Evaluation of MALDI Biotyper interpretation criteria for the accurate identification of nontuberculous mycobacteria

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Running Head: MALDI-TOF score interpretation for NTM identification

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ABSTRACT

Mycobacteria identification by MALDI-TOF MS requires not only a good protein extraction protocol but also an adequate cut-off score in order to provide reliable results. The aim of this study was to assess the cut-off scores proposed by the MALDI-TOF MS system for mycobacteria identification. A total of 693 clinical isolates from liquid media and 760 from solid media were analysed, encompassing 67 different nontuberculous mycobacteria (NTM) species. MALDI-TOF MS identified 558 (80.5%) isolates from liquid media and 712 (93.7%) isolates from solid media with a score≥1.6 0. Among these, four (0.7%) misidentifications were obtained from liquid media and four (0.5%) from solid media. Regarding species diversity, MALDI-TOF MS was able to identify successfully 64 (95.5%) different species, whereas PCR-reverse hybridization (GenoType CM/AS) identified 24 (35.8%) different species. With a MALDI-TOF MS score ≥2 all isolates were correctly identified, as well as most isolates in the range 1.60-1.99, except *M. angelicum*, *M. parascrofulaceum*, *M. peregrinum*, *M. porcinum* and *M. gastri*. In conclusion, MALDI-TOF MS is a useful method for identifying a large diversity of NTM species. A score threshold of 1.60 proved useful for identifying almost all the isolates tested; only a few species required a higher score (≥2 .00) to obtain a valid definitive identification.

INTRODUCTION

Currently, 199 species of mycobacteria have been described (http://www.bacterio.net/mycobacterium.html), and most of them are classified as nontuberculous mycobacteria (NTM). Although many of these species are environmental, around one third may cause important human infections in both immunocompetent and immunocompromised patients (1). For this reason, accurate identification to species level is required, as recommended by the American Thoracic Society and the Infectious Disease Society of America (ATS/IDSA) (2).

Traditionally, the identification of NTM was carried out by phenotypic and biochemical tests. However, these laborious methods were unable to identify a high number of new species described and required long periods of time to obtain results. For this reason, they have been replaced by molecular techniques such as PCR-reverse hybridization and gene sequencing. With the implementation of these methods at clinical microbiology laboratories, the characterization and identification of NTM became more reliable, accurate, and rapid. However, PCR-reverse hybridization is limited to a certain number of NTM species; as several closely related species are indistinguishable from each other, they are identified together as a group (3). Moreover, in some cases the interpretation of the results is subjective and can lead to confusion. Although gene sequencing techniques are highly accurate, they require specific infrastructure and are time-consuming and expensive.

The implementation of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) at clinical microbiology laboratories to identify conventional bacteria is one of the latest breakthroughs in bacterial identification. This technique achieved a more rapid and precise identification and a significant cost saving as well (4). In the case of mycobacteria, the application of mass spectrometry is not yet

totally validated, in part due to the characteristics of the cell wall which make it mandatory to perform a special protein extraction procedure prior to analysis. Moreover, the use of MALDI-TOF MS to identify these microorganisms raises other critical issues, such as the type of culture media used (5), the level of updating of the database (6, 7) and the criteria applied to interpret the results.

The MALDI-TOF Biotyper system (Bruker Daltonics) provides a numerical score for the interpretation of the results which is classified globally into several categories. According to the manufacturer, currently, the score thresholds for mycobacteria identification are as follows: a score $\geq$1.80 represents high confidence, a score of 1.60-1.79 low confidence, and a score <1.60 is considered unreliable. Several studies have reported that these lower cut-offs may be suitable for some groups of microorganisms, such as *Corynebacterium*, *Gordonia* and others (8, 9, 10, 11). In the case of mycobacteria, some authors have used these thresholds in order to increase the identification rate (6, 12, 13, 14). However, there is no established cut-off point for species level identification of the entire range of mycobacteria.

The aim of this study was to determine the best MALDI-TOF MS cut-off scores for the reliable identification of the most frequent and clinically relevant NTM species.

MATERIALS AND METHODS

Mycobacterial strains and growth conditions

A total of 693 clinical isolates from liquid media and 760 from solid media were studied. They were isolated in the Department of Microbiology of the Hospital Universitari de Bellvitge-IDIBELL (Barcelona, Spain) and in the Clinical Microbiology and Infectious Diseases Department of the Hospital General Universitario Gregorio Marañón (Madrid, Spain). The strains were classified in 67 different species: 36 slow-

growing mycobacteria (SGM) and 31 species of rapid-growing mycobacteria (RGM), as shown in Table 1. All strains were cultured in liquid media (MGIT; Becton Dickinson, Towson, MD) and/or solid media (Löwenstein-Jensen, bioMérieux, Marcy-l'Etoile, France). The MGIT media were incubated following the instructions of the manufacturer in BACTEC MGIT960 system (Becton Dickinson). Once positive, they were processed for MALDI-TOF MS analysis after 0-5 days.

PCR-reverse hybridization and gene sequencing

Identification by PCR-reverse hybridization was performed on all clinