JOURNAL OF CLINICAL MICROBIOLOGY, May 2011, p. 1790–1794 Vol. 49, No. 5
0095-1137/11/$12.00 doi:10.1128/JCM.02135-10

Identification of Mycobacteria in Solid-Culture Media by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry▽

Paul G. Saleeb,[1] Steven K. Drake,[2] Patrick R. Murray,[3] and Adrian M. Zelazny[3]*

Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland[1]; Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland[2]; and Microbiology Service, Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland[3]

Received 21 October 2010/Returned for modification 27 November 2010/Accepted 3 March 2011

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has recently been introduced into the clinical microbiology laboratory as a rapid and accurate method to identify bacteria and yeasts. In this paper we describe our work on the use of MALDI-TOF MS for the identification of mycobacterial isolates. We developed a protocol for protein extraction from mycobacteria and utilized it to construct a database containing 42 clinically relevant type and reference strains of mycobacteria. The database was used to identify 104 clinical isolates of mycobacteria. All members of the *Mycobacterium tuberculosis* complex were identified accurately at the complex level but could not be separated at the species level. All other organisms were identified at the species level, with the exception of one strain of *M. kansasii* (accurately identified but with a low spectral score) and three pairs of closely related strains: *M. abscessus* and *M. massiliense*, *M. mucogenicum* and *M. phocaicum*, and *M. chimaera* and *M. intracellulare*. These pairs of organisms can currently be identified only by multilocus gene sequence analysis. We conclude that MALDI-TOF MS analysis can be incorporated into the work flow of the microbiology laboratory for rapid and accurate identification of most strains of mycobacteria isolated from solid growth media.

The genus *Mycobacterium* consists of nearly 150 species, many of which are clinically significant. These organisms cause significant morbidity in humans, including pulmonary infections, skin and soft tissue infections, and disseminated disease. Rapid and accurate diagnosis of mycobacterial infections is of utmost importance due to the fact that inappropriate treatment may lead to drug resistance or unnecessary exposure to drug toxicities. Accurate identification in the microbiology laboratory is challenging because biochemical testing is slow and unable to identify less common species, molecular probes are available for a limited number of species, and gene sequencing is technically cumbersome and relatively expensive.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is recognized as a powerful tool for the identification of bacteria and yeasts in the clinical laboratory (2, 16, 17, 18, 21). This technique allows identification of organisms on the basis of unique spectral fingerprints produced by extracted proteins (9). The method is relatively simple, rapid, and associated with significantly lower consumable costs than traditional microbiological identification methods (16). Although the MALDI-TOF MicroFlex LT mass spectrometer (Bruker Daltonics Inc., Billerica, MA) and associated software are expensive initially (approximately $200,000), the continuing consumable costs are inexpensive (less than $1 per isolate).

There has been limited work thus far on the use of MALDI-TOF MS for the identification of mycobacteria (5, 6, 10). This paper describes our efforts to develop and validate MALDI-TOF MS for the identification of mycobacterial organisms. In this study we developed a protein extraction protocol specifically designed for mycobacteria, created an identification database of clinically relevant type and reference strains of mycobacteria, and challenged the database with clinical isolates from our institution. We demonstrate that MALDI-TOF MS can be used in the clinical laboratory for rapid, accurate identification of mycobacteria from solid culture media.

MATERIALS AND METHODS

Mycobacterial type and reference strains and clinical isolates. The NIH mycobacterial database library was created from type and reference strains obtained from the American Type Culture Collection (ATCC; Manassas, VA), the Culture Collection of the University of Göteborg (CCUG; Göteborg, Sweden), and the Deutsche Sammlung von Mikroorganismen (DSM; Braunschweig, Germany). The library was then challenged with clinical isolates recovered in the NIH Clinical Microbiology Laboratory. Clinical isolates for all mycobacterial species were obtained from patients at the NIH Clinical Center, a research hospital where studies are conducted with patients from diverse regions of the United States and worldwide, with the exception of 9 *Mycobacterium tuberculosis* strains that were isolated in South Korea as part of an ongoing NIH study. Identification of all clinical isolates was performed by use of the AccuProbe test (Gen-Probe, San Diego, CA) or by sequencing of *secA1* (23), 16S rRNA (4, 12), or *hsp65* (11) genes. The reference strains and clinical isolates were frozen at −80°C until they were tested. Most strains were thawed at room temperature, subcultured onto Middlebrook 7H11 agar, and incubated at 37°C. *M. marinum* was incubated at 30°C, *M. xenopi* was incubated at 42°C, and *M. haemophilum* was cultured onto sheep blood agar and incubated at 30°C.

