

1-1-2018

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Recommended Citation

AKYAR, IŞIN; ÇAVUŞOĞLU, CENGİZ; AYAS, MELTEM; SÜRÜCÜOĞLU, SÜHEYLA; İLKİ, ZEYNEP ARZU; KAYA, DENİZ ECE; and BEŞLİ, YEŞİM (2018) "Evaluation of the performance of MALDI-TOF MS and DNA sequence analysis in the identification of mycobacteria species," *Turkish Journal of Medical Sciences*: Vol. 48: No. 6, Article 37. <https://doi.org/10.3906/sag-1801-198>
Available at: <https://journals.tubitak.gov.tr/medical/vol48/iss6/37>

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Evaluation of the performance of MALDI-TOF MS and DNA sequence analysis in the identification of mycobacteria species

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Received: 24.01.2018 • Accepted/Published Online: 08.09.2018 • Final Version: 12.12.2018

Background/aim: Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is an alternative way of identifying mycobacteria via the analysis of biomolecules. It is being increasingly used in routine microbiology practice since it permits early, rapid, and cost-effective identification of pathogens of clinical importance. In this study, we aimed to evaluate the efficacy of phenotypic identification of mycobacteria by the MALDI-TOF MS MBT Mycobacteria Library (ML) 4.0 (Bruker, Daltonics) compared to standard sequence analysis.

Materials and methods: A total of 155 *Mycobacterium* clinical and external quality control isolates, comprising nontuberculous mycobacteria (NTM) (n = 95) and the *Mycobacterium tuberculosis* complex (MTC) (n = 60), were included in the study.

Results: Identification by MBT ML4.0 was correctly performed in 100% of MTC and in 91% of NTM isolates. All of the MTC isolates were correctly differentiated from NTM isolates.

Conclusion: Based on our results, MBT ML4.0 may be used reliably to identify both NTM and MTC.

Key words: MALDI-TOF MS, identification, *Mycobacterium*, *Mycobacterium tuberculosis* complex, nontuberculous mycobacteria

1. Introduction

Mycobacterium species constitute an important group of microorganisms that thrive in different natural environments. The taxonomy of *Mycobacterium* consists of more than 177 species, which have been evolving continuously in the recent past, and some species in the genus are human pathogens. The *Mycobacterium tuberculosis* complex (MTC) is responsible for tuberculosis infection, which is associated with high morbidity and mortality rates (1). The MTC includes species such as *Mycobacterium tuberculosis*, *Mycobacterium africanum*, and *Mycobacterium bovis*. Other less known species within the MTC are *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, *Mycobacterium mungi*, *Mycobacterium suricattae*, *Mycobacterium orygis*, and *Mycobacterium canettii* (2). By contrast, nontuberculous mycobacteria (NTM) are a group of *Mycobacterium* species commonly found in the environment, although some

NTM species are opportunistic pathogens that can cause critical infectious diseases (3). The mycobacterial species associated with NTM disease are *Mycobacterium avium-intracellulare*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium kansasii*, *Mycobacterium xenopi*, *Mycobacterium marinum*, *Mycobacterium scrofulaceum*, and *Mycobacterium szulgai* (1,3–6). Discriminating between MTC and NTM species is crucial for infection control and guidance of antimicrobial therapy and species-level identification of clinical NTMs is recommended by the American Thoracic Society (ATS) in order to anticipate the clinical features, permit epidemiological analysis, and guide both infection control strategies and therapeutic options (7,8).

Until recently, *Mycobacterium* species have been identified by traditional methods based on biochemical profiling, morphological characteristics, growth rates, and other phenotypic techniques (5,6). However,

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identification based on these phenotypic traits is time-consuming, meaning that the final diagnosis may be delayed until after therapy has started, and may result in misidentification (6,9). Molecular methods involving PCR-based hybridization and sequencing are routinely used in advanced laboratories and have become the new gold standard for mycobacterial identification. Although these techniques offer a fast and specific way to identify major *Mycobacterium* species, they remain expensive and require high-level technical expertise (1). On the other hand, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is accepted as a simple, powerful, rapid, and cost-effective tool for the routine identification of microorganisms in clinical laboratories. Furthermore, multiple studies have corroborated the fact that MALDI-TOF MS can effectively identify mycobacteria. The limitations of current methods have led some authors to suggest that MALDI-TOF MS is an alternative that is well worth considering (1,9–11).

