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Comparison of the Bruker Biotyper and Vitek MS Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Systems for Identification of Mycobacteria Using Simplified Protein Extraction Protocols

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has recently been described as a fast and inexpensive method for the identification of mycobacteria. Although mycobacteria require extraction prior to MALDI-TOF MS analysis, previously published protocols have been relatively complex, involving significant hands-on time and materials not often found in the clinical laboratory. In this study, we tested two simplified protein extraction protocols developed at the University of Washington (UW) and by bioMérieux (BMX) for use with two different mass spectrometry platforms (the Bruker MALDI Biotyper and the bioMérieux Vitek MS, respectively). Both extraction protocols included vortexing with silica beads in the presence of ethanol. The commercial Bruker database was also augmented with an in-house database composed of 123 clinical *Mycobacterium* strains. A total of 198 clinical strains, representing 18 *Mycobacterium* species, were correctly identified to the species level 94.9% of the time when extracted using the UW protocol and compared to the augmented database. The BMX protocol and Vitek MS system resulted in correct species-level identifications for 94.4% of these strains. In contrast, only 79.3% of the strains were identified to the species level by the nonaugmented Bruker database, although the use of a lower identification score threshold (≥1.7) increased the identification rate to 93.9%, with two misidentifications that were unlikely to be clinically relevant. The two simplified protein extraction protocols described in this study are easy to use for identifying commonly encountered *Mycobacterium* species.

Mycobacterium species are a significant cause of morbidity and mortality worldwide (1–3). There were an estimated 8.7 million new cases of *Mycobacterium tuberculosis* infection in 2011, which is second only to HIV/AIDS as an infectious cause of death worldwide (2). The incidence of infection with nontuberculous *Mycobacterium* spp. (NTM) is also on the rise, in part due to an ever-increasing population of immunocompromised individuals (4). In a 2007 official joint statement by the American Thoracic Society and Infectious Disease Society of America (ATS/IDSA) on the diagnosis, treatment, and prevention of NTM disease, it was specifically recommended that clinically significant NTM isolates be identified to the species level whenever possible (5). However, because there are >160 currently recognized species of NTM (see Euzéby's list of mycobacterial names with standing in nomenclature [http://www.bacterio.cict.fr/m/mycobacterium.html]), this recommendation may be challenging for many clinical microbiology laboratories to implement.

The identification of *Mycobacterium* spp. has traditionally relied upon phenotypic traits (e.g., growth rates and pigmentation) as well as biochemical methods (e.g., nitrate reduction and semiquantitative catalase activity) (6). Unfortunately, many of these methods are not only time-consuming but they have poor accuracy. As a result, these have been augmented by molecular-based methods in the United States, which include commercially available DNA probes (7–9) and DNA sequencing of a variety of target genes (e.g., 16S rRNA genes and *rpoB*) (10–12). While accurate, these methods are expensive to perform and may not necessarily be cost-effective in populations of low mycobacterial prevalence (13).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is increasingly used for the identification of routine bacterial and yeast isolates using intact or minimally extracted cells (14–16). Although less extensively published, the method has also been described as a rapid and cost-effective method for the identification of *Mycobacterium* spp. (17–21). However, to minimize the potential exposure of laboratory personnel to *Mycobacterium tuberculosis* complex and to maximize the quality of the spectra obtained, inactivation of the mycobacterial cells and extraction of mycobacterial proteins are necessary prior to the performance of MALDI-TOF MS (19, 20). Previously published procedures for mycobacterial protein extraction have either been technically complex, using materials not often found in a clinical microbiology laboratory (18, 19), or have involved multiple centrifugation and washing steps prior to organism inactivation (21, 22). Finally, the relative performances of the two commercially available MALDI-TOF MS platforms for the identification of *Mycobacterium* spp. have not been established.

Therefore, the goals of this study were 3-fold: (i) to develop a simplified extraction procedure for mycobacterial testing us-

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ing the Bruker MALDI Biotyper system (University of Washington [UW] procedure), (ii) to evaluate the performance of the bioMérieux (BMX) extraction procedure, and (iii) to evaluate the relative performances of the Bruker Biotyper and Vitek MS MALDI-TOF MS systems for the identification of *Mycobacterium* spp.

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MATERIALS AND METHODS

***Mycobacterium* clinical isolates.** A total of 199 *Mycobacterium* strains were tested in this study. All *Mycobacterium* strains were clinical isolates that had previously been identified at the University of Washington Medical Center Clinical Microbiology Laboratory by DNA sequencing of the 16S rRNA, *rpoB*, and/or *hsp65* genes (10–12). The isolates were stored in fetal calf serum at −80°C and subcultured on Middlebrook 7H11 agar (Remel, Lenexa, KS), and *Mycobacterium haemophilum* isolates were subcultured on chocolate agar (Remel). With the exception of isolates of *Mycobacterium marinum*, *M. haemophilum*, and *Mycobacterium xenopi*, all isolates were incubated at 35°C with 5% CO_2. Isolates of *M. marinum* and *M. haemophilum* were incubated at 30°C without CO_2, whereas isolates of *M. xenopi* were incubated at 42°C, also without CO_2. Rapidly growing strains were extracted for MALDI-TOF MS after 3 to 7 days of growth, and slowly growing strains were extracted after 14 to 21 days of growth.

UW *Mycobacterium* extraction procedure. A 1-μl loopful of mycobacterial biomass was collected in a 1.5-ml screw-top microcentrifuge tube (VWR International, Radnor, PA) containing 300 μl of deionized water and approximately 200 μl of 1-mm silica beads (BioSpec Products, Barlesville, OK). Following heat inactivation at 95°C for 30 min, 900 μl of absolute ethanol (Decon Labs, Inc., King of Prussia, PA) was added to each tube. The tubes were then vortexed for 10 min in a horizontal position at maximum speed using a Vortex-Genie 2 with a 24-tube adaptor (MoBio Laboratories, Inc., Carlsbad, CA). The liquid contents were then transferred to a new 1.5-ml snap-top microcentrifuge tube and centrifuged at 13,000 × *g* for 2 min. The supernatant was removed using a narrow-gauge pipette and the pellet allowed to air dry for 10 min. Following resuspension of the pellet by repeated aspiration and expulsion in 10 μl of 70% formic acid (Sigma-Aldrich, St. Louis, MO), 10 μl of 98% acetonitrile (Sigma-Aldrich) was then added to each tube and the tubes were vortexed for 20 s. The extracts were then centrifuged at 10,000 × *g* for 1 min, and the supernatant was used for analysis by MALDI-TOF MS.

BMX extraction procedure. Extractions were performed according to the manufacturer's recommended procedure. Briefly, a 1-μl loopful of mycobacterial biomass was collected in a 1.5-ml screw-top microcentrifuge tube containing 500 μl of 70% ethanol (Decon Labs, Inc.) and 200 μl of 0.5-mm silica beads (MoBio Laboratories, Inc.). All tubes were vortexed for 15 min in a horizontal position using a Vortex-Genie 2 with a 24-tube adaptor at maximum speed (MoBio Laboratories, Inc.). The tubes were then allowed to sit at room temperature for 10 min to complete inactivation of the mycobacteria. The liquid contents (excluding beads) were then transferred to a new 1.5-ml snap-top microcentrifuge tube and centrifuged at 10,000 × *g* for 2 min. The supernatant was decanted and the excess removed with a narrow-gauge pipette. The pellet was resuspended in 10 μl of 70% formic acid by repeated aspiration and expulsion. The extracts were allowed to sit for 2 to 5 min in formic acid (Sigma-Aldrich), after which time 10 μl of 98% acetonitrile (Sigma-Aldrich) was added to each tube. Following mixing by vortexing for 20 s and subsequent centrifugation at 10,000 × *g* for 2 min, the supernatant was used for analysis by MALDI-TOF MS.

Confirmation of organism inactivation. To assess the efficacy of inactivation, the samples were inoculated on Middlebrook 7H11 agar (Remel) and into VersaTREK Myco bottles supplemented with mycobacterial growth supplement (Trek Diagnostic Systems, Cleveland, OH). For the UW extraction procedure, the samples were divided equally (approximately 100 μl each) for inoculation immediately after the inactivation step. For the BMX extraction procedure, the liquid contents were then transferred to a new 1.5-ml snap-top microcentrifuge tube immediately following inactivation and were centrifuged at 10,000 × *g* for 2 min. The supernatant was decanted, with the excess removed with a narrow-gauge pipette. The pellet was resuspended in 300 μl of sterile water and divided equally for inoculation. The agar plates were examined daily for growth during the first week and then weekly thereafter for a total of 6 weeks under the appropriate incubation conditions. The VersaTREK bottles were incubated on the VersaTREK instrument for 6 weeks.

MALDI-TOF MS analysis: Bruker MALDI Biotyper. One microliter of supernatant from each sample was deposited onto a spot on a polished steel MSP 96-target plate (Bruker Daltonics, Inc., Billerica, MA). After samples had dried, 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 2.5% trifluoroacetic acid (Bruker Daltonics) was overlaid onto each spot. Spectra were acquired in a linear positive ion mode at a laser frequency of 60 Hz across a mass/charge ratio (*m/z*) of 2,000 to 20,000 Da using the MicroFlex LT mass spectrometer (Bruker Daltonics). For each spectrum, 240 laser shots in 40-shot steps from different areas of the sample spot were accumulated and analyzed (in automatic mode using default settings). The Bruker bacterial test standard (Bruker Daltonics), an extract of *Escherichia coli* supplemented with RNase A and myoglobin, was used for daily calibration according to the manufacturer's recommendations. If no spectrum was obtained, 2 μl of sample was deposited to a new spot with 1 μl of matrix solution. Any samples that subsequently did not generate a spectrum were reextracted. The spectra were analyzed using the MALDI Biotyper software package (version 3.1) with the following reference databases: Bruker's *Mycobacterium* 2.0 database, containing 313 main spectrum profiles (MSPs) from 131 species, and a combined database consisting of the Bruker *Mycobacterium* 2.0 database augmented with MSPs generated from 123 of the isolates extracted with the UW protocol (the combined UW/Bruker database; see "Creation of the UW *Mycobacterium* database" below). Of note, self-MSPs were excluded whenever the combined UW/Bruker database was used to analyze strains used to generate the database. Using the Bruker Biotyper software 3.1 (Bruker Daltonics), isolates with scores of ≥2.0 were considered to be definitively identified to the species level, whereas isolates with scores of ≥1.7 were considered to be identified to the genus level. Scores of <1.7 were considered invalid for identification purposes.