Protein extraction protocol. The MALDI-TOF MS protein extraction protocol for mycobacteria was as follows: a disposable 10-μl inoculating loop was used to obtain mycobacterial colonies grown on Middlebrook agar. The organisms were suspended in 500 μl of distilled water in a 1.5-ml screw-cap Eppendorf tube and heated to 95°C for 30 min to kill the mycobacteria. The tube was briefly vortexed, and a plastic micropestle (pellet pestle, 749521-1500; Kimble Chase, Vineland, NJ) was subsequently used to disperse the suspension in the Eppendorf tube for approximately 30 s. The tube was centrifuged in an Eppendorf 5415D centrifuge at 13,000 rpm for 2 min, and the supernatant was completely removed. The pellet

* Corresponding author. Mailing address: Microbiology Service, Department of Laboratory Medicine, Clinical Center, National Institutes of Health, 10 Center Drive, MSC 1508, Bethesda, MD 20892-1508. Phone: (301) 496-4433. Fax: (301) 402-1886. E-mail: azelazny@mail.nih.gov.

▽ Published ahead of print on 16 March 2011.

was washed with 300 μl of distilled water and centrifuged at 13,000 rpm for 2 min, and the liquid was removed. The pellet was then suspended in 1.2 ml of 70% ethanol, vortexed briefly, and centrifuged at 13,000 rpm for 2 min. The supernatant was decanted, the pellet was briefly spun in the centrifuge, and all residual liquid was completely removed with a pipette. Fifty microliters of 70% formic acid and 100 to 200 μl of 0.1-mm-diameter glass beads (zirconia-silica beads, 36270-62; Cole-Parmer Instrument Co., Vernon Hills, IL) were added to the pellet. The suspension was vortexed for 10 min, 50 μl of 100% acetonitrile was added, and the sample was vortexed for another 10 min. The sample was centrifuged for 2 min at 13,000 rpm, and the supernatant was utilized for analysis by MALDI-TOF MS.

MALDI-TOF mass spectrometry. One microliter of supernatant from each test sample was pipetted onto a spot on the MALDI-TOF steel target plate, and 1 μl of the calibration standard (protein calibration standards I and peptide calibration standards II; Bruker Daltonics Inc., Billerica, MA) was pipetted onto a separate spot. After the samples had dried, 2 μl of the MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid prepared daily) was pipetted onto the samples and calibration standard. A slide warmer (Premiere slide warmer, XH-2002; Daigger, Vernon Hills, IL) heated to 35°C was used to expedite uniform drying of the samples and matrix. The samples and matrix routinely dried within approximately 5 min. Spectra were acquired over a mass/charge (*m*/*z*) ratio of 2,000 to 20,000 using a MALDI-TOF MicroFlex LT mass spectrometer (Bruker Daltonics Inc., Billerica, MA) in linear mode and summing 1,000 laser shots over 20 sites on each sample.

Spectral analysis. Criteria established by Bruker Daltonics for construction of the reference database are that each spectrum must contain at least 25 peaks with a resolution greater than 400, of which 20 of the peaks must have a resolution greater than 500. The NIH mycobacterial database was created with spectra meeting these standards, with a minimum of 8 quality spectra per organism required for entry of the mycobacterial strain into the reference database. During this study clinical isolates were run in quadruplicate, and the spectral patterns were matched with those in the NIH and the manufacturer's databases. The BioTyper software (version 2.0.4) assigned scores of 0 to 3, with higher scores indicating a greater probability of correct organism identification. The scoring software is a proprietary algorithm, calculating a logarithmic score on the basis of spectral matching patterns. On the basis of our previous experience with bacterium and yeast identification by MALDI-TOF MS, accurate identification is obtained with scores of 1.8 or greater (17, 18).

RESULTS AND DISCUSSION

Optimization of extraction protocol for mycobacteria. Working with mycobacteria in the open work area of the laboratory poses significant risks to laboratory personnel. To address this, we evaluated a heat-killing protocol. Mycobacterial growth from the surface of an agar plate was collected with a disposable 10-μl loop, transferred to a 1.5-ml Eppendorf tube with 500 μl of distilled water, and heated to 95°C for 30 min. The heated suspension was vortexed, subcultured onto Middlebrook 7H11 agar, incubated, and checked weekly for growth. The entire procedure was performed for 20 different mycobacterial species, including *M. tuberculosis*. None of the species grew after 6 weeks of incubation, indicating that the extraction protocol could be performed safely in the open laboratory after this heat-killing step.

Initial attempts at protein extraction using the protocol for bacteria and yeasts (18) did not yield adequate spectra for MALDI-TOF MS analysis. Acid-fast staining of the heated samples showed that the mycobacteria had clumped together. Different methods for dispersing the clumps were compared, including repeatedly passing the suspension through a narrow-gauge needle into a syringe, grinding the specimen in a 15-ml conical tube with a tissue grinder, and grinding the specimen in a 1.5-ml Eppendorf tube with a micropestle. Acid-fast staining demonstrated that the micropestle had dispersed the clumps more than the other techniques. In addition, the micropestle was the least cumbersome and the most cost-effective (approximately $0.70 per micropestle).

Despite the successful disruption of clumps with the micropestle, the quality of spectra for several species was still poor and precluded adequate organism identification. It was noted that the problematic isolates (e.g., selected strains of *M. abscessus* and *M. fortuitum*) produced smooth colonies on solid growth media. We recognized that mycobacterial colony morphology may be a function of the presence of glycopeptidolipids (GPLs) in the cell wall, as has been shown in the case of the *M. avium* complex and *M. smegmatis* (14). We then hypothesized that the extraction reagents were unable to penetrate the GPL-rich cell envelope for adequate protein extraction in these isolates. Several methods for physically breaking up the cell envelopes were considered. We hypothesized that zirconia-silica beads should be able to accomplish this without destroying the proteins needed for accurate identification by MALDI-TOF MS. We compared the quality of the spectra produced by beads of different diameters (0.1 mm, 0.2 mm, and 0.4 to 0.6 mm) for analysis of a smooth strain of *M. abscessus* and *M. fortuitum*. Beads were added to the first extraction reagent (70% formic acid) in an Eppendorf tube containing one of these organisms, and the tube was vortexed for 10 min. The best results were obtained with 0.1-mm-diameter beads. After incorporation of the use of the micropestle and silica beads into the mycobacterial protein extraction protocol, we were able to generate reproducible high-quality spectra for all species of mycobacteria that were analyzed.

One issue that arose while the use of silica beads with formic acid for protein extraction was considered was the possibility that heat generated during vortexing may lead to formylation of the proteins and subsequently result in misidentification of the organisms. We addressed this concern by vortexing 10 μl of peptide standards with the beads in 70% formic acid for 10, 20, or 60 min. The negative control consisted of 1 μl of untreated peptide standards plated directly onto the MALDI-TOF steel well. For the positive control, we boiled 10 μl of the standards in 50 μl of formic acid at 55°C for 60 min prior to plating on the target. MALDI-TOF spectra were acquired and analyzed. Compared with the negative control, spectra of the positive control had shifted by multiples of 28 *m*/*z* (the mass of a formyl group), indicating that formylation had occurred. No formylation occurred in the samples that were vortexed for 10 or 20 min, and a minimal amount of formylation occurred in the sample that was vortexed for 60 min. Consequently, we concluded that no changes were needed in our 10-min extraction protocol.

Creation of mycobacterial database. Our novel protein extraction protocol allowed us to create our own database of mycobacterial signatures. Forty-two type and reference strains comprising 37 species of mycobacteria were analyzed by MALDI-TOF MS and produced spectra meeting the requirements for incorporation into the NIH mycobacterial database (Table 1). The NIH database contains three species in the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*), 24 other species of slowly growing mycobacteria, and 10 species of rapidly growing mycobacteria. The Bruker database (version 3.0.2.0), on the other hand, contains 50 strains of mycobacteria, comprising 18 species. The NIH database is more comprehensive, containing additional clinically relevant

TABLE 1. Reference strains incorporated into the NIH mycobacterial MALDI-TOF database