Recently, a new enhanced database for mycobacteria identification to use with the MALDI Biotyper, the Mycobacteria Library, has been introduced. The newly enhanced database for mycobacteria identification with the MALDI Biotyper, the Mycobacteria Library 4.0 (ML4.0), covers as many as 159 of the 169 mycobacteria species currently known (12). In this study, our aim was to evaluate the diagnostic performance of MALDI-TOF MS utilizing the MBT ML4.0 library and to compare the results with sequence analysis with respect to discriminating between MTC and NTM isolates, and allowing species-level identification of NTMs.

2. Materials and methods

2.1. *Mycobacterium* isolates

In this study, 155 mycobacteria strains consisting of 60 MTC and 95 NTM isolates were analyzed. The 155 mycobacteria isolates consisted of 74 clinical strains that had been isolated in four different centers (Acıbadem Labmed Medical Laboratories, Ege University School of Medicine, Celal Bayar University School of Medicine, and Marmara University School of Medicine) between 2007 and 2017 and that could be successfully recovered from frozen stocks, 79 external quality control strains, *M. tuberculosis* ATCC 25177/H37Ra, and *M. fortuitum* ATCC 6842.

2.2. Growth conditions and identification

Archived mycobacteria strains were subcultured in solid medium (Lowenstein–Jensen). After 21 days of incubation at 37 °C, specific mycobacteria colonies were grown and processed. Gram staining was also performed to ensure lack of any microbial contamination.

Initially, the MPT64 immunochromatographic test (SD Biotline, Yongin, Korea), which has been reported to

possess a sensitivity and specificity above 98% and 95%, respectively, was used to differentiate the *M. tuberculosis* complex from the NTM species (13). Afterwards, identification to species level was performed by using MALDI-TOF MS (Microflex LT, Bruker Daltonics, Germany) and by DNA sequencing of genes encoding 16S rRNA and the 65-kDa heat-shock protein (hsp65) using the AB3500 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. MycoEX (mycobacteria extraction) protocol for MALDI-TOF MS

Fresh culture from Lowenstein medium with sufficient biomass was used to undertake the process. The grown colonies were transferred to a microcentrifuge tube with 300 µL of water (HPLC grade) in a class II type A2 biological safety cabinet (Heraeus, Germany) under biosafety level III. Then they were inactivated by boiling for 30 min at 100 °C in a thermoblock. Next, 900 µL of 100% ethanol was added to the microcentrifuge tube and mixed with the contents, then centrifuged at 13,500 rpm for 2 min. The supernatant was discarded and the pellet was left to dry for 5 min, with the tube open, to ensure complete removal of the ethanol. Silica beads (BioSpec Products Inc., Bartlesville, OK, USA) and 20–50 µL (according to the strength of the pellet) of pure acetonitrile were added to the microcentrifuge tube, which was vortexed thoroughly for 1 min. Then 70% formic acid (with the same volume of acetonitrile) was added to the microcentrifuge tube and centrifuged at 13,500 rpm for 2 min. One microliter of each supernatant was then placed in three of the 96 spots of the steel target plate and they were allowed to dry at room temperature. Finally, 1 µL of HCCA matrix solution (α -cyano-4-hydroxycinnamic acid) was added to each of the spots and left to dry before further analysis by MALDI-TOF MS (14). Each sample was analyzed in duplicate.

2.4. MALDI-TOF MS analysis

Spectra were acquired in linear positive ion mode at a laser frequency of 60 Hz across a mass/charge ratio (m/z) of 2000 to 20,000 Da using the Microflex LT MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany). The protein profile was obtained by the software FlexControl 3.4 (Bruker Daltonik GmbH) and analyzed by the application FlexAnalysis 3.4 (Bruker Daltonik GmbH). The MBT ML 4.0 Library, representing 159 species, was also used (12,15).