Creation of the UW *Mycobacterium* database (Bruker). A reference database consisting of 123 clinical strains was created using manufacturer-recommended criteria (Table 1). These criteria require that each spectrum contain at least 25 peaks with a resolution of >400, of which 20 of the peaks must have a resolution of >500. Each MSP was created using at least 20 spectra from at least 5 different MALDI spots. The spectra were visually inspected as normalized spectra and in "gel mode" to evaluate reproducibility prior to inclusion in the MSP. Each individual spectrum was then run against the MSP for that isolate (self-MSP) to ensure that the identification scores were >2.4 for all spectra incorporated into the MSP.

MALDI-TOF MS analysis: bioMérieux Vitek MS. One microliter of supernatant from each sample was deposited onto a single well of a disposable barcode-labeled target slide (Vitek MS-DS; bioMérieux SA, Marcy l'Etoile, France) and allowed to dry. The samples were then overlaid with 1.0 μl of a saturated solution of CHCA matrix in 50% acetonitrile and 2.5% trifluoroacetic acid (Vitek MS-CHCA; bioMérieux SA). The spectra were acquired in linear positive ion mode at a laser frequency of 50 Hz across *m/z* 2,000 to 20,000 Da using the Vitek MS system (bioMérieux SA). For each target slide, the *E. coli* reference strain ATCC 8739 was used for instrument calibration according to the manufacturer's specifications. For each test, up to 100 mass profiles were produced from 500 laser shots at different areas of the target that were then summed into a single raw mass spectrum. Each spectrum was then processed by baseline correction, denoising, and peak detection to identify well-defined peaks.

TABLE 1 *Mycobacterium* species tested in this study

Mycobacterium sp.	No. of strains	
	UW database	Bruker database[a]
M. abscessus	12	10
M. avium	9	10
M. chelonae	10	10
M. fortuitum complex	7	21
M. gordonae	8	10
M. haemophilum	4	1
M. immunogenum	4	3
M. intracellulare	5	9
M. kansasii	10	14
M. lentiflavum	5	3
M. marinum	8	8
M. mucogenicum/*M. phocaicum* group	10	3
M. scrofulaceum	1	6
M. simiae	5	3
M. szulgai	5	5
M. terrae complex	3	12
M. tuberculosis complex	13	13
M. xenopi	4	5
All species	123	146

[a] The Bruker *Mycobacterium* Library 2.0 database contains 313 MSPs from 131 species in total.

After spectrum acquisition, the data were transferred from the Vitek MS acquisition station to the SARAMIS analysis server, where the data were reported as the number of peaks. In the event that <50 peaks were present for a given spectrum, the sample was respotted using 2 μl of sample and 1 μl of matrix and rerun. Samples that failed to generate >50 peaks after this process were reextracted.

Deidentified spectra generated on the Vitek MS were downloaded from the analysis server and compared to the SARAMIS 4.12 research use only (RUO) database in France. All spectra generated on the Vitek MS were compared to both SuperSpectra and ReferenceSpectra in the SARAMIS 4.12 database. ReferenceSpectra are the spectral fingerprints generated from a single isolate. In contrast, SuperSpectra are computed from multiple (15+) reference spectra by extracting conserved mass signals and weighting those signals according to their specificities for different taxonomic levels of identification (i.e., family, genus, species, and subspecies). Identifications with confidence scores of >90% obtained by species-specific SuperSpectra were considered to be definitively identified to the species level. In the event that a definite identification was not obtained using SuperSpectra, organisms were considered to be definitively identified using ReferenceSpectra if the following cutoff values applied: sample spectra had to match the ReferenceSpectra at >40% of the peaks and 35 peaks in absolute numbers, with a >10% peak difference between the most closely matching species and second most closely matching species.

Evaluation of spectral quality. The following previously published parameters were used to assess spectral quality (23): base peak signal-to-noise (S/N) ratio, base peak resolution, number of peaks, and mass range of peaks. For spectra generated on the MicroFlex LT, peaks were detected using the Centroid algorithm in FlexAnalysis 3.3 (Bruker Daltonics) with a S/N threshold of 3, minimum relative intensity threshold of 5%, 500 as the max number of peaks, *m/z* 4 peak width, and 80% height. Baseline subtraction was performed using the TopHat algorithm. For spectra generated on the Vitek MS, peaks were detected using Launchpad software version 2.9.3 according to the manufacturer's predetermined algorithm.

To evaluate the effect of culture time on spectral quality, one strain each of five species, *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium kansasii*, and *Mycobacterium gordonae*, was randomly selected for evaluation. For rapidly growing species, extractions of the subculture were performed at 72 h, 96 h, and 7 and 10 days. For the slowly growing species, extractions of the subculture were performed at 9, 14, 21, and 28 days. A minimum of 10 spectra were generated from each extract. Strains extracted using the UW protocol were run on the MicroFlex LT and compared to the combined UW/Bruker database using the Biotyper 3.1 software (Bruker Daltonics). Strains extracted using the BMX protocol were run on the Vitek MS and identified using the SARAMIS 4.12 database.

RESULTS

Evaluation of simplified extraction procedures. The quality of spectra generated by the UW and Vitek MS extraction procedures was evaluated for 20 strains according to previously published criteria (23). These strains consisted of two strains each of *M. abscessus*, *Mycobacterium avium*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *Mycobacterium intracellulare*, *M. kansasii*, *M. marinum*, *Mycobacterium szulgai*, and *M. tuberculosis*. As shown in Fig. 1, both extraction procedures produced high-quality spectra on the two MALDI-TOF MS platforms, and we did not observe any statistically significant differences in the numbers of peaks, peak ranges, base peak S/N ratios, or base peak resolution between the two procedures ($P \geq 0.05$ for all parameters, paired *t* test). However, extracts tested on the Vitek MS had higher maximum intensities than those tested on the Bruker Biotyper ($P < 0.005$, paired *t* test). To ensure that complete organism inactivation occurred prior to manipulation in the open laboratory, we tested strains from five different NTM species (*M. abscessus*, *M. fortuitum*, *M. kansasii*, *M. intracellulare*, and *M. marinum*), as well as five strains of *M. tuberculosis* for viability following the inactivation step for both the UW and BMX extraction procedures. Importantly, no growth was observed on either solid medium or in liquid broth for any of the organisms tested after 6 weeks of incubation.

Creation and validation of the UW *Mycobacterium* database. The Bruker *Mycobacterium* 2.0 database contains spectra from 131 *Mycobacterium* species, although only a limited number of reference spectra are included for many of these organisms. We therefore chose to create a custom database that had a greater number of reference spectra per species, focusing on those species specifically referenced in the previously published ATS/IDSA guidelines as being clinically relevant and/or frequently isolated (5). To this end, we created a database consisting of 123 clinical strains spanning 18 *Mycobacterium* species that had been definitively identified by DNA sequencing (Table 1). A total of 97.5% of the strains used to construct the database were correctly identified to the species level by the UW database when each strain's self-MSP was excluded from the analysis, with 99.1% correctly identified to the genus level (data not shown). We then used an additional 76 clinical strains representing the species covered in the UW database to validate the database. Unfortunately, we were unable to test additional strains of *M. haemophilum*, *Mycobacterium immunogenum*, and *Mycobacterium scrofulaceum*, as we lacked additional isolates in our strain collection. Nevertheless, 90.7% of the validation strains were correctly identified to the species level when tested against the UW database, and 96.1% of strains were identified to the genus level (data not shown).

Comparison of *Mycobacterium* sp. identification using the Bruker *Mycobacterium* 2.0, combined UW/Bruker, and SARAMIS version 4.12 databases. We directly compared the performances of the UW and BMX extraction procedures on the two MALDI-TOF MS platforms for 198 clinical *Mycobacterium* strains, 122 of which

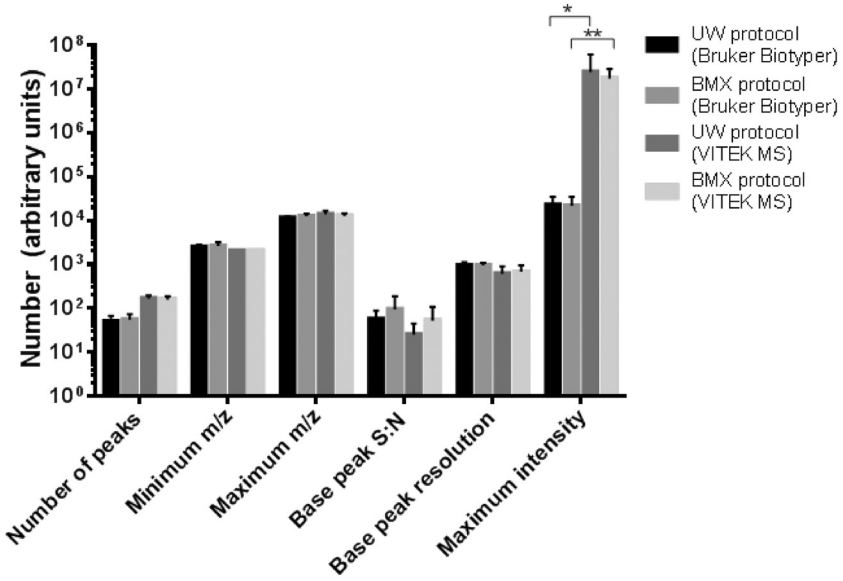

FIG 1 Comparison of spectral quality generated using the UW and BMX extraction protocols. Two isolates each of 10 different *Mycobacterium* spp. (*M. abscessus*, *M. avium*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. szulgai*, and *M. tuberculosis*) were selected for evaluation. The number of peaks, minimum and maximum *m/z*, base peak S/N ratio, base peak resolution, and maximum intensity were evaluated on each instrument. The error bars represent the 95% confidence intervals around the mean. *, $P = 0.005$; **, $P < 0.001$ (paired *t* test).

were used for construction of the UW database. Both procedures generated spectra in the majority of cases. Adequate spectra were generated on both instruments for 179 of the 198 strains extracted with the UW procedure when one microliter of extract was used, and an additional two strains produced spectra when two microliters of extract was applied (91.4% overall success rate). With the BMX extraction procedure, adequate spectra were generated on both instruments for 150 of the 198 strains using one microliter extract, and an additional 37 strains produced adequate spectra when two microliters of extract was applied (94.4% overall success rate). All strains that initially failed to produce adequate spectra did so upon reextraction for both extraction methods tested.

