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Identification of Mycobacteria from Solid and Liquid Media by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry in the Clinical Laboratory

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Mycobacteria cause significant morbidity in humans. Rapid and accurate mycobacterial identification is important for improvement of patient outcomes. However, identification may be challenging due to the slow and fastidious growth of mycobacteria. Several diagnostic methods, such as biochemical, sequencing, and probe methods, are used for mycobacterial identification. We compared the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) Biotyper system (Bruker Daltonics) to 16S rRNA/*hsp65* sequencing and/or DNA probes (Gen-Probe) for mycobacterial identification. One hundred seventy-eight mycobacterial isolates grown on solid and/or broth medium were included in the study. MALDI-TOF MS identified 93.8% of the mycobacteria isolates accurately to the species level and 98.3% to the genus level, independent of the type of medium used for isolation. The identification of mycobacteria directly from cultures using MALDI-TOF MS allows for precise identification in an hour compared to traditional biochemical and phenotypic methods that can take weeks or probes and sequencing that may take a few hours. Identification by MALDI-TOF MS potentially reduces the turnaround time and cost, thereby saving resources within the health care system.

Mycobacterial infections are a worldwide public health problem causing significant morbidity and mortality (1, 2). Mycobacteria have a spectrum of virulence and antibiotic susceptibility. The major causes of morbidity and mortality are tuberculous mycobacteria belonging to the *Mycobacterium tuberculosis* complex (2). A World Health Organization report indicates that 9.4 million people are newly infected with *M. tuberculosis* and 1.7 million people die of tuberculosis every year (3). Nontuberculous mycobacteria (NTM) comprise a large group of organisms that are widely present in the environment and commonly inhabit soil and water (4). NTM are considered to be opportunistic pathogens causing pulmonary, skin, soft tissue, lymphatic, and disseminated infections, as well as nosocomial outbreaks related to inadequate disinfection/sterilization of medical devices. The NTM are grouped into slow growers and rapid growers. Of clinical importance are the slowly growing mycobacteria, namely the *M. avium-M. intracellulare* complex (MAIC), *M. kansasii*, *M. marinum*, *M. xenopi*, *M. simiae*, and *M. ulcerans*, and the rapidly growing mycobacteria, namely *M. abscessus*, *M. chelonae*, and *M. fortuitum*. The number of NTM has increased rapidly over the past few decades, largely due to advances in identification techniques (5). The American Thoracic Society and the Infectious Diseases Society of America recommend that clinically significant NTM be identified to the species level in order to determine their clinical significance and select appropriate treatments (6).

It is important to differentiate mycobacterial isolates to the species level in order to define them as true pathogens or environmental contaminants. The identification of mycobacterial species has traditionally been based on conventional biochemical tests and phenotypic characteristics, such as growth rate and pigmentation. Although these tests are simple to perform, they are laborious, cumbersome, and require extensive incubation periods, thereby delaying prompt and accurate mycobacterial identification, which can be of serious consequence for good patient care.

Molecular methods, namely DNA probes and DNA sequencing, have provided fast and reproducible ways of identifying *Mycobacterium* species. Highly sensitive DNA probes are available for the *M. tuberculosis* complex, MAIC, *M. kansasii*, and *M. gordonae* (AccuProbe, Gen-Probe, San Diego, CA, and Inno-LiPA Mycobacteria, Innogenetics, Ghent, Belgium). However, DNA probes for less-frequently encountered species of mycobacteria often lack specificity, including problems with misidentification (7). 16S rRNA-based mycobacterial identification has been the primary technique for molecular taxonomic studies (8). Other targets for molecular technique-based species identification of mycobacteria include the heat shock protein (*hsp65*) gene, the intergenic gene between 16S and 23S, and the gene coding for the beta subunit of RNA polymerase (*rpoB*) (9–11). Molecular techniques have helped greatly to improve the turnaround time; however, these require a high level of expertise and a special laboratory setup (13–15). High-pressure liquid chromatography (HPLC) for the analysis of mycolic acids (β-hydroxy-α-fatty acids) is also used to identify mycobacteria from culture; however, this technique is available in only a few specialized laboratories (12).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid, powerful, and relatively inexpensive tool that is being increasingly used for the identification of bacteria and yeasts in the clinical laboratory setting (12a). In this study, we validated the Biotyper MALDI-

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TABLE 1 *Mycobacterium* species included in the study and media used for culture

Mycobacterium species	No. of isolates tested	No. of isolates obtained with medium type:			No. of isolates obtained from:		
		MGIT	7H10/11	LJ	Patients	ATCC (no.[a])	Reference laboratory
M. tuberculosis complex	36	18	15	3	22	3 (25177)[b], 1 (35747)	10
MAIC	31	16	13	2	28	2 (25291)	1
M. chelonae	24	5	17	2	18	1 (35752)	5
M. abscessus	23	7	15	1	18	1 (19977)	4
M. fortuitum group	16	4	12		14	1 (6841), 1 (700686)	
M. gordonae	15	10	5		15		
M. kansasii	14	5	8	1	13	1 (12478)	
M. mucogenicum group	11	2	9		10		1
M. xenopi	3		3		3		
M. marinum	2	2					2
M. scrofulaceum	1		1			1 (19981)	
M. lentiflavum	1	1			1		
M. kumamotonense	1		1		1		
Total no. of isolates	178	70	99	9	143	12	23

[a] The ATCC numbers are shown in parentheses.
[b] Inoculated with different media.

TOF MS (Bruker Daltonics, Billerica, MA) for the identification of *Mycobacterium* species grown on solid or broth medium.

MATERIALS AND METHODS

Reference strains and clinical isolates. A total of 178 mycobacterial isolates comprising 35 reference strains (obtained from the American Type Culture Collection [ATCC] [$n = 12$] and reference laboratories [Cleveland Clinic, Center for Disease Control, Ohio Department of Health] [$n = 23$]), 2 isolates from environmental water sources (MAIC and the *M. mucogenicum* group), and 141 clinical strains previously isolated from patient specimens submitted to the Ohio State University Wexner Medical Center were used in this study (Table 1). These mycobacterial isolates were previously identified using DNA probes or 16S rRNA and/or *hsp65* DNA sequencing. These isolates were grown on Middlebrook 7H10/7H11 plates (Becton, Dickinson Microbiology Systems, Cockeysville, MD), Löwenstein-Jensen (LJ) medium (Bio-Rad, Hercules, CA), or mycobacteria growth indicator tubes (MGIT) (Becton, Dickinson Microbiology Systems, Cockeysville, MD) (Table 1). MGITs were supplemented with oleic acid-albumin-dextrose-catalase (OADC) and antimicrobials (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin [PANTA]), and cultures were incubated at 37°C in a 10% CO_2 incubator. The cultures were handled inside a biosafety level 2 (BSL2) cabinet wearing N95 respiratory masks, disposable gloves, and a gown.

Identification by DNA probes. Acridinium-ester-labeled DNA probes (AccuProbe, Gen-Probe, San Diego, CA) were used to identify *M. tuberculosis* complex, *M. avium* complex, *M. kansasii*, and *M. gordonae*. Briefly, a 1-μl loopful of an isolate growing on 7H10/7H11 medium or 100 μl of MGIT medium was placed in tubes containing glass beads and lysis reagents and was sonicated. The tubes were incubated at 95°C for 10 min, and 100 μl of the lysate was added to the hybridization tubes containing the lyophilized DNA probe. Tubes were incubated for 15 min at 60°C, and 300 μl of the selection reagent was added. Tubes were vortexed three times for 15 s and incubated at 60°C for 5 min (for MAIC and *M. gordonae*), 8 min (for *M. kansasii*), and 10 min (for *M. tuberculosis* complex). The assay results were read in a luminometer. Samples producing signals of ≥30,000 relative light units were considered to be positive for the tested organism (16).

Identification by 16S rRNA and *hsp65* gene sequencing. DNA was extracted from organisms in pure culture using the PrepMan Ultra reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR amplification of the 16S rRNA and *hsp65* genes was performed as previously described (10, 17). The amplicons were then purified using the QIAquick PCR purification kit (Qiagen, Germany) and sequenced (http://www.seattlebiomed.org/sequencing). Sequences were compared to those available in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi), and the Ribosomal Database Project (http://rdp.cme.msu.edu). A homology identity of ≥99% with the database sequence was used.

Protein extraction. Isolates growing on 7H10/7H11 and LJ media. Using a sterile 10-μl loop, mycobacterial colonies growing on solid medium (Middlebrook 7H10/7H11 plates and LJ slants) were transferred into a 1.5-ml screw-cap microcentrifuge tube containing 300 μl of distilled water to a turbidity approximately equaling a 2.0 McFarland standard, measured visually by comparing it with standards. On average, colonies were 3 to 10 days old, depending on the growth rate of the mycobacteria. Nine hundred microliters of 100% ethanol was added to the suspension, and the tubes were vortexed and centrifuged at 13,000 rpm for 2 min. The supernatant was discarded and the pellet was resuspended in 500 μl of distilled water, vortexed, and centrifuged again at 13,000 rpm for 2 min. The supernatant was discarded, the tubes were centrifuged at 13,000 rpm for 2 min, and all residual liquid was removed. The pellet was resuspended in 50 μl of water and heat inactivated at 100°C for 30 min. The tubes were allowed to cool at room temperature for 2 min, and 1.2 ml of precooled 100% ethanol was added to the suspension. Following vortexing, the tubes were centrifuged at 13,000 rpm for 2 min and the supernatant was discarded. The tubes were centrifuged again at 13,000 rpm for 2 min and all residual liquid was completely removed with a pipette. The pellet was allowed to dry at room temperature for 2 to 3 min. The pellet was suspended in 10 to 50 μl of acetonitrile, depending on the pellet size, and approximately 50 to 100 0.5-mm-diameter glass beads (Research Products International Corp., Mount Prospect, IL) were added to the pellet. The tubes were vortexed for 1 min and 70% formic acid (same volume as acetonitrile) was added, and the sample was vortexed again for 1 min. The tubes were centrifuged at 13,000 rpm for 2 min, and the supernatant was used for analysis by MALDI-TOF MS. All steps requiring open manipulation of mycobacteria before heat inactivation were performed inside a biological safety cabinet in a biosafety level 2 setup.

Isolates growing in MGITs. MGITs were processed within 48 h after a positive result was achieved (Bactec MGIT 960, mycobacterial detection system; BD, Franklin Lakes, NJ). MGITs were centrifuged at 4,100 rpm for

TABLE 2 MALDI-TOF MS scores for *Mycobacterium* species included in the study

Mycobacterium species	No. of isolates tested	No. (%) detected to the species level with a MALDI score of:		
		≥2.000	1.700–1.999	<1.700
M. tuberculosis complex	36	36 (100)		
MAIC	31	26 (83.8)	3 (9.7)	2 (6.5)
M. chelonae	24	23 (95.8)	1 (4.2)	
M. abscessus	23	23 (100)		
M. fortuitum group	16	14 (87.5)	2 (12.5)	
M. gordonae	15	14 (93.7)	1 (6.2)	
M. kansasii	14	14 (100)		
M. mucogenicum group	11	9 (82)	1 (8)	1 (8)
M. xenopi	3	3 (100)		
M. marinum	2	2 (100)		
M. scrofulaceum	1	1 (100)		
M. lentiflavum	1	1 (100)		
M. kumamotonense	1	1 (100)		
Total isolates	178	167 (93.8)	8 (4.5)	3 (1.7)

15 min (Heraeus/Baxter Megafuge 1.0; Thermo Scientific), and 100 to 200 μl of the sediment from the bottom was transferred into a 1.5-ml screw-cap microcentrifuge tube. The tubes were centrifuged at 13,000 rpm for 2 min and the supernatant was discarded. The tubes were centrifuged again at 13,000 rpm for 2 min and the liquid was completely removed. The pellet was resuspended in 300 μl of water and vortexed, and 900 μl of 100% ethanol was added. The remaining steps were the same as described above for isolates from 7H10/7H11 or LJ slants.

MALDI-TOF MS. One microliter of extracted protein (supernatant) was spotted in duplicate onto a steel target (MTP 384 target plate polished steel BC; Bruker Daltonics GmbH, Bremen, Germany) and air dried at room temperature. One microliter of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid (Bruker Daltonics) was pipetted onto each of the spotted samples. The bacterial test standard (Bruker Daltonics) was used as a positive control and the matrix alone as a negative control. After drying briefly, the target was inserted into the MALDI-TOF MS microflex LT (Bruker Daltonics), and the nitrogen laser was fired at the target, resulting in ionization of the sample to achieve a mass-to-charge ratio (*m*/*z*) for each ionized protein between 2,000 and 20,000 Da. Each spot was measured using 1,000 laser shots at 60 Hz in groups of 40 shots per sampling area. MALDI-TOF MS analysis was performed in automatic mode, and a minimum of 240 laser shots were collected for each spot.

Analysis of spectra. Spectra were analyzed against those in the Bruker database (Mycobacteria database v1; Bruker Daltonics) using the Biotyper software (v3.0; Bruker Daltonics), which assigned a logarithmic score ranging from 0 to 3 and determined the best match based on the *m*/*z* ratio and relative peak size of each ionized protein. Scores were interpreted as ≥2.000 for genus- and species-level identification, 1.700 to 1.999 for genus-level identification, and <1.7 as unreliable for identification. Isolates with low scores (<2.0) were reextracted from the same MGIT or solid medium and reanalyzed. The higher score of the two reads was used.

RESULTS

A total of 178 mycobacterial isolates, including strains from ATCC, reference laboratories, and patient isolates, were used to assess the performance and clinical validity of the Bruker Biotyper MALDI-TOF MS (Table 1). The isolates were grown on either Middlebrook 7H10/7H11 ($n = 99$), MGIT ($n = 70$), or LJ slants ($n = 9$), the media commonly used for mycobacteria isolation in the mycobacteriology laboratory (Table 1). All isolates were heat inactivated at 100°C for 30 min prior to extraction. A total of 57 isolates of mycobacteria belonging to different species were checked for viability following protein extraction. These included 10 isolates each from the *M. tuberculosis* complex and MAIC, 5 isolates each from *M. chelonae*, *M. abscessus*, the *M. fortuitum* group, *M. kansasii*, *M. gordonae*, and the *M. mucogenicum* group, two isolates from *M. xenopi* and *M. marinum*, and one isolate each from *M. scrofulaceum*, *M. lentiflavum*, and *M. kumamotonense*. Nonviability was confirmed by culturing the inactivated cultures on Middlebrook 7H10/7H11 medium and incubating for 8 weeks. Of the 178 isolates, 167 (93.8%) and 175 (98.3%) were correctly identified to the species and genus levels, respectively (Table 2). Three isolates (1.7%) had scores of <1.700 and did not yield any identification.

TABLE 3 Mycobacterial isolates with MALDI scores of ≤2.000

Patient isolate/ATCC no.	Medium	DNA probe/sequencing identification result	MALDI results	
			Identification	Score
1	MGIT	MAIC	Not reliable	1.490
2	MGIT	MAIC	Not reliable	1.330
3	MGIT	MAIC	*M. intracellulare*	1.720
4	7H10	MAIC	*M. intracellulare*	1.770
5	7H10	MAIC	*M. avium*	1.940
6	MGIT	*M. gordonae*	*M. gordonae*	1.960
7	7H11	*M. mucogenicum* group	*M. phocaicum*	1.910
8	7H11	*M. mucogenicum* group	Not reliable	1.670
9	MGIT	*M. fortuitum* group	*M. porcinum*	1.950
10	7H11	*M. fortuitum* group	*M. septicum*	1.940
ATCC 35752	7H10	*M. chelonae*	*M. chelonae*	1.960

Of the different *Mycobacterium* species analyzed, a MALDI score of ≥2.000 was obtained for 36/36 species from the *M. tuberculosis* complex, 26/31 from MAIC, 23/24 from *M. chelonae*, 23/23 from *M. abscessus*, 14/16 from the *M. fortuitum* group, 14/15 from *M. gordonae*, 14/14 from *M. kansasii*, 9/11 from the *M. mucogenicum* group, 3/3 from *M. xenopi*, 2/2 from *M. marinum*, 1/1 from *M. lentiflavum*, 1/1 from *M. kumamotonense*, and 1/1 from *M. scrofulaceum*. A score of 1.700 to 1.999 was obtained for 3/32 from MAIC, 2/16 from the *M. fortuitum* group, 1/24 from *M. chelonae*, 1/15 from *M. gordonae*, and 1/11 from the *M. mucogenicum* group. A score of <1.700 was obtained for 2/32 from MAIC and 1/11 from the *M. mucogenicum* group (Table 2). Upon repeating the procedure, similar results were obtained. All MALDI-TOF MS identifications with scores of ≥2.000 correlated with the identifications obtained with DNA probes and/or sequencing (data not shown).

Eleven mycobacterial species that gave an identification score of <2.000 were reanalyzed using species-level cutoffs of ≥1.900. With the ≥1.900-score cutoff, we obtained correct species identification on an additional 6/11 isolates of mycobacteria (Table 3). Overall, 173/178 (97.2%) isolates were identified to the genus and species levels, an increase of 3.4% compared to the results when a cutoff MALDI score of ≥2.000 was used.

DISCUSSION

In our study, the MALDI-TOF MS correctly identified 93.8% of mycobacterial isolates to the species level and 98.3% to the genus level when the manufacturer-recommended cutoff of 2.0 was used. Eleven out of 178 mycobacterial isolates were not identified to the species level (Table 2). Of these, 5 were from the MAIC, 2

from the *M. fortuitum* complex, 2 from the *M. mucogenicum* group, one from *M. chelonae*, and one from *M. gordonae*; we speculate that the lower MALDI scores might be due to the heterogeneity of a complex or group and that by increasing the number of spectra for these mycobacteria, we may be able to obtain higher identification scores. By lowering the MALDI score for the species level from 2 to 1.9, we were able to resolve 97.2% mycobacterial identifications to the species level (Table 3). The use of different isolation media commonly used in the laboratory for the recovery of mycobacteria gave similar results (Table 2).

Previous reports have shown the potential of MALDI-TOF MS for mycobacterial identification; however, these reports differ in the type of isolation medium, extraction methodology, and libraries used (18–21). In this study, besides solid medium, we also included MGITs, since they often become positive before growth on solid culture medium. To our knowledge, this is one of the first studies to utilize the Mycobacteria database v1 for mycobacterial identification in the clinical microbiology laboratory, especially from MGIT tubes.

Additionally, for safe processing and to minimize the risk of transmission, the mycobacteria were heat inactivated and treated with ethanol and mechanical disruption in the mycobacteriology laboratory in a biosafety cabinet in a BSL2 setup prior to analysis. This completely inactivated the mycobacteria, as evidenced by the subsequent lack of growth. The plates spotted with the extracted protein could safely be taken outside the mycobacteriology laboratory to be processed on the MALDI-TOF MS instrument in the general laboratory area.

The spectra were matched against the Bruker Mycobacteria library v1 that includes reference and clinical isolates representing 94 species and 173 strains. This represents a remarkable increase in spectra over the previous version of the library (v3.0.2.0), which contained 18 species comprising 18 strains. With the new library update, we were able to identify the majority of mycobacterial isolates. The database can also be enhanced by the testing laboratory by adding new spectra when additional *Mycobacterium* species are encountered for which there is no identified good match.

Rapid and accurate diagnosis of mycobacterial infections is important for initiating early treatment and thus preventing drug resistance (13). The clinical microbiology laboratory plays an important role in the rapid identification of mycobacterial species. However, the identification and differentiation of mycobacteria pose various challenges. Identification of mycobacteria based on phenotypic tests is time-consuming, labor-intensive, expensive, and often provides erroneous or inconclusive results (6). DNA probes are available for only a limited number of species and may misidentify a number of less-common mycobacteria (16). Gene sequencing and chromatographic analysis of lipids using HPLC and gas-liquid chromatography methods are technically cumbersome, relatively expensive, and available in only a few specialized clinical laboratories (12). DNA sequencing using 16S rRNA alone cannot differentiate between closely related rapidly growing mycobacterial species, including *M. chelonae* and the *M. abscessus* group and between *M. mucogenicum* and *M. phocaicum*, and it requires the use of *hsp65* or *rpoB* sequencing (15). While MALDI-TOF MS can differentiate between *M. chelonae* and the *M. abscessus* group, and between *M. avium* and*M. intracellulare*, it cannot differentiate *M. intracellulare* from *M. chimaera* (18). Moreover, it cannot differentiate between *M. marinum*, *M. shottsii*, and *M. pseudoshottsii* based on the spectra currently available in the database.

The Bruker library additionally groups to the complex level for the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti*, *M. caprae*, *M. microti*, and *M. pinnipedii*), the MAIC (*M. avium*, *M. intracellulare*, *M. chimera/M. colombiense*, *M. hominissuis*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum*, *M. timonense*, *M. arosiense*, *M. marseillense*, *M. bouchedurhonense*), the *M. fortuitum* complex (*M. fortuitum*, *M. farcinogenes*, *M. porcinum*, *M. farcinogenes* subsp. *senegalense*, *M. peregrinum*, *M. setense*, *M. septicum*, *M. houstonense*, *M. boenickei*, *M. brisbanense*, *M. neworleansense*, and *Mycobacterium* sp. [1]), the *M. abscessus* group (*M. abscessus* sensu stricto, *M. abscessus* subsp. *bolletii*, and *M. massiliense*). Furthermore, we were unable to distinguish between *M. mucogenicum*, *M. phocaicum*, and *M. aubagnense* and between *M. marinum*, *M. shottsii*, and *M. pseudoshottsii* (18, 22). Further studies are warranted with additional mycobacterial strains, especially those belonging to the groups and complexes so as to improve the discrimination capabilities for these species. Differentiation of these species may be important in the future, since virulence differences have been observed between *M. avium* and *M. intracellulare*, with the latter causing disease in immunocompetent patients (22). Likewise, *M. abscessus* subsp. *bolletii* has been reported to show increased resistance to clarithromycin (23). It is therefore recommended that susceptibility testing to clarithromycin be performed to provide guidance on its use in treatment.

Overall, MALDI-TOF MS provides accurate identification that compares well with that of genomic sequencing. Moreover, the MALDI-TOF MS is quicker, providing identification within 1 to 2 h. The cost of identification is significantly less compared to other methods, including genomic sequencing, HPLC, and biochemical techniques. MALDI-TOF MS generates less waste than other methods that are based on molecular and biochemical tests that use many disposable materials. This study confirms the use of MALDI-TOF MS as a reliable technique for the accurate identification of most clinical strains of mycobacteria growing on solid and liquid media.

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