2.5. 16S rDNA and hsp65 sequencing

Double-stranded DNA sequences from the strains included in the study were determined using BigDye Terminator chemistry on an AB3500 DNA sequencer (Applied Biosystems) following the standard protocol of the supplier. The 1524-bp fragment of the 16S rRNA gene was sequenced by using

primers P1 (5'-AGAGTTTGATCCTGGCTCAG-3'; corresponding to positions 8–28) and P2 (5'-TGCAACAAGGCCACAAGGA-3'; corresponding to positions 1046–1026) and primers P3 (5'-GTGTGGGTTTCCTTCCTTGG-3'; corresponding to positions 830–847) and P4 (5'-AAGGAGGTGATCCAGCCGCA-3'; corresponding to positions 1542–1522). The 441-bp fragment of the hsp65 gene was sequenced by using primers Tb11 (5'-ACCAACGATGGTGTGCCAT-3') and Tb12 (5'-CTTGTCGAACCGCATACCCT-3'). Afterwards, the sequences thus obtained were compared with reference sequences in the GenBank database using the Finch TV and BLAST programs (16,17).

3. Results

Analysis of the results obtained here according to 16S rRNA and hsp65 sequence analysis reveals that MALDI-TOF MS in conjunction with the MBT ML4.0 library was successful in identifying 94% of the mycobacteria isolates and in differentiating 100% of MTC isolates from NTM isolates. MBT ML4.0 managed to identify 91% of NTM isolates to the species level and also to identify 82% of MAC isolates. All the results are shown in the Table.

4. Discussion

MALDI-TOF MS is in use by clinical laboratories for the identification of bacteria as it is successful, rapid, user-friendly, and cost-effective to implement. The profitable outcomes obtained with this technique have attracted researchers' interest in utilizing this technology to identify mycobacterial species (18). In this study, we compared the usefulness of the newly enhanced database for mycobacteria identification with MBT ML4.0. Our results show that MBT ML4.0 was successful in identifying all of the MTC isolates as such by 16S rRNA and hsp65 sequence analysis. With regard to the NTM isolates, MBT ML4.0 achieved the identification of 91% of the isolates identified as such by 16S rRNA and hsp65 sequence analysis. Identification by MBT ML 4.0 was performed accurately for 100% of MTC isolates. However, while all of the MTC isolates were correctly differentiated from the NTM isolates and positively identified as *M. tuberculosis* complex, subspecies of the isolates could not be defined by this method. The MTC encompasses 10 different species, but MBT ML4.0 contains only five of them (*M. tuberculosis*, *M. africanum*, *M. bovis*, *M. caprae*, *M. microti*) (12). The mass spectra of MTC member species have similarity. This similarity prevents successful discrimination between subspecies of MTC.

The identification results of 5% of the NTM isolates by MBT ML4.0 were not in accord with 16S rRNA and hsp65 sequence analysis. This result was due to the close