The UW and BMX extraction procedures correctly identified 94.9% and 94.4% of strains to the species level when analyzed with the combined UW/Bruker and SARAMIS databases, respectively (Table 2). Importantly, both of these databases outperformed the Bruker *Mycobacterium* Library 2.0 database, which correctly identified only 79.3% of strains to the species level using the UW extraction protocol and 59.6% of strains using the BMX protocol. No misidentifications occurred with the combined UW/Bruker or Bruker databases with either of the extraction procedures. In the case of the BMX extraction procedure and Vitek MS system, three strains were misidentified when no match was obtained with SuperSpectra but ReferenceSpectra were used instead for identification (Table 3): one *M. immunogenum* strain was misidentified as *M. chelonae*, one *Mycobacterium europaeum* strain was misidentified as *M. scrofulaceum*, and one *Mycobacterium terrae* strain was misidentified as *Mycobacterium arupense*. None of these three strains were identified when extracted with the UW procedure and tested with the combined UW/Bruker or Bruker databases. There were 3 additional strains that could not be identified by any of the databases tested (Table 3). Resequencing of the 6 misidentified and/or unidentified strains resulted in the reclassification of three of these strains as *Mycobacterium* spp. that were closely related, but not identical to, the original species identification. The *M. terrae* complex organism was identified as *Mycobacterium heraklionense*, which is a member of the *M. terrae* complex (24) and is not represented in any of the tested databases.

Interestingly, 87.4% of strains extracted using the UW procedure but that were subjected to MALDI-TOF MS using the Vitek MS system were correctly identified to the species level. Similarly, identification by ReferenceSpectra in cases where no match was given with SuperSpectra resulted in two misidentifications: *M. immunogenum* was misidentified as *M. chelonae*, and *Mycobacterium lentiflavum* was misidentified as *M. kansasii*. When the BMX extraction procedure was tested on the Bruker instrument, the combined UW/Bruker database correctly identified 81.8% of strains to the species level.

Impact of culture time on identification. To evaluate the effect of culture time on identification, three rapidly growing *Mycobacterium* spp. (*M. abscessus*, *M. chelonae*, and *M. fortuitum*) were extracted at 72 h, 96 h, and 7 and 10 days after subculture, and two slowly growing *Mycobacterium* spp. (*M. kansasii* and *M. gordonae*) were extracted at 9, 14, 21, and 28 days after subculture. All strains tested were successfully identified for the first three time points when extracted using the UW extraction procedure and tested against the combined UW/Bruker database (Fig. 2A). However, both of the slowly growing strains showed markedly reduced rates of identification at the latest time point. With the exception of *M. fortuitum*, we observed a statistically significant difference between the identification scores obtained at the earliest time points and those obtained at the latest time point for the remaining strains tested ($P < 0.001$, paired *t* test; data not shown).

In the case of the BMX extraction procedure and Vitek MS system, we observed improved levels of identification at earlier time points than at later time points for two of the rapidly growing strains tested (Fig. 2B). In contrast to what we observed with the UW extraction and combined UW/Bruker database, none of the

TABLE 2 Comparison of databases for 198 clinical *Mycobacterium* strains extracted with both the UW and BMX extraction protocols[a]

Species (no. of strains tested)	UW protocol (no. [%])			BMX protocol (no. [%])		
	Combined: correctly identified with score of ≥2.0, excluding self-MSPs	Bruker: correctly identified with score of ≥2.0	SARAMIS: correctly identified by SuperSpectra or ReferenceSpectra	Combined: correctly identified with score of ≥2.0, excluding self-MSPs	Bruker: correctly identified with score of ≥2.0	SARAMIS: correctly identified by SuperSpectra or ReferenceSpectra
M. abscessus (26)	26 (100)	24 (92)	25 (96)	23 (88)	11 (42)	26 (100)
M. avium (14)	14 (100)	13 (93)	13 (93)	13 (93)	11 (79)	14 (100)
M. chelonae (14)	14 (100)	7 (50)	11 (79)	0 (0)	0 (0)	14 (100)
M. fortuitum complex (12)	12 (100)	10 (83)	10 (83)	12 (100)	12 (100)	12 (100)
M. gordonae (12)	9 (75)	9 (75)	9 (75)	7 (58)	5 (42)	12 (100)
M. haemophilum (4)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)
M. immunogenum (5)	4 (80)	2 (40)	4 (80)	4 (80)	0 (0)	4 (80)
M. intracellulare (12)	11 (92)	11 (92)	10 (83)	10 (83)	9 (75)	11 (92)
M. kansasii (14)	14 (100)	7 (50)	12 (86)	12 (86)	4 (29)	10 (71)
M. lentiflavum (9)	9 (100)	8 (89)	7 (78)	9 (100)	8 (89)	8 (89)
M. marinum (15)	15 (100)	14 (93)	15 (100)	15 (100)	15 (100)	15 (100)
M. mucogenicum (14)	14 (100)	9 (64)	14 (100)	13 (93)	6 (43)	14 (100)
M. scrofulaceum (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
M. simiae (9)	8 (89)	5 (56)	8 (89)	8 (89)	1 (11)	8 (89)
M. szulgai (9)	8 (89)	8 (89)	5 (56)	7 (78)	7 (78)	8 (89)
M. terrae complex (4)	2 (50)	2 (50)	2 (50)	2 (50)	2 (50)	2 (50)
M. tuberculosis (16)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)
M. xenopi (8)	8 (100)	8 (100)	8 (100)	7 (88)	7 (88)	8 (100)
All species (198)	188 (94.9)	157 (79.3)	173 (87.4)	162 (81.8)	118 (59.6)	187 (94.4)

