

# Comparison of MALDI-TOF MS With HPLC and Nucleic Acid Sequencing for the Identification of *Mycobacterium* Species in Cultures Using Solid Medium and Broth

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## ABSTRACT

**Objectives:** The genus *Mycobacterium* contains over 150 species including pathogenic and nonpathogenic strains. Accurate species level identification can aid in differentiating environmental contamination from true infection, and also can aid in selection of antimicrobial therapy.

**Methods:** We evaluated the performance of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the routine identification of clinical isolates of mycobacteria using 2 commercially available spectral reference libraries, and also assessed the impact of mycobacterial culture using solid medium and broth on MALDI-TOF MS-based identification.

**Results:** All results were compared with those obtained on high-pressure liquid chromatography and nucleic acid sequencing. Optimal results were obtained with a mycobacterium-specific reference library (*Mycobacterium* Library v1.0). The identification rate was 89.8% (79/88) for isolates cultured on solid medium and 98.8% (85/86) for isolates analyzed directly from broth. Among these, the proportion identified with a high confidence level was 50.0% (44/88) for isolates cultured on solid medium and 80.2% (69/86) for isolates cultured in broth.

**Conclusions:** Agreement with nucleic acid sequencing for species present in *Mycobacterium* Library v1.0 was 97.6% (81/83) for isolates cultured on solid medium and 97.5% (79/81) for those cultured in broth.

Mycobacterial infections, including tuberculosis, affect millions of people worldwide each year. Most of the mycobacteria described belong to species other than *Mycobacterium tuberculosis* complex and are collectively referred to as nontuberculous mycobacteria (NTM). The NTM are commonly isolated from natural and municipal (treated) water sources and are particularly well suited to colonize household, industrial, and hospital water supply lines.<sup>1-5</sup> Of the more than 150 species of NTM, only a relatively small number of species are strongly associated with human disease.<sup>6,7</sup> The ubiquity of NTM in the environment, including health care facilities, and the variable pathogenicity within the genus can pose a problem for clinicians when differentiating among asymptomatic colonization, environmental contamination, and true infection.<sup>8-10</sup> Species-level identification of NTM isolated from respiratory sources can aid in patient care by differentiating species with increased pathogenic potential, such as *M avium*/*M intracellulare* (MAI) or *M abscessus*, from other species with low pathogenic potential such as *M gordonae*.<sup>7,11</sup> Likewise, identifying NTM in postsurgical wound infections or indwelling venous catheters can aid in differentiating NTM associated with invasive infection from those that are environmental or commonly associated with colonization of catheters such as *M mucogenicum*.<sup>12</sup> In both respiratory and cutaneous specimens, species-level identification of NTM can guide decisions about the necessity of antimicrobial therapy, and in some cases, can assist in selecting appropriate antimicrobial therapy because of the predictable susceptibility profiles in specific NTM species.<sup>6,11</sup>

Historically, NTM were identified using the Runyon scheme, based on physical and biochemical properties of

isolates including growth rate, development of color, and substrate utilization.<sup>13</sup> High-performance liquid chromatography (HPLC) analysis of mycolic acids in the mycobacterial cell wall has been used to provide better discrimination between species and a more specific identification.<sup>14</sup> Although more rapid than phenotypic-based methods, HPLC still requires initial culture of isolates on solid medium before analysis. This can be problematic because the slow growth rate of mycobacteria delays full identification and leaves treating physicians with little useful information after the initial report of an acid-fast bacilli (AFB)-positive broth culture. In addition, HPLC is insufficient to accurately differentiate between closely related species such as *M chelonae*/*M abscessus*, *M avium*/*M intracellulare*, and species within the *M mucogenicum* group, which may be important to patient care decisions.<sup>10-12,15-17</sup> Nucleic acid sequencing has emerged as the most rapid and accurate method for identifying *Mycobacterium* species; however, species-level discrimination may require comparison of sequence obtained from several genes including 16S rDNA, *rpoB*, and *hsp65*.<sup>6,18</sup> In addition, similar to HPLC, sequence analysis requires a pure isolate obtained from solid medium, which delays turnaround time, and instruments required for sequence reactions and analysis are not widely available in many clinical laboratories.

More rapid methods for identifying *Mycobacterium* species directly from broth culture have been developed; however, they are limited to few specific *Mycobacterium* species.<sup>19-21</sup> Specifically, tests based on hybridization of chemiluminescent nucleic acid probes are available for *M tuberculosis* complex, *M avium*/*M intracellulare*, *M gordonae*, and *M kansasii*. These tests enable these species to be identified after the processing of positive broth cultures, with a sensitivity rate of more than 95% and specificity of more than 88%.<sup>19-21</sup>

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is increasingly being used by clinical laboratories for identifying bacterial and fungal isolates obtained from solid medium as well as directly from blood culture broth and urine.<sup>22-29</sup> Studies demonstrate advantages including rapid identification, high accuracy, and low reagent cost associated with the use of MALDI-TOF MS in clinical laboratories. Comparatively few reports have described the application of MALDI-TOF MS to the identification of *Mycobacterium* species. However, a majority of current studies found that MALDI-TOF MS identified 95% to 99% of isolates tested from solid medium.<sup>30-33</sup> Importantly, these results required the de novo creation of reference libraries before analysis, and few of these studies examined the impact of broth culture on identification using MALDI-TOF MS.

We evaluated the performance of MALDI-TOF MS for the routine identification of clinical isolates of mycobacteria using 2 commercially available reference libraries, the

standard MALDI Biotyper 3.1 library (Bruker, Billerica, MA) and the mycobacteria-specific Mycobacteria Library v1.0 (Bruker). We also report differences in identification rate and accuracy when isolates are obtained from solid medium or broth culture.