relatedness of these isolates, such as *Mycobacterium avium-intracellulare*, the *Mycobacterium chelonae-Mycobacterium abscessus* complex, *Mycobacterium gordonae*, *Mycobacterium paragordoniae*, *Mycobacterium peregrinum*, and *Mycobacterium genavense*. The complexity of the differentiation between phylogenetically close species, which, in any case, remains unresolved by standard methods, explains the difficulty in identifying these species (19–36). The *M. avium-intracellulare* complex (MAC) consists of *M. avium*, *M. intracellulare*, *M. arosiense*, *M. bouchedurhonense*, *M. chimaera*, *M. colombiense*, *M. marseillense*, *M. timonense*, *M. vulneris*, and *M. yongonense*. *M. avium*, *M. intracellulare*, and *M. chimaera* are apart from the others in being the most significant human pathogens. For demonstrating sources, revealing pathogenicity traits, and observing differences in treatment outcome between MAC species, identification to the species level has particular importance. Nonetheless, it is hard to differentiate between the many species and subspecies in most laboratories (19–23). In the present study, MBT ML4.0 was apparently successful in identifying *M. avium* species, while it failed to identify *M. intracellulare chimaera*, which is one of the subspecies of the MAC. The *M. chelonae-M. abscessus* complex is currently accepted as including six species: *M. chelonae*; *M. abscessus* with three subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*; *M. immunogenum*; *M. salmoniphilum*; *M. franklinii*; and *M. saopaulense* (24–33). MBT ML4.0 includes *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. chelonae* (15). MBT ML4.0 accurately identified all of the *M. chelonae* isolates and 91% of *M. abscessus* isolates. Not only MBT ML4.0 but also the older versions are capable of identifying correctly both *M. chelonae* and *M. abscessus* species (37,38). *Mycobacterium gordonae*, which is usually considered a laboratory contaminant and only rarely is a proven human pathogen, was also included in this study (39). MBT ML4.0 yielded identification of 17 of 18 *M. gordonae* isolates and failed to identify at subspecies level only one of the 18 *M. gordonae* isolates. Nevertheless, this isolate was defined as *Mycobacterium* sp. *nontuberculous* (confidence score ≥ 2.0). *M. gordonae* used to be difficult to identify confidently using the older databases, but the procedure has been greatly improved by using updated libraries, such as Mycobacteria Library v3.0 and ML4.0, the results from which are outlined in this study (37–40). *Mycobacterium paragordoniae* was identified by Kim et al. in 2014 as a novel species, phenotypically and genetically clearly related to *M. gordonae*, but with some dissimilarity in terms of certain phenotypic characteristics (34). Accordingly, only ML4.0, the latest database, includes the reference spectra of *M. paragordoniae*, which then allowed us to identify 4 out of 5 *M. paragordoniae* isolates (12). As for

Table. Comparison of identification results of MBT Mycobacteria Library ver4.0 with mycobacterial DNA sequencing.

hsp65/16S rDNA Sequencing	Total of isolates (n)	MBT Mycobacteria Library ver 4.0											
		Score ≥2		Score 1.9–1.7		Score <1.7 (no identification)		Identification to genus level		Total of identification to species level			
		n	%	n	%	n	%	n	%	n	%		
<i>M. tuberculosis complex</i>	60	56	93	4	7	0	0	0	0	0	0	60	100
<i>M. goodii</i>	18	14	77	3	17	0	0	0	0	1*	6	17	94
<i>M. avium intracellulare complex (MAC)</i>	11	8	73	1	9	2	18	0	0	0	0	9	82
<i>M. avium</i>	4	3	75	1	25	0	0	0	0	0	0	4	100
<i>M. intracellulare chimera</i>	2	1	50	0	0	1	0	0	0	0	0	1	50
<i>M. chimera</i>	2	2	100	0	00	0	0	0	0	0	0	2	100
<i>M. avium intracellulare</i>	1	1 ^b	100	0	0	0	0	0	0	0	0	1	100
<i>M. avium intracellulare/M. paraintracellulare</i> (100%)	1	1 ^c	100	0	0	0	0	0	0	0	0	1	100
<i>M. intracellulare</i> (100%), <i>M. indicus pranii</i> (100%)													
<i>Mycobacterium sp. MOTT36Y</i> (100%), <i>M. chimera</i> (99%)													
<i>M. yongonense/M. paraintracellulare</i> (100%)													
<i>M. intracellulare</i> (100%), <i>M. indicus pranii</i> (100%), <i>Mycobacterium sp. MOTT36Y</i> (100%), <i>M. yongonense</i> (99%)	1	0	0	0	0	1	0	0	0	0	0	0	0
<i>M. abscessus</i>	11	8	73	2	18	1	9	0	0	0	0	10	91
<i>M. kansasii</i>	8	8	100	0	0	0	0	0	0	0	0	8	100
<i>M. chelonae</i>	7	4	57	3	43	0	0	0	0	0	0	7	100
<i>M. fortuitum</i>	6	4	66	1	17	0	0	0	0	1 ^d	17	5	83
<i>M. simiae</i>	6	4	67	2	33	0	0	0	0	0	0	6	100
<i>M. paragordoniae</i>	5	3	60	1	20	1	20	0	0	0	0	4	80
<i>M. lentiflavum</i>	4	3	75	1	25	0	0	0	0	0	0	4	100
<i>M. peregrinum</i>	4	2	50	0	0	0	0	0	0	2 ^e	50	2	50
<i>M. szulgai</i>	3	3	100	0	0	0	0	0	0	0	0	3	100
<i>M. marinum</i>	2	2	100	0	0	0	0	0	0	0	0	2	100
<i>M. mucogenicum phocaicum group</i>	2	2	100	0	0	0	0	0	0	0	0	2	100
<i>M. canariense</i>	1	1	100	0	0	0	0	0	0	0	0	1	100
<i>M. cerefilavum</i>	1	1	100	0	0	0	0	0	0	0	0	1	100
<i>M. nonchromogenicum</i>	1	0	0	1	100	0	0	0	0	0	0	1	100
<i>M. porcinum</i>	1	1	100	0	0	0	0	0	0	0	0	1	100
<i>M. scrofulaceum</i>	1	1	100	0	0	0	0	0	0	0	0	1	100
<i>M. shimoidei</i>	1	1	100	0	0	0	0	0	0	0	0	1	100
<i>M. senegalense</i>	1	1	100	0	0	0	0	0	0	0	0	1	100
<i>M. genavense</i>	1	0	0	0	0	0	0	0	0	1 ^f	100	0	0
Total	155	127	82	19	12	4	3	5	3	5	3	146	94