[a] Combined, combined UW/Bruker database; Bruker, Bruker *Mycobacterium* 2.0 database; SARAMIS, SARAMIS 4.12 database.

spectra generated at 10 days for *M. fortuitum* were identified. Although the *M. abscessus* strain was successfully identified at all four time points, the average identification confidence scores decreased from 99.9% at 72 h and 96 h to 98.6% at 10 days (data not shown). The *M. kansasii* strain was successfully identified at all four time points, although the number of peaks present reached a maximum at 14 days (data not shown). In contrast, the rates of identification for *M. gordonae* varied at each time point tested (Fig. 2B).

Impact of lowering score thresholds on identification using Biotyper software. Previous studies have reported improved species-level identification rates without an increase in false identifications for a variety of different organisms by decreasing the identification score threshold to <2.0 for the Bruker Biotyper system (15, 25–27). Decreasing the species-level score threshold to 1.9 resulted in 95.9% of strains being correctly identified to the species level using the UW extraction protocol with the combined UW/Bruker database, but it resulted in a single strain originally identified as *M. intracellulare* being reidentified as the closely related

TABLE 3 Unidentified and misidentified mycobacterial strains

Original ID[a]	Identification results using:		Revised/updated ID
	UW/Bruker database	SARAMIS database	
M. immunogenum	No ID	*M. chelonae* (ReferenceSpectra)	*M. franklinii*
M. terrae	No ID	*M. arupense* (ReferenceSpectra)	*M. terrae*
M. simiae	No ID	*M. scrofulaceum* (ReferenceSpectra)	*M. europaeum*
M. intracellulare	No ID	No ID	*M. timonense*
M. szulgai	No ID	No ID	*M. szulgai*
M. terrae complex	No ID	No ID	*M. heraklionense*

[a] ID, identification.

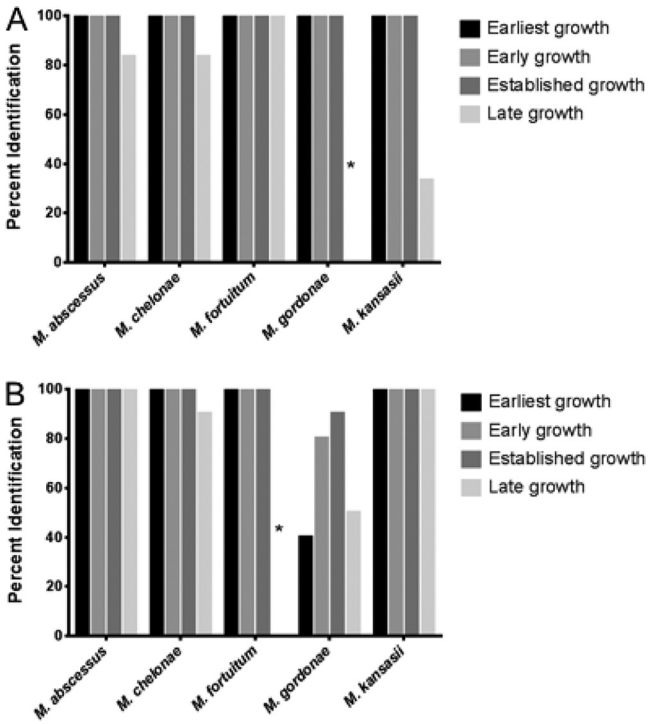

FIG 2 Impact of organism age on identification. At each time point, at least 10 individual spectra were compared against the combined UW/Bruker database (UW extraction, Bruker Biotyper) (A) or the SARAMIS version 4.12 database (BMX extraction, Vitek MS) (B). Earliest growth was at 72 h/9 days, early growth was at 96 h/14 days, established growth was at 7 days/21 days, and late growth was at 10 days/28 days for rapidly/slowly growing strains, respectively. *, no spectra were identified at this time point.

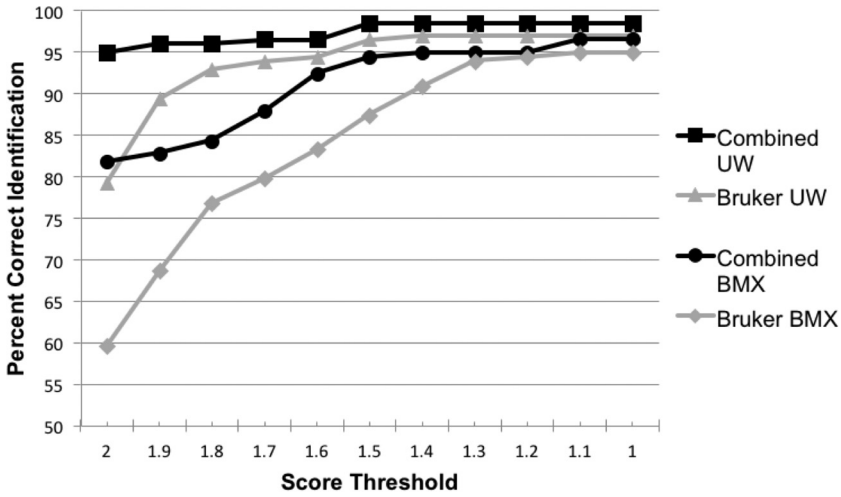

FIG 3 Impact of lowering score thresholds on identification rates. The rates of correct identification at score thresholds between 1.0 and 2.0 were calculated using increments of 0.1 for spectra generated on the MicroFlex LT and evaluated using Biotyper 3.1 software. Spectra generated using the UW protocol were compared to the combined UW/Bruker and the Bruker 2.0 databases (combined UW and Bruker UW, respectively), as were spectra generated using the BMX protocol (combined BMX and Bruker BMX, respectively).

species *Mycobacterium marseillense* (28). Upon resequencing of the strain's 16S rRNA gene, it was reclassified as *Mycobacterium timonense*, which is a member of the *M. avium* complex that is also very closely related to *M. marseillense* (28). The Bruker *Mycobacterium* 2.0 database correctly identified 89.4% of strains when a score threshold of 1.9 was applied, with the same *M. marseillense* strain being misidentified. Interestingly, decreasing the score threshold to 1.8 did not change the identification rate using the combined UW/Bruker database. At this cutoff, the identification rate increased to 92.9% for the Bruker 2.0 database, albeit resulting in a second misidentified strain (*M. immunogenum* misidentified as *M. abscessus*). At a cutoff of ≥1.7, 96.5% of strains were identified using the combined UW/Bruker database, and 93.9% of strains were identified using the Bruker 2.0 database. Further decreases in the threshold raised the identification rate without further loss of accuracy until scores of ≤1.3 were considered (Fig. 3).

In contrast, decreasing the score threshold for identification using the BMX extraction procedure with the combined UW/Bruker or Bruker 2.0 databases did not substantially increase the rate of species-level identifications with the Bruker Biotyper system. At a score threshold of ≥1.9, 82.8% of isolates were identified to the species level using the combined UW/Bruker database, and only 68.7% of isolates were identified using the Bruker 2.0 database. Misidentification of the same *M. timonense* isolate mentioned above occurred at this threshold, though it was called *Mycobacterium mantenii* (which is more distantly related to *M. intracellulare* than *M. marseillense* [29]). At a cutoff of ≥1.7, 87.9% of the strains generated using the BMX protocol were identified using the combined database, and 79.8% of these strains were identified using the Bruker 2.0 database. A further decrease in the cutoff threshold to 1.0 raised the identification rate to a maximum of 96.5% using the combined database and 94.9% using the Bruker 2.0 database but had a misidentification rate of 3% for both databases (Fig. 3 and data not shown). Decreasing the score threshold for SuperSpectra identification to 80% using the SARAMIS version 4.12 database did not affect the overall identification rate when ReferenceSpectra were also accepted for identification (data not shown).

DISCUSSION

In this study, we successfully developed a simplified extraction procedure for the analysis of mycobacteria using the Bruker Biotyper MALDI-TOF MS system. This procedure involves substantially less manipulation and hands-on time than either the manufacturer-recommended procedure (21, 22) or other previously published procedures (18, 19). Furthermore, we showed that the simplified protein extraction procedures developed by bioMérieux and UW are highly effective at identifying clinically relevant *Mycobacterium* spp.

In contrast to the procedure recommended by Bruker (22), both the UW and BMX extraction procedures include an organism inactivation step that occurs prior to any centrifugation of the samples. These simplified procedures therefore reduce the risk for the potential exposure of laboratory personnel to aerosol droplets and thus reduce the risk for laboratory-acquired infections with *M. tuberculosis* (30). The BMX procedure inactivates mycobacteria by lysing the cells using silica beads, followed by ethanol inactivation, while the UW procedure inactivates mycobacteria using heat. Even though heat denaturation and ethanol have been previously reported to decrease spectral quality (18, 31), both extraction procedures produced high-quality spectra with an average of >50 peaks per spectrum. This is more than four times greater than what was reported with the extraction protocol described by El Khéchine et al. (18). While the maximum intensity reported by the Vitek MS was observed to be significantly higher than that of the Bruker Biotyper, it is important to note that this parameter is reported in arbitrary units and the two instruments have different operating parameters.

The overall performances of the databases were similar to that reported by Balada-Llasat and colleagues in a recently published study (21) but substantially better than that reported by Buchan et al. in another recent study (32). In the former study, 93.8% of the *Mycobacterium* strains tested were correctly identified to the species level using the extraction procedure recommended by Bruker (including a preinactivation turbidity adjustment step) tested against the *Mycobacterium* 1.0 database. The latter study tested

strains against the same database using a similar procedure except that 3-mm beads were used in the extraction. Only 50% of isolates were identified to the species level using a score threshold of ≥2.0 for strains grown on solid medium, with 80.2% identification for strains grown in broth. Other previously published studies that used more technically involved bead-based extraction methods than those used in this study have demonstrated identification rates ranging from 86.5% to 100% when analyzed with custom-generated databases (18, 19). Importantly, our study shows that an equivalent efficacy of identification is possible using simplified extraction procedures that involve significantly less manipulation and hands-on time than other published protocols.

The combined UW/Bruker and SARAMIS databases reliably differentiated *M. tuberculosis* from NTM species with their respective extraction procedures. As has been previously reported (18, 19, 21), *M. abscessus* and *M. chelonae* were readily distinguished from one another, potentially enabling earlier initiation of appropriate antimicrobial therapy compared with traditional identification methodologies for these closely related species that differ in their antimicrobial susceptibilities (33). In contrast, it was previously reported that MALDI-TOF MS was unable to distinguish *M. abscessus* subsp. *abscessus* from *M. abscessus* subsp. *bolletii* (*M. massiliense*) (19). This may represent a clinically important shortcoming, as these organisms have been reported to have differing antibiotic susceptibility patterns (34). However, a recent study demonstrated the ability of MALDI-TOF MS to separate these subspecies using cluster analysis (35). It is unknown whether any of the databases tested in this study can reliably separate *M. abscessus* subsp. *abscessus* from *M. abscessus* subsp. *bolletii*, as our strains had been originally identified to the species level using *rpoB* sequencing and not by the more recently recommended multilocus sequencing approach (36). However, dendrograms of the MSPs created from our strains did separate into two clades (data not shown).

The Bruker 2.0 database could not reliably differentiate *Mycobacterium mucogenicum* from *Mycobacterium phocaicum* (reporting instead *M. mucogenicum/M. phocaicum* group), a shortfall that was previously noted by Saleeb et al. in 2011 (19). As neither the SARAMIS nor the UW database contains isolates of *M. phocaicum*, it is unknown whether these databases can differentiate these species. However, because the two organisms have similar antibiotic susceptibilities (37), distinguishing them by mass spectrometry may not be clinically relevant. Of the six strains that were unidentified or misidentified by the UW and BMX extraction procedures analyzed with the combined UW/Bruker and SARAMIS databases, respectively, four were reclassified after resequencing. In all four cases, the new identity was a species closely related to that of the original identification. The failure of any of the databases we tested to identify the three unidentified strains suggests that MALDI-TOF MS may be highly sensitive to species-specific variations in spectra for *Mycobacterium* spp.

Our data highlight the importance of consistency between the extraction method used for database generation and that used for testing. It is likely that this accounts for the relatively poor performance of the Bruker 2.0 database alone using the manufacturer-recommended identification score thresholds with our simplified extraction procedure. It is important to note, however, that the impact of inconsistency between the extraction method used for testing and that used for database generation may be ameliorated by using score thresholds lower than those recommended by the manufacturer. Specifically, we observed a 93.5% species-level identification rate using a ≥1.7 threshold, albeit with two misidentifications. Although neither misidentification was likely to be clinically relevant (*M. timonense* was misidentified as *M. marseillense* and *M. immunogenum* was misidentified as *M. abscessus*), the effects of employing a lower threshold on the identification of *Mycobacterium* species beyond those tested in this study are unknown.

Our data suggest that the age of the colonies used for testing should closely match the age of those colonies used for database creation. We observed important differences between the rates of identification for *M. fortuitum* identified with the combined UW/Bruker compared with the SARAMIS database. Although this strain was successfully identified across all four time points tested with the former database, none of the *M. fortuitum* spectra generated at 10 days were identified using the SARAMIS database. These data suggest that the SARAMIS database may lack representation of this species at the late stages of growth compared with the other rapidly growing mycobacterial species tested. Interestingly, when the UW database alone was used to identify this strain, we observed an 85% identification rate at the 10-day time point (data not shown). This may reflect the presence of an MSP or multiple MSPs in the Bruker 2.0 database generated at a late stage of growth that compensate for the change in spectra that occurs over time. For slowly growing strains, we observed a maximal number of peaks at 14 to 21 days (data not shown), perhaps corresponding to the higher likelihood of identification of the *M. gordonae* strain at those time points. As all of the databases tested generally performed better at earlier stages of growth, we propose that identification by MALDI-TOF MS be attempted as soon as colonies of a single morphotype are visible to ensure the highest likelihood of finding a match in the existing databases.

Our study was associated with a number of limitations. First, we limited our analysis to the *Mycobacterium* species that are most commonly encountered in clinical microbiology laboratories. Thus, the performances of these simplified protocols for less commonly encountered species are unknown. In addition, we did not examine the performances of these protocols for the identification of *Mycobacterium* species directly from liquid culture medium. However, previous studies have demonstrated the ability of MALDI-TOF MS to identify mycobacteria directly from positive MGIT broth cultures (21, 32). Similarly, we did not include organisms grown on Lowenstein-Jensen agar and thus cannot draw any conclusions regarding the performance of the databases tested in this study for organisms grown on that medium type. Nevertheless, our study is the first study to examine the performance of the Vitek MS system for mycobacterial identification and is the first study to utilize the Bruker *Mycobacterium* Library 2.0. It is also the first study to directly compare the performances of the Bruker Biotyper and Vitek MS systems for mycobacterial identification. In addition, the custom database that we developed is composed exclusively of clinical strains, a feature that has been previously shown to improve identification rates compared to databases composed of reference strains alone in the case of *M. marinum* (19).

In conclusion, this study definitively shows that the simplified protein extraction protocols developed by UW and bioMérieux can be used to identify *Mycobacterium* spp. on the Biotyper or Vitek MS system for strains grown on solid medium (7H11). Both protocols offer substantial advantages over previously published

protocols with regard to safety and ease of use. The use of simplified extraction protocols is likely to increase the use of MALDI-TOF MS in clinical laboratories for the identification of *Mycobacterium* species.

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