisted of glass beads, instrument settings of 40W PIP, 50%DF (2 min), followed by centrifugation and addition of acetonitrile and 70% formic acid.
- A six week inactivation study using 26 species including *Mycobacterium tuberculosis complex* determined the USM successfully inactivated all Mycobacteria tested after 2 minutes exposure.
- The optimum number of spots analyzed was determined to be 2 for the USM (88.6%) and 4 for the CM (77.5%).
- The optimal confidence score for both the USM and CM was ≥ 1.7.
- Addition of 2 spectra from a **single** JHH clinical isolate of *M. chelonae* and *M. mucogenicum* resulted in an increase in % agreement for all strains of the same species (before average log confidence score: *M. chelonae* = 1.64 and *M. mucogenicum* = 1.94; after: 2.19 and 2.12, respectively).

Conclusions

The USM provides for simultaneous inactivation/protein extraction of Mycobacteria in ≤ 5 minutes with accurate identification to the species level using MALDI-ToF MS. As a result, it has the potential to significantly decrease turn-around-time for reporting and initiation of more rapid, appropriate therapy.

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