n: Number. ^a: One isolate identified as *Mycobacterium sp. nontuberculosis* (score: 2.2). ^b: One isolate identified as *M. chimera-intracellulare* group (score: 2.17). ^c: One isolate identified as *M. chimera-intracellulare* group (score: 2.18) d: One isolate was identified as *M. septicum* (score: 2.04). e: One isolate identified as *M. septicum* (score: 2.32) and other one identified as *M. porcinum* (score: 2.31). ^f: One isolate identified as *M. lentiflavum* (score: 2.13).

the other species, such as *M. canariensis*, *M. cerefilavum*, *M. nonchromogenicum*, *M. porcinum*, *M. scrofulaceum*, *M. shimoidei*, *M. senegalense*, and *M. genavense*, it is hard to conclude that MALDI-TOF MS was effective in identifying these isolates due to the limited number of isolates in the present study.

In the identification of microorganisms by MALDI-TOF MS, the cutoff score is another substantial issue in interpreting the results. Identification results scoring at least 2.0 are recommended to allow identification at the species level in bacteriology, while results with lower scores are reported to be in accordance with the gold standard methods for identifying mycobacteria (41,42). It is worth noting that higher confidence scores did not, however, correlate with a higher rate of correct identification, and mycobacteria isolates for which lower confidence scores (1.7–2.0) were obtained had a similar concordance rate with nucleic acid sequencing results to those with higher confidence scores (>2.0). Actually, the exact concordance rate depends on the reference library used (43). In this

study, by using MBT ML4.0, concordant identification with nucleic acid sequencing for MTC isolates was 93% with scores of ≥ 2.0 and 100% with scores of ≥ 1.7 . Ultimately, MBT ML4.0 covering 159 of the currently known 169 *Mycobacterium* species provided us with high sensitivity in mycobacteria identification (12).

In conclusion, our results proved that MALDI-TOF MS, which has already been used in routine laboratory practice as a rapid and cost-effective tool in the long term, represents a reliable identification technique and is the method of choice for the identification of clinically important *Mycobacterium* species for a routine laboratory. The MALDI-TOF MS Biotyper library, MBT ML4.0, which was under evaluation, was 100% successful in identifying MTC species with regard to DNA sequencing. Rapid and accurate diagnosis of mycobacterial infections is essential for commencing early treatment and the prevention of disease spread from person to person. The results of this study show that MBT ML4.0 can be used reliably to identify both MTC and NTM.

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