Strain
Mycobacterium abscessus ATCC 19977^T
Mycobacterium abscessus ATCC 23040
Mycobacterium africanum ATCC 25420^T
Mycobacterium asiaticum CCUG 29115^T
Mycobacterium avium subsp. *avium* ATCC 25291^T
Mycobacterium bolletii CCUG 50184^T
Mycobacterium bovis ATCC 19219^T
Mycobacterium celatum ATCC 51131^T
Mycobacterium chelonae ATCC 35749
Mycobacterium chelonae ATCC 35751
Mycobacterium chelonae ATCC 35752^T
Mycobacterium chimaera CCUG 50989^T
Mycobacterium colombiense DSM 45105^T
Mycobacterium flavescens ATCC 14474^T
Mycobacterium fortuitum ATCC 35931
Mycobacterium fortuitum ATCC 6841^T
Mycobacterium gastri ATCC 15754^T
Mycobacterium gordonae ATCC 14470^T
Mycobacterium haemophilum ATCC 29548^T
Mycobacterium intracellulare ATCC 13950^T
Mycobacterium kansasii ATCC 35775
Mycobacterium mageritense CCUG 37984^T
Mycobacterium malmoense ATCC 29571^T
Mycobacterium mantenii DSM 45255^T
Mycobacterium marinum 2006 1152[a]
Mycobacterium marseillense CCUG 56325
Mycobacterium massiliense CCUG 48898^T
Mycobacterium mucogenicum ATCC 49650^T
Mycobacterium nonchromogenicum ATCC 19530^T
Mycobacterium peregrinum ATCC 14467^T
Mycobacterium peregrinum ATCC 700686
Mycobacterium phocaicum CCUG 50185^T
Mycobacterium scrofulaceum ATCC 19981^T
Mycobacterium shimoidei ATCC 27962^T
Mycobacterium simiae ATCC 25275^T
Mycobacterium smegmatis CCUG 21002^T
Mycobacterium szulgai ATCC 35799^T
Mycobacterium triplex ATCC 700071^T
Mycobacterium triviale ATCC 23292^T
Mycobacterium tuberculosis H37RV ATCC 27294^T
Mycobacterium ulcerans ATCC 35839
Mycobacterium xenopi ATCC 19250^T

[a] The type strain for *M. marinum*, *M. marinum* ATCC 927, was replaced by a clinical isolate of *M. marinum*, *M. marinum* 2006 1152, in the database.

TABLE 2. Clinical isolates identified by MALDI-TOF MS

Species (no. of isolates tested)	No. of isolates with the following highest score:			
	<1.8	1.8–<1.9	1.9–<2.0	≥2.0
M. abscessus (6)[a]			1	5
M. asiaticum (2)				2
M. avium (7)			1	6
M. chelonae (5)				5
M. chimaera (2)[b]				2
M. fortuitum (6)				6
M. gordonae (5)				5
M. intracellulare (6)[b]				6
M. kansasii (15)	1	3	4	7
M. marinum (5)				5
M. massiliense (6)[a]			1	5
M. mucogenicum (5)[c]		1	1	3
M. peregrinum (4)				4
M. smegmatis (1)			1	
M. szulgai (2)				2
M. tuberculosis complex (26)				26
M. xenopi (1)				1
Total (104)	1	4	9	90

[a] *M. abscessus* and *M. massiliense* were not differentiated from each other by MALDI-TOF MS.
[b] *M. chimaera* and *M. intracellulare* were not differentiated from each other by MALDI-TOF MS.
[c] *M. mucogenicum* was identified as *M. mucogenicum* and *M. phocaicum* by MALDI-TOF MS.

species, including *M. bolletii*, *M. haemophilum*, *M. massiliense*, *M. scrofulaceum*, and *M. szulgai*. Similar to the Bruker database, our database contains *M. avium*; however, we were able to incorporate a more complete catalogue of *M. avium* complex organisms that includes *M. chimaera*, *M. colombiense*, *M. intracellulare*, *M. mantenii*, and *M. marseillense* (Table 1). By creating our own database, we have opened the possibility for the future addition of other species of mycobacteria into the database as needs arise. This is of special importance, because the number of species within the genus *Mycobacterium* is increasing rapidly (19) and clinical laboratories will be challenged to remain abreast of this ever-expanding taxonomy.

Testing the library. One hundred four clinical isolates comprising 17 species of mycobacteria were analyzed by the NIH mycobacteria library (Table 2). Only one isolate, an *M. kansasii* strain, had a spectral score of <1.8. It was correctly identified but had scores ranging from 1.61 to 1.78. All *M. tuberculosis* complex isolates attained scores of >2.0 and were unambiguously differentiated from nontuberculous mycobacteria. An example of the spectral patterns obtained for these organisms is shown in Fig. 1. The 26 *M. tuberculosis* complex organisms tested included 7 isolates of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Beijing and non-Beijing strains of *M. tuberculosis* from patients in South Korea. The remainder of the isolates were obtained from native and foreign-born patients referred to the NIH Clinical Center, including two strains of *M. bovis*. Although MALDI-TOF MS accurately classified all isolates as members of *M. tuberculosis*

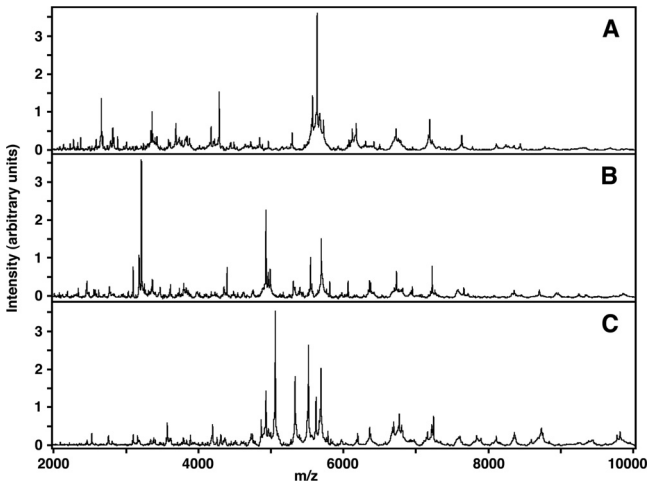

FIG. 1. Expanded view of MALDI spectra representing 3 clinical isolates. (A) *M. abscessus* (BioTyper score, >2.0); (B) *M. kansasii* (BioTyper score, >1.8); (C) *M. tuberculosis* (BioTyper score, >2.0).

complex, it was not able to separate them into the individual species. Likewise, three other pairs of closely related organisms could not be distinguished: *M. abscessus* and *M. massiliense*, *M. mucogenicum* and *M. phocaicum*, and *M. chimaera* and *M. intracellulare*.

Clinical isolates of *M. abscessus* and *M. massiliense*, identified in the microbiology laboratory by sequencing of the *secA*, *rpoB*, or *hsp65* genes, could not be discriminated from each other by MALDI-TOF. This is not surprising because members of the *M. abscessus* group (i.e., *M. abscessus* sensu stricto, *M. bolletii*, and *M. massiliense*) are closely related phylogenetically and cannot be differentiated readily on the basis of single-gene sequencing (7). Multilocus sequencing, as performed in our laboratory, is necessary for proper identification of these organisms (22).

MALDI-TOF MS was also unable to differentiate *M. mucogenicum* from *M. phocaicum*, another set of closely related species of rapidly growing mycobacteria (3). Similar to *M. abscessus* and *M. massiliense*, multilocus gene analysis is necessary for accurate differentiation of these two organisms (3). *M. mucogenicum* and *M. phocaicum* have similar clinical presentations: both organisms usually cause catheter-related infections (1, 3) and have similar antibiotic susceptibility patterns (1). Thus, there are few, if any, clinical implications in the inability of MALDI-TOF MS to differentiate between these two closely related species.

The third pair of organisms that could not be differentiated by MALDI-TOF MS was *M. chimaera* and *M. intracellulare*. These two organisms are closely related, differing by only 1 base pair in their 16S rRNA gene regions (15, 20); however, differentiation is possible on the basis of their nucleotide differences (20 bp) in the 16S to 23S internal transcribed spacer (ITS) region. Thus, the ambiguity encountered when these organisms are identified by MALDI-TOF MS is not surprising. Finally, there are conflicting reports as to whether or not *M. chimaera* is a more virulent species than *M. intracellulare* (15, 20).

In our initial work developing the mycobacterial database, we experienced problems with low spectral scores when the database was challenged with the *M. marinum* clinical isolates. To resolve this, we replaced the *M. marinum* type strain, ATCC 927, with a recent clinical isolate, *M. marinum* 2006 1152. When the clinical strains, as well as the original type strain, were retested all were correctly identified with spectral scores of >2.0. This observation underscores the value in using a diverse collection of strains for building the database. The type strain of this species was originally described in 1926, and it is possible that significant changes in the protein profile of this species have occurred in subsequent years.

Aside from the limitations noted above, MALDI-TOF MS analysis of mycobacteria worked quite well for the identification of mycobacterial isolates in our laboratory. During the study, each clinical isolate was assayed in quadruplicate for MALDI-TOF identification so we could determine the minimum number of tests per sample necessary for accurate identification. Of the clinical isolates with spectral scores of >2.0, a score of 1.8 or greater was achieved for 99% of the strains with the first spot tested. Thus, a single test is required for the identification of most organisms. Two strains of *M. kansasii* had consistently low spectral scores (e.g., range, 1.7 to 1.9), which may reflect the genetic diversity of this species. The heterogeneity of *M. kansasii* has also been encountered during analysis by sequence analysis of the 16S rRNA gene (13). Again, supplementation of the database with additional strains of *M. kansasii* may resolve this issue.

Few previous studies have addressed the utilization of MALDI-TOF MS for accurate identification of mycobacteria. Hettick et al. (6) compared the utilization of whole-cell preparations with protein extracts for the identification of six species of *Mycobacterium*. They observed that the extraction reagents (trifluoroacetic acid and acetonitrile) but not the matrix solution inactivated mycobacteria. Since similar results were obtained with both whole cells and protein extracts, the authors concluded that the utilization of protein extracts is preferable to whole-cell preparations, as the mycobacteria are rendered inactive by the extraction reagents. We concur with the authors regarding the importance of using protein extracts for MALDI-TOF MS identification. Our protocol differs, however, in that methods to disrupt clumps and enhance protein extraction (i.e., the utilization of the micropestle and glass beads) are incorporated into the procedure to allow accurate identification of a wide variety of mycobacteria. Furthermore, the organisms are rendered inactive at an early step in our extraction protocol by heat killing to allow all subsequent processes to be conducted in the open laboratory.

Similar to our analysis, Pignone et al. (10) were able to identify correctly a large number of mycobacteria (36 of 37 strains tested). Unlike our study, though, whole cells were used for MALDI-TOF MS analysis. We reiterate our preference in utilizing protein extracts to minimize exposure of laboratory workers to viable mycobacteria.

In a later work, Hettick et al. (5) describe their attempts to discriminate among strains of mycobacteria using MALDI-TOF MS. They conclude that strain differences are likely due to differences in relative abundance of shared m/z values rather than the presence or absence of specific peaks. Although we did not attempt to discriminate among strains of the same species in our study, we do anticipate that MALDI-TOF MS can be utilized for strain differentiation of mycobacteria.

Overall, our work extends these studies by describing a specific protein extraction protocol that is useful for all types of mycobacteria, as it accounts for this genus's hardy cell envelope and tendency to form clumps. We also demonstrate that our extraction protocol can be utilized to create a mycobacterial database into which different species of mycobacteria can be incorporated indefinitely. Our results indicate that it is quite feasible to incorporate MALDI-TOF MS for routine identification of mycobacteria into the work flow of the clinical microbiology laboratory. With some minor exceptions noted above, identifications were accurate to the species level. Results were obtained rapidly: the turnaround time from sample preparation to results was approximately 90 min, a significant improvement when one considers the time necessary for identifying mycobacteria using phenotypic tests, gene probes, or sequencing. In short, we believe that this study justifies the use of MALDI-TOF MS for the routine identification of isolated colonies of *Mycobacterium* species. Studies of the use of this technology for identification of mycobacteria from liquid growth media are under way.

ACKNOWLEDGMENTS

We thank Steven M. Holland for his mentorship throughout the project and his review of the manuscript. We also thank Clifton E. Barry and Isdore C. Shamputa for providing clinical strains and Yvonne R. Shea and the staff of the mycobacteriology laboratory for assisting in specimen preparation.

This research was supported by the Laboratory of Clinical Infectious Diseases and the Intramural Research Program of the NIH Clinical Center, Department of Laboratory Medicine.