## Materials and Methods

### Collection of Isolates

A collection of mycobacterial isolates (n = 88) obtained from clinical specimens submitted to Dynacare Laboratories (Milwaukee, WI) was used for this study. Original sources included sputum, bronchial alveolar lavage, blood, and various surgical soft-tissue specimens. Routine identification was conducted using Accuprobe nucleic acid probes (Gen-Probe, San Diego, CA) for presumptive identification of *M tuberculosis* complex and *M avium*/*M intracellulare*. Probe-negative broth cultures or isolates were subcultured on Middlebrook 7H11 medium and sent to the Wisconsin State Laboratory of Hygiene (Madison, WI) for HPLC analysis.

### Preparation of Isolates for MALDI-TOF MS Analysis

A fresh subculture of each isolate on 7H11 medium was used as the starting material. Following sufficient incubation to obtain adequate biomass (3-5 days for rapid growers and up to 3 weeks for slower growing AFB), a 2.0 to 3.0 McFarland standard was made in molecular grade water. A 300- $\mu$ L aliquot of this suspension was used in the extraction process for identification from solid medium (see below). A 1.0-mL aliquot of the suspension was added to a VersaTREK Myco Broth bottle (ThermoFisher Scientific, Lenexa, KS) along with 1.0 mL of growth supplement (ThermoFisher Scientific). Inoculated broths were then incubated using the VersaTREK-system and analyzed within 1 to 2 days of culture positivity. A 2.0-mL aliquot was removed from positive broth cultures and centrifuged for 2 minutes at 14,000 rpm. The supernatant was then removed from the pellet; 500  $\mu$ L of molecular grade water was added to the pellet and centrifuged for 2 minutes at 14,000 rpm (centrifuge model Mikro-22R, Hettich, Germany). The supernatant was then removed and the pellet was suspended in 300  $\mu$ L of molecular grade water before 900  $\mu$ L of 100% ethanol (final concentration 75% ethanol) was added. For isolates cultured on solid medium, a 300- $\mu$ L aliquot of the 2.0 to 3.0 McFarland was centrifuged and the resulting pellet was resuspended in 75% ethanol as previously described.

The steps of the extraction process were performed as follows. The ethanol suspension was vortexed and centrifuged for 2 minutes at 14,000 rpm. The supernatant was then removed and the pellet was suspended in 500  $\mu$ L of molecular grade water, vortexed, and centrifuged for 2 minutes at

14,000 rpm. The supernatant was removed and the pellet was suspended in 50  $\mu$ L of molecular grade water by vortex, and then put in a  $95^{\circ}\text{C} \pm 5^{\circ}\text{C}$  heat block for 30 minutes to inactivate the organism. After heat inactivation, the suspension was allowed to cool to room temperature before adding 1,200  $\mu$ L of chilled 100% ethanol. This suspension was then vortexed to homogenize and centrifuged for 2 minutes at 14,000 rpm. The supernatant was removed and the pellet was dried completely. Once the pellet was completely dry, 25 to 50  $\mu$ L (depending on size of the pellet) of 100% acetonitrile and glass beads (3 mm, Kimble Glass, Gerresheimer Glas AG, Dusseldorf, Germany) was added to the tube. The tube was vortexed for 1 minute to disrupt any aggregates, and then an equal amount of 70% formic acid was added to the tube. This acetonitrile/formic acid suspension was again vortexed and then centrifuged for 2 minutes at 1,400 rpm. A 1- $\mu$ L portion of supernatant was added to a well on a 96-spot stainless steel target plate (Bruker) and allowed to dry. When dry, 1  $\mu$ L of alpha-cyano-4-hydroxy cinnamic acid matrix was used to overlay the analyte and allowed to dry before analyzed using MALDI-TOF MS. Each extract was analyzed in duplicate and the higher of the 2 scores was used for data analysis.

#### MALDI-TOF Analysis

A target plate prepared with protein extracts (described earlier) was inserted into the Bruker Microflex LT MALDI-TOF MS (Bruker) for analysis. A composite profile of proteins with a mass-to-charge ratio ( $m/z$ ) of 2,000 to 20,000 was generated based on a minimum of 240 measurements (laser shots) for each analyte. The composite profile was analyzed using either MALDI Biotyper 3.1 standard reference library or Mycobacteria Library v1.0. MALDI Biotyper 3.1 library is a general identification library containing 4,110 unique bacterial spectra (at the time of analysis) including 79 spectra corresponding to 42 species of mycobacteria. Mycobacteria Library v1.0 is a mycobacterium-specific library that contains 173 unique mycobacterial spectra representing 94 species (at the time of this study Mycobacteria Library v1.0 was under development and contained only 119 spectra). Query of either reference library returned the top 10 identification matches along with confidence scores ranging from 0.0 to 3.0. Confidence scores greater than 2.0 were considered high (secure species) identification, scores of 1.7 to 2.0 were considered intermediate identification, and scores of less than 1.7 were considered unreliable identification.

#### Nucleic Acid Sequencing

A heat-inactivated preparation of each isolate in 75% ethanol was sent to Accugenix (Newark, DE) for identification with nucleic acid sequencing. Analysis was conducted using the *16S rDNA* gene as primary target for sequencing. Sequence data from the *16S rDNA* gene was compared with

an independently validated library (Accugenix). This library was constructed using replicate type strains obtained from multiple sources to enable cross comparison and identification of sequence errors or point mutations. The library is regularly validated using common and novel isolates, and sequences are compared with those available in GenBank. If identification results were ambiguous based on 16S sequence, the *rpoB* and/or *hsp65* genes were analyzed and the sequence was compared with GenBank entries. Confidence calls were made using the CLSI MM-18-A Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing: Approved Guideline.

## Results

### Analysis of Isolates Using Standard MALDI Biotyper 3.1 Reference Library

A total of 88 clinical isolates were analyzed using HPLC, nucleic acid sequencing (16S rDNA or *rpoB*), and MALDI-TOF MS. HPLC analysis of 88 clinical isolates included in this study identified 15 different species or groups of mycobacteria. Notably, HPLC was unable to discriminate among the *M tuberculosis* complex, *M avium/M intracellulare* complex, and *M abscessus/M chelonae* complex species. In contrast, nucleic acid sequence analysis of these same isolates identified 24 unique species or groups. In addition, sequence data revealed multiple unique species that were identified with HPLC as *M mucogenicum* group or *M fortuitum* group.

Analysis of isolates cultured on solid medium (Middlebrook 7H11) using MALDI-TOF MS and Biotyper 3.1 generated acceptable confidence scores ( $>1.7$ ) for only 50.6% (44/87) of isolates. This included only 10.3% (9/87) with high secure species confidence scores ( $>2.0$ ). Analysis of the same isolates directly from broth culture increased the overall acceptable identification rate to 68.2% (60/88) with 39.7% (35/88) reaching the secure species designation **Table 1**.

A comparison of MALDI-TOF MS identification results with nucleic acid sequencing results indicated that the level of concordance was dependent on confidence score category and culture media. When cultured on solid medium or in broth, the lowest concordance between sequencing and MALDI-TOF MS (67.4% solid medium, 74.1% broth culture) was observed for isolates with confidence scores less than 1.7. The concordance increased when confidence scores reached 1.7 to 2.0 (94.4% solid, 84.0% broth) and concordance was 100% for isolates with confidence scores greater than 2.0. When considering all isolates with scores at or higher than 1.7, identification was concordant for 95.5% (42/44) of isolates cultured on solid medium and 93.3%

(56/60) for isolates cultured in broth. Importantly, broth culture resulted in 16 additional isolates with scores higher than 1.7 compared with solid medium culture.

With the Biotyper 3.1, 16 discordant results were obtained from isolates cultured on solid medium and 12 discordant results for isolates cultured in broth. For both growth conditions, the majority (14/16 solid, 8/12 broth) were isolates with confidence scores less than 1.7. Isolates with confidence scores less than 1.7 would be considered unacceptable identifications; therefore, we focused on isolates with discordant

identification results whose confidence scores were greater than 1.7. Two such isolates were found to have discordant identifications from solid culture as well as from broth. The first was identified as *M smegmatis* with HPLC, *M porcinum* with nucleic acid sequencing, and *M fortuitum* with MALDI-TOF MS. The second was identified as *M mucogenicum* with HPLC and MALDI-TOF MS, but as *M phocaicum* with nucleic acid sequencing. Two additional isolates with scores greater than 1.7 generated discordant identifications from broth culture only. These were identified as *M mucogenicum*

**Table 1**  
Identification of *Mycobacterium* Species Cultured on Middlebrook 7H11 Agar or Directly From VersaTREK Myco Broth Using MALDI Biotyper 3.1 Standard Reference Library

HPLC Identification	Sequence ID	No.	Middlebrook 7H11			Concordant, No. (%)	VersaTREK Myco Broth			Concordant, No. (%)
			<1.7	1.7-2.0	>2.0		<1.7	1.7-2.0	>2.0	
MAI	<i>M avium</i>	12		10	2	12 (100)		4	8	12 (100)
	<i>M intracellulare</i>	1	1 <sup>a</sup>			0 (0)	1 <sup>b</sup>			0 (0)
MTBc	<i>M bovis/tuberculosis</i>	5	5 <sup>c</sup>			5 (100)	4 <sup>d</sup>	1 <sup>d</sup>		5 (100)
	<i>M abscessus/chelonae</i>	8	4	3	1	8 (100)	2	2	4	8 (100)
	<i>M chelonae</i>	7	4 <sup>e</sup>	3		6 (85.7)		3	4	7 (100)
	<i>M immunogenum</i>	1	1 <sup>f</sup>			0 (0)	1 <sup>f</sup>			0 (0)
	<i>M fortuitum</i> group	13	2	8	3	13 (100)	1	3	9	13 (100)
	<i>M peregrinum</i>	1		1		1 (100)			1	1 (100)
	<i>M porcinum</i>	1	1 <sup>g</sup>			0 (0)	1 <sup>g</sup>			0 (0)
<i>M gordonae</i>	<i>M gordonae</i>	4	1	2	1	4 (100)			4	4 (100)
<i>M kansasii</i>	<i>M kansasii</i>	3	2	1		3 (100)		1	2	3 (100)
<i>M mucogenicum</i>	<i>M mucogenicum</i>	1		1		1 (100)	1			1 (100)
	<i>M phocaicum</i>	3	2 <sup>h</sup>	1 <sup>h</sup>		0 (0)	2 <sup>i</sup>	1 <sup>j</sup>		0 (0)
	<i>M mucogenicum/phocaicum</i>	6	6 <sup>k</sup>			6 (100)	4 <sup>k</sup>	2 <sup>k</sup>		6 (100)
	<i>M llatzerense</i>	4	4 <sup>k</sup>			0 (0)	2 <sup>k</sup>	2 <sup>k</sup>		0 (0)
	<i>M aubagnense</i>	1	1 <sup>k</sup>			0 (0)	1 <sup>k</sup>			0 (0)
	<i>M peregrinum</i>	1			1	1 (100)			1	1 (100)
	<i>M xenopi</i>	6	1	5		6 (100)	2	3	1	6 (100)
<i>M marinum</i>	<i>M pseudoshottsii</i>	2	1 <sup>l</sup>			0 (0)		1	1	2 (100)
<i>M szulgai</i>	<i>M szulgai</i>	2	2 <sup>m</sup>			1 (50.0)	2			2 (100)
<i>M neoaurum</i>	<i>M neoaurum</i>	2	2 <sup>n</sup>			1 (50.0)	1	1		2 (100)
<i>M malmoense</i>	<i>M malmoense</i>	1	1			1 (100)	1			1 (100)
<i>M phlei</i>	<i>M phlei</i>	1	1			1 (100)	1			1 (100)
<i>M smegmatis</i>	<i>M porcinum</i>	1		1 <sup>o</sup>		0 (0)		1 <sup>o</sup>		0 (0)
<i>M terrae</i>	<i>M arupense</i>	1	1 <sup>p</sup>			0 (0)	1 <sup>p</sup>			0 (0)
	Total	88	43	36	8		28	25	35	
	% in score range		49.4	41.4	9.2		31.8	28.4	39.7	
	% concordant <sup>q</sup>		67.4	94.4	100	81.6	71.4	84.0	100	85.2

HPLC, high-pressure liquid chromatography; MAI, *M avium/M intracellulare*; MTBc, *M tuberculosis* complex.

<sup>a</sup> Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) identified as *Pseudomonas aeruginosa*, score 1.20.

<sup>b</sup> MALDI-TOF MS identified as *Staphylococcus succinus*, score 1.33.

<sup>c</sup> MALDI-TOF MS identifications included two *M bovis* and three *M tuberculosis*.

<sup>d</sup> MALDI-TOF MS identified all as *M tuberculosis*.

<sup>e</sup> MALDI-TOF MS identified one isolate as *S lentus*, score 1.53.

<sup>f</sup> MALDI-TOF MS identified as *M abscessus*, score 1.68 (Middlebrook 7H11), 1.66 (VersaTREK Myco Broth).

<sup>g</sup> MALDI-TOF MS identified as *M peregrinum*, score 1.60 (Middlebrook 7H11), *M conceptionense*, score 1.54 (VersaTREK Myco Broth).

<sup>h</sup> MALDI-TOF MS identified all three as *M mucogenicum*, score 1.56, 1.26, 1.81.

<sup>i</sup> MALDI-TOF MS identified as *M mucogenicum*, score 1.62, *C mastidis*, score 1.38.

<sup>j</sup> MALDI-TOF MS identified as *M mucogenicum*, score 1.90.

<sup>k</sup> MALDI-TOF MS identified all as *M mucogenicum*. *M llatzerense*, *M aubagnense* absent from reference library.

<sup>l</sup> Only one of two isolates tested. MALDI-TOF MS identified as *S aureus*, score 1.31.

<sup>m</sup> MALDI-TOF MS identified one isolate as *Novosphingobium* Species, score 1.34.

<sup>n</sup> MALDI-TOF MS identified one isolate as *Lactobacillus fructivorans*, score 1.47.

<sup>o</sup> MALDI-TOF MS identified as *M fortuitum*, score 1.80 (Middlebrook 7H11), 1.95 (VersaTREK Myco Broth).

<sup>p</sup> MALDI-TOF MS identified as *L paracasei*, score 1.45 (Middlebrook 7H11), 1.36 (VersaTREK Myco Broth).

<sup>q</sup> Compared with sequencing result.

with HPLC and MALDI-TOF MS, but as *M llatzerense* with nucleic acid sequencing. The same two isolates were also misidentified when cultured on solid medium; however, confidence scores were less than 1.7 and they were considered unacceptable identifications rather than discordant. Review of the MALDI Biotyper standard reference database revealed the absence of any spectra corresponding to this species.

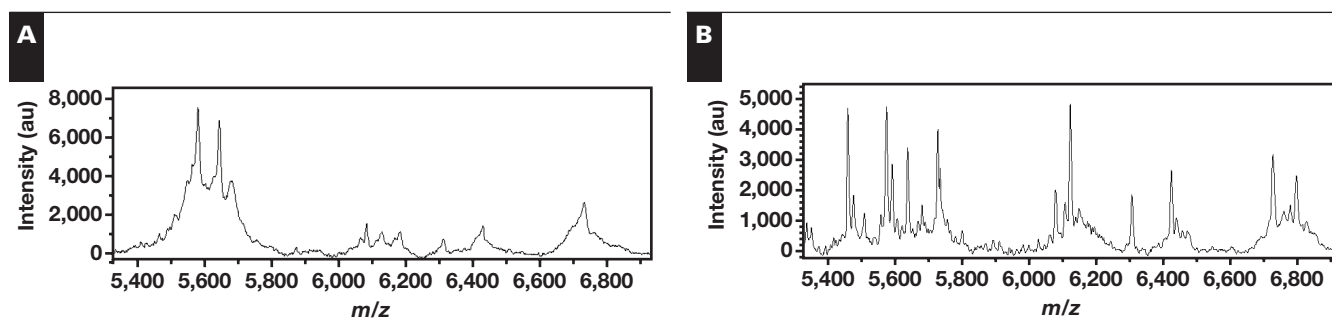
### Analysis of Isolates Using Mycobacterium Library v1.0 Reference Library

Mycobacterium Library v1.0 is a supplementary, mycobacterium-specific, spectral library for use with the MALDI Biotyper standard software. At the time of this study, Mycobacterium Library v1.0 contained 119 unique *Mycobacterium* species isolates. In addition to expanding the number of strains used to construct this library, all strains were processed using an improved method for extraction of mycobacterial proteins involving a bead-based disruption step (see “Materials and Methods” section). This is a departure from the MALDI Biotyper standard library in which mycobacteria were processed using an extraction protocol obviously less robust for mycobacteria. Addition of bead-based disruption of mycobacterial isolates resulted in better defined spectra, which likely improve the accuracy of identification using MALDI-TOF MS. The impact of the new extraction protocol on quality of spectra is demonstrated in **Figure 1**. In this study, we reanalyzed our original set of 88 isolates using the Mycobacterium Library v1.0 to assess the effect of an expanded reference library on identification rate, confidence scores, and concordance of identification with nucleic acid sequence results.

The proportion of isolates generating acceptable confidence scores ( $\geq 1.7$ ) increased from 50.6% (Biotyper standard library) to 89.8% (79/88) when using Mycobacterium Library v1.0 for isolates cultured using solid medium **Table 2**. A further increase was observed for broth-grown

cultures, in which 98.8% (85/86) yielded confidence scores greater than 1.7. In addition, a larger proportion of these identifications attained high confidence scores ( $>2.0$ ). This included 50.0% (44/88) of isolates cultured on solid medium and 80.2% (69/86) of isolates cultured in broth. Concordance of the MALDI-TOF MS identification with nucleic acid sequence results was also high using Mycobacterium Library v1.0. In total, 92.4% (73/79) of isolates cultured on solid medium and 91.8% (78/85) cultured in broth with scores greater than 1.7 were concordant with the species level identified with nucleic acid sequencing. Among isolates with confidence scores greater than 2.0, concordance was 95.5% (42/44) for isolates cultured on solid medium and 92.8% (64/69) for broth-grown isolates (Table 2). Interestingly, the percentage of concordance was similar to that obtained using MALDI Biotyper standard library for all isolates with scores greater than 1.7, but was slightly lower for isolates with confidence scores greater than 2.0. This can be explained by the observation that a larger proportion of isolates reached the greater than 2.0 score threshold using Mycobacterium Library v1.0, including species that are absent from both reference libraries and thus cannot be identified correctly (as discussed later in this article). Of note, when using Mycobacterium Library v1.0, even among isolates with unreliable confidence scores less than 1.7, the concordance with sequencing results was 88.9% (8/9) and 100% (1/1) for isolates cultured on solid medium or broth, respectively.

Fewer discordant results were obtained when using Mycobacterium Library v1.0 compared with Biotyper 3.1. Both broth culture and solid medium culture yielded seven isolates with identifications discordant with nucleic acid sequencing results (Table 2). All but one of these generated confidence scores greater than 1.7 that were acceptable. Of note, two of seven isolates cultured on solid medium and five of seven cultured in broth with discordant identification



**Figure 1** Mass spectrum generated for an isolate of *Mycobacterium abscessus*. The spectrum in panel **A** was generated from an extraction without bead-based disruption. The spectrum in panel **B** was generated using the same extraction method with the addition of a bead-based disruption step. au, Arbitrary units.

results had confidence scores greater than 2.0, indicating a secure species identification with MALDI-TOF MS. Four of the seven discordant results were attributable to isolates identified as *M mucogenicum* with HPLC and MALDI-TOF MS, but identified as closely related *M llatzerense* with nucleic acid sequencing. This included four of five discordant results with scores greater than 2.0 from broth culture and two of two with scores greater than 2.0 obtained from solid medium. *M llatzerense* is also absent from Mycobacterium Library v1.0, preventing identification of these strains using this reference library as previously discussed. Similarly, another strain generating discordant results was identified as *M aubagnense* using nucleic acid sequencing.

This species is also absent from Mycobacterium Library v1.0. This isolate was identified as *M mucogenicum* with HPLC and *M phocaicum* (solid medium) or *M mucogenicum* (broth) with MALDI-TOF MS. The remaining two isolates generating discordant results were identified as *M marinum* with HPLC and MALDI-TOF MS, but as *M pseudoshottsii* with nucleic acid sequencing. Interestingly, one of two was identified correctly when cultured on solid medium but both were incorrectly identified when cultured in broth (Table 2).

Taken together, these data indicate that the majority of discordant results were due to the absence of those species from the reference databases. If these strains are excluded from analysis, the total concordance of MALDI-TOF using

**Table 2**  
Identification of *Mycobacterium* Species Cultured on Middlebrook 7H11 Agar or Directly From VersaTREK Myco Broth Using Mycobacterium Library v1.0

HPLC Identification	Sequence ID	No.	Middlebrook 7H11			Concordant, No. (%)	VersaTREK Myco Broth			Concordant, No. (%)
			<1.7	1.7-2.0	>2.0		<1.7	1.7-2.0	>2.0	
MAI	<i>M avium</i>	12		2	10	12 (100)		2	10	12 (100)
	<i>M intracellulare</i>	1		1		1 (100)			1	1 (100)
MTBc	<i>M bovis/tuberculosis</i>	5		3 <sup>a</sup>	2 <sup>a</sup>	5 (100)			5 <sup>a</sup>	5 (100)
	<i>M abscessus/chelonae</i>	8		2	6	8 (100)		3	5	8 (100)
<i>M fortuitum</i> group	<i>M chelonae</i>	7		3	4	7 (100)		4	3	7 (100)
	<i>M immunogenum</i>	1		1		1 (100)			1	1 (100)
	<i>M fortuitum</i>	13	1	4	8	13 (100)	1	12	13 (100)	
	<i>M peregrinum</i>	1		1		1 (100)			1	1 (100)
<i>M gordonae</i>	<i>M porcinum</i>	1		1		1 (100)			1	1 (100)
	<i>M gordonae</i>	4		1	3	4 (100)		4	4	4 (100)
<i>M kansasii</i>	<i>M kansasii</i>	3		2	1	3 (100)		3	3 (100)	
<i>M mucogenicum</i>	<i>M mucogenicum</i>	1		1 <sup>b</sup>		0 (0)			1	1 (100)
	<i>M phocaicum</i>	3		3		3 (100)		3	3 (100)	
	<i>M mucogenicum/phocaicum</i>	6 <sup>c</sup>	2	3	1	6 (100)	1	5	6 (100)	
	<i>M llatzerense</i>	4	1 <sup>d</sup>	1 <sup>d</sup>	2 <sup>d</sup>	0 (0)			4 <sup>e</sup>	0 (0)
	<i>M aubagnense</i>	1		1 <sup>f</sup>		0 (0)		1 <sup>g</sup>		0 (0)
<i>M xenopi</i>	<i>M peregrinum</i>	1			1	1 (100)			1	1 (100)
	<i>M xenopi</i>	6 <sup>h</sup>	1	2	3	6 (100)		2	2	4 (100)
<i>M marinum</i>	<i>M pseudoshottsii</i>	2		2 <sup>i</sup>		1 (50.0)	1 <sup>j</sup>	1 <sup>j</sup>	0 (0)	
<i>M szulgai</i>	<i>M szulgai</i>	2	1	1		2 (100)		2	2 (100)	
<i>M neoaurum</i>	<i>M neoaurum</i>	2	1		1	2 (100)		1	1	2 (100)
<i>M malmoense</i>	<i>M malmoense</i>	1			1	1 (100)			1	1 (100)
<i>M phlei</i>	<i>M phlei</i>	1	1			1 (100)	1		1	1 (100)
<i>M smegmatis</i>	<i>M porcinum</i>	1			1	1 (100)			1	1 (100)
<i>M terrae</i>	<i>M arupense</i>	1				1 (100)			1	1 (100)
	Total	88	9	35	44		1	16	69	
	% in score range		10.2	39.8	50.0		1.2	18.6	80.2	
	% concordant <sup>k</sup>		88.9	91.4	95.5 <sup>l</sup>	92.0	100	87.5	92.8 <sup>m</sup>	91.9

HPLC, high-pressure liquid chromatography; MAI, *M avium/M intracellulare*; MTBc, *M tuberculosis* complex.

<sup>a</sup> Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) identified all isolates as *M tuberculosis*.

<sup>b</sup> MALDI-TOF MS identified as *M phocaicum*.

<sup>c</sup> MALDI-TOF MS identified five of six as *M phocaicum*. One isolate identified as *M mucogenicum*, score 1.77 (Middlebrook 7H11), 2.05 (VersaTREK Myco Broth).

<sup>d</sup> MALDI-TOF MS identified all isolates as *M phocaicum*. *M llatzerense* absent from reference library.

<sup>e</sup> MALDI-TOF MS identified three of four isolates as *M mucogenicum* and one as *M phocaicum*. *M llatzerense* absent from reference library.

<sup>f</sup> MALDI-TOF MS identified isolate as *M phocaicum*. *M aubagnense* absent from reference library.

<sup>g</sup> MALDI-TOF MS identified isolate as *M mucogenicum*. *M aubagnense* absent from reference library.

<sup>h</sup> Two of six isolates not tested from VersaTREK Myco Broth.

<sup>i</sup> MALDI-TOF MS identified one isolate as *M marinum*.

<sup>j</sup> MALDI-TOF MS identified as *M marinum*.

<sup>k</sup> Compared with sequencing result.

<sup>l</sup> Both incorrect identifications in this category involve *M llatzerense*, which is absent from the reference library.

<sup>m</sup> Four of five incorrect identifications in this category involve *M llatzerense*, which is absent from the reference library.

Mycobacteria Library v1.0 with nucleic acid sequencing is 97.6% (81/83) for isolates cultured on solid medium and 97.5% (79/81) for those cultured in broth.

### Effect of Medium on Confidence Score

The increased identification rate observed when analyzing isolates from broth culture using either the standard MALDI Biotyper 3.1 reference library or Mycobacterium Library v1.0 was the result of overall higher average confidence scores obtained from broth culture (Table 3). This may be because of more reproducible growth or biomass in broth culture specimens compared with preparation of suspensions from isolates cultured on solid medium. Using MALDI Biotyper 3.1 standard library, mean confidence scores for solid medium-cultured isolates in five of 11 groups/species were 1.7 to 2.0 (acceptable, genus only) and mean confidence scores for the remaining six groups/species were less than 1.7 (unreliable identification). In contrast, when analyzed directly from broth culture, eight of 11 groups/species generated mean confidence scores greater than 1.7 including four groups/species (*M avium*/*M intracellulare*, *M fortuitum*, *M gordonae*, *M kansasii*) with mean scores greater than 2.0.

Use of Mycobacterium Library v1.0 coincided with higher overall mean confidence scores for all groups/species tested (Table 3). For isolates cultured on solid medium, nine of 11 groups/species generated mean confidence scores greater than 1.7 including four groups/species with scores greater than 2.0. For isolates cultured in broth all 11 groups/species generated high, secure species mean confidence scores greater than 2.0. Confidence scores for isolates of *M tuberculosis* complex were among the most highly affected, increasing from a mean confidence score of  $1.59 \pm 0.08$  (unreliable identification) using the standard reference library to  $2.24 \pm 0.11$  (secure species identification) using the mycobacterium-specific library (Table 3).

## Discussion

Infection with pathogenic NTM can be asymptomatic or result in pulmonary, skin and soft tissue, bone, and/or disseminated disease.<sup>7,9,11</sup> The ubiquity of NTM in the natural and health care environment makes recovery of NTM in clinical specimens difficult to interpret. Water supply lines in the hospital environment are often colonized with NTM including *M gordonae* (52.9%-100%) and *M mucogenicum* (5.9%-80.5%) and can be a significant source of intraoperative or postsurgical infection.<sup>2-4</sup> Indeed, isolates of *M gordonae* and *M terrae* have been identified as the cause of pseudo-infection and pseudo-outbreak in hospitals.<sup>5</sup> Rapid identification of isolates obtained from surgical cultures may aid in differentiating “contaminants” of low pathogenic potential from species with well-established roles in surgical site or skin and soft tissue infection. The American Thoracic Society has offered criteria for the microbiologic diagnosis of NTM pulmonary disease, which includes multiple culture-positive sputum specimens or a single culture-positive specimen obtained via bronchial washing or lavage.<sup>11</sup> Unfortunately, this guideline may be inappropriate for individuals at higher risk for NTM infection or with comorbidities such as tuberculosis or human immunodeficiency virus infection.<sup>15</sup> For example, NTM were isolated from multiple cultures in only 27% of individuals with clinical symptoms consistent with NTM pulmonary disease; however, clinical and radiologic features were consistent with NTM disease in 27% to 97% of individuals not meeting American Thoracic Society criteria for disease (ie, NTM isolated from single culture).<sup>15</sup> In addition, a stronger correlation was found between clinical findings and patients infected with *M kansasii* than with those infected with *M fortuitum*.<sup>15</sup> In these cases rapid and accurate species-level identification may aid in guiding management decisions based on the pathogenic potential of the species isolated.

**Table 3**  
Mean Confidence Scores for Groups/*Mycobacterium* Species Using the Standard MALDI Biotyper 3.1 Standard Reference Library and Mycobacterium Library v1.0

HPLC Identification	No.	Biotyper 3.1 Standard Library		Mycobacterium v1.0	
		7H11, Mean (SD)	Myco Broth, Mean (SD)	7H11, Mean (SD)	Myco Broth, Mean (SD)
MAI	13	1.88 (0.22)	2.01 (0.25)	2.03 (0.27)	2.14 (0.15)
MTBc	5	1.46 (0.08)	1.59 (0.08)	2.00 (0.13)	2.24 (0.11)
<i>M abscessus/chelonae</i>	16	1.73 (0.22)	1.81 (0.28)	2.05 (0.17)	2.05 (0.17)
<i>M fortuitum</i>	15	1.85 (0.19)	2.04 (0.22)	2.04 (0.26)	2.16 (0.16)
<i>M gordonae</i>	4	1.84 (0.16)	2.05 (0.04)	1.99 (0.29)	2.22 (0.07)
<i>M mucogenicum</i>	16	1.57 (0.21)	1.66 (0.19)	1.87 (0.18)	2.08 (0.13)
<i>M xenopi</i>	6	1.84 (0.12)	1.71 (0.36)	1.69 (0.21)	2.09 (0.18)
<i>M marinum</i>	2	1.31 (ND)	1.89 (ND)	1.73 (ND)	2.25 (ND)
<i>M kansasii</i>	3	1.64 (0.04)	2.04 (0.07)	1.98 (0.16)	2.27 (0.02)
<i>M szulgai</i>	2	1.46 (ND)	1.44 (ND)	1.66 (ND)	2.14 (ND)
<i>M neoaurum</i>	2	1.60 (ND)	1.75 (ND)	1.94 (ND)	2.11 (ND)

HPLC, high-pressure liquid chromatography; MAI, *M avium*/*M intracellulare*; MTBc, *M tuberculosis* complex; ND, not done.

A major obstacle to timely identification of *Mycobacterium* species is the inherently slow growth rate of these organisms, which can require weeks to grow on solid medium. Growth rate and time to detection can be enhanced using broth culture,<sup>34</sup> and both solid and liquid cultures are currently recommended for optimal recovery of mycobacteria in clinical specimens.<sup>11</sup> Rapid methods such as the AccuProbe test can identify *M tuberculosis* complex, *M avium* complex, *M gordonae*, and *M kansasii* from broth culture.<sup>19-21</sup> The performance of these tests can be high, reaching a sensitivity of more than 95% and specificity of 88%; however, sensitivity can be affected by cultures with a low organism concentration; sensitivity and specificity are both dependent on the luminescent cutoff value used to define a positive result.<sup>19-21</sup> An immunochromatographic direct test to identify *M tuberculosis* complex in broth cultures has been evaluated and has demonstrated a sensitivity of 85.4% to 98.5% and specificity of 99.2% to 100% but is not cleared by the Food and Drug Administration for use in the United States.<sup>35,36</sup>

We demonstrated the ability of MALDI-TOF MS to both rapidly and accurately identify mycobacteria to the species level from solid medium or directly from broth culture. Identification rate and accuracy compared with nucleic acid sequencing was enhanced using Mycobacteria Library v1.0. This library was improved through the addition of 54 reference spectra (119 at the time of this study, currently including 173 spectra representing 94 species) as well as an improved extraction method to generate more well-defined spectral profiles. It is difficult to assess which modification had a greater impact on identification of isolates; however, previous studies have demonstrated excellent results using a similar extraction process with a considerably smaller reference library.<sup>31</sup> We also observed better performance in the form of increased mean confidence scores for all species or groups of mycobacteria when analyzed directly from broth culture. This finding is in contrast to an earlier report by Lotz et al,<sup>32</sup> who noted a 77% identification rate from broth compared with 97% from solid medium. These studies had two major differences. First, broth culture was conducted using the BACTEC MGIT system (BD Diagnostics, Sparks, MD) in the previous study, whereas the current study used the VersaTREK system. Second, Lotz et al<sup>32</sup> analyzed isolates directly on ethanol-inactivated biomass without any processing or extraction. In the present study, all isolates obtained from solid medium or broth were subjected to an optimized extraction procedure using formic acid and acetonitrile in addition to physical disruption using silica beads. A comparison of spectra obtained with and without silica bead-based disruption demonstrated superior quality spectra when using bead-based disruption.

A specific advantage of MALDI-TOF MS-based identification of mycobacteria is the ability to differentiate among closely related species. This was clearly demonstrated by the

high level of agreement between MALDI-TOF MS identification and gold standard nucleic acid sequencing using 16S rDNA or *rpoB* gene targets. Of note, all 15 *M chelonae/M abscessus* and 13 *M avium/M intracellulare* were accurately identified to species level with MALDI-TOF MS. Sixteen isolates were identified as *M mucogenicum* with HPLC. This group is made up of a number of closely related strains including *M mucogenicum*, *M phocaicum*, *M aubagnense*, and *M llutzerense*.<sup>12,37</sup> Genetic similarity among these species is extremely high for all targets including *gyrB*, *hsp65*, *recA*, *rpoB*, and *sodA* genes.<sup>37</sup> Sequencing of the 16S rDNA, which can be identical between *M mucogenicum* and *M phocaicum*, was insufficient to differentiate between *M mucogenicum/M phocaicum* in six of ten isolates. MALDI-TOF MS identified five of these as *M phocaicum* and the remaining isolate as *M mucogenicum*. The remaining four isolates in this group generated definitive identification with nucleic acid sequence (three *M phocaicum*, one *M mucogenicum*), and sequence data and MALDI-TOF MS identification results were in agreement.

Of all isolates generating discordant results on direct broth culture analysis, five of seven were in the *M mucogenicum* group. Four of these were identified as *M llutzerense* and one was identified as *M aubagnense* with nucleic acid sequencing. All four isolates identified as *M llutzerense* generated subjectively good peaks and attained high confidence scores when identified from broth and solid medium culture using Mycobacteria Library v1.0. This suggests a high level of similarity between spectra obtained from isolates identified as *M llutzerense* with nucleic acid sequencing and the matching spectrum in Mycobacteria Library v1.0 (listed as *M mucogenicum*). This apparent misidentification could be the result of highly similar spectral profiles between these closely related species, but may also be the result of misidentification of the *M mucogenicum* isolate used to create the reference spectrum. This is particularly feasible in this instance because *M llutzerense* is a newly described species and may not have been differentiated from *M mucogenicum* at the time the reference library was constructed.<sup>37</sup> The two final discordant results were identified as *M pseudoshottsii* with nucleic acid sequencing but as *M marinum* with HPLC and MALDI-TOF MS. *M pseudoshottsii* is present in Mycobacteria Library v1.0, and therefore should be identified with MALDI-TOF MS. *M pseudoshottsii* is a relatively newly described species and is closely related to *M marinum* and *M ulcerans* based on 16S rDNA sequence, differing by as few as four nucleic acids.<sup>38</sup> One study using nucleic acid probes found cross reactivity between *M pseudoshottsii* and *M marinum*, suggesting that the identification of *M pseudoshottsii* with 16S rDNA sequencing may be incorrect.<sup>39</sup>

The strengths of this study included: (1) the evaluation of two commercially available reference spectrum libraries (one general, one mycobacteria specific) and (2) the analysis



of isolates cultured on solid medium and in broth in parallel. These comparisons demonstrated that the identification rate and strength of confidence score, ie, the number of isolates attaining “acceptable” identification scores ( $\geq 1.7$ ) and the proportion of isolates that are identified with “high” scores ( $> 2.0$ ), are significantly higher when isolates are identified directly from broth culture. This was true regardless of which reference library was used. Interestingly, higher mean confidence scores did not correlate with a higher correct identification rate. Isolates generating lower confidence scores (1.7-2.0) had a similar concordance rate with nucleic acid sequencing results as those with higher confidence scores ( $> 2.0$ ). This phenomenon has been reported in previous studies using MALDI-TOF MS to identify bacteria and yeast, suggesting that the manufacturer-driven score thresholds may be overly conservative.<sup>23,24,40,41</sup> The concordance rate was affected by the reference library used. We observed a 10.4% increase in correct identification rate for isolates cultured on solid medium and 6.7% increase for those cultured in broth when using Mycobacteria Library v1.0 compared with MALDI Biotyper standard library. This is likely the results of the expansion of Mycobacteria Library v1.0 to include more reference spectra as well as the optimized extraction methods used to create the spectra.

A potential weakness of this study was the use of banked clinical isolates rather than prospective clinical specimens. Though all isolates will be extracted to purify the mycobacterial proteins for analysis, components present in clinical specimens have the potential to negatively affect identification rate.<sup>23,42</sup> Further, an established weakness of MALDI-TOF MS is the inability to identify individual components of a polymicrobial culture.<sup>23</sup> A study is currently under way to examine the performance of MALDI-TOF MS using prospectively collected specimens. A second potential weakness is the limited number of isolates representing some of the species in this study. Because of this weakness, we could not adequately assess the impact of strain diversity on the rate or accuracy of identifying some species.

In conclusion, we describe the use of MALDI-TOF MS as a reliable method for identifying *Mycobacteria* species from solid and liquid culture when used in conjunction with a robust reference library. Additional studies using clinical specimens are needed to validate the efficacy of MALDI-TOF MS for identifying *Mycobacteria* species. This technology holds promise for routine use in the clinical laboratory.

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