REFERENCES

1. **Adekambi, T.** 2009. *Mycobacterium mucogenicum* group infections: a review. Clin. Microbiol. Infect. **15:**911–918.
2. **Bizzini, A., C. Durussel, J. Bille, G. Greub, and G. Prod'hom.** 2010. Performance of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J. Clin. Microbiol. **48:**1549–1554.
3. **Cooksey, R. C., et al.** 2008. Multiphasic approach reveals genetic diversity of environmental and patient isolates of *Mycobacterium mucogenicum* and *Mycobacterium phocaicum* associated with an outbreak of bacteremias at a Texas hospital. Appl. Environ. Microbiol. **74:**2480–2487.
4. **Hall, L., K. A. Doerr, S. L. Wohlfiel, and G. D. Roberts.** 2003. Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J. Clin. Microbiol. **41:**1447–1453.
5. **Hettick, J. M., et al.** 2006. Discrimination of intact mycobacteria at the strain level: a combined MALDI-TOF MS and biostatistical analysis. Proteomics **6:**6416–6425.
6. **Hettick, J. M., et al.** 2004. Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. **76:**5769–5776.
7. **Macheras, E., et al.** 2009. Inaccuracy of single-target sequencing for discriminating species of the *Mycobacterium abscessus* group. J. Clin. Microbiol. **47:**2596–2600.
8. Reference deleted.
9. **Marvin, L. F., M. A. Roberts, and L. B. Fay.** 2003. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in clinical chemistry. Clin. Chim. Acta **337:**11–21.
10. **Pignone, M., K. M. Greth, J. Cooper, D. Emerson, and J. Tang.** 2006. Identification of mycobacteria by matrix-assisted laser desorption ionization-time-of-flight spectrometry. J. Clin. Microbiol. **48:**1520–1526.
11. **Ringuet, H., et al.** 1999. *hsp65* sequencing for identification of rapidly growing mycobacteria. J. Clin. Microbiol. **37:**852–857.
12. **Rogall, T., T. Flohr, and E. C. Bottger.** 1990. Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. J. Gen. Microbiol. **136:**1915–1920.
13. **Ross, B. C., K. Jackson, M. Yang, A. Sievers, and B. Dwyer.** 1992. Identification of a genetically distinct subspecies of *Mycobacterium kansasii*. J. Clin. Microbiol. **30:**2930–2933.
14. **Schorey, J. S., and L. Sweet.** 2008. The mycobacterial glycopeptidolipids: structure, function, and their role in pathogenesis. Glycobiology **18:**832–841.
15. **Schweickert, B., et al.** 2008. Occurrence and clinical relevance of *Mycobacterium chimaera* sp. nov., Germany. Emerg. Infect. Dis. **14:**1443–1446.
16. **Seng, P., et al.** 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption time-of-flight mass spectrometry. Clin. Infect. Dis. **49:**543–551.
17. **Stevenson, L. G., S. K. Drake, and P. R. Murray.** 2010. Rapid identification of bacteria in positive blood culture broths by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. **48:**4444–4447.
18. **Stevenson, L. G., S. K. Drake, Y. R. Shea, A. M. Zelazny, and P. R. Murray.** 2010. Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically important yeast species. J. Clin. Microbiol. **48:**3482–3486.
19. **Tortoli, E.** 2006. The new mycobacteria: an update. FEMS Immunol. Med. Microbiol. **48:**159–178.
20. **Tortoli, E., et al.** 2004. Proposal to elevate the genetic variant MAC-A, included in the *Mycobacterium avium* complex, to species rank as *Mycobacterium chimaera* sp. nov. Int. J. Syst. Evol. Microbiol. **54:**1277–1285.
21. **Van Veen, S. Q., E. C. J. Claas, and E. J. Kuijper.** 2010. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J. Clin. Microbiol. **48:**900–907.
22. **Zelazny, A. M., et al.** 2009. Cohort study of molecular identification and typing of *Mycobacterium abscessus*, *Mycobacterium massiliense*, and *Mycobacterium bolletii*. J. Clin. Microbiol. **47:**1985–1995.
23. **Zelazny, A. M., L. B. Calhoun, L. Li, Y. R. Shea, and S. H. Fischer.** 2005. Identification of *Mycobacterium* species by *secA1* sequences. J. Clin. Microbiol. **43:**1051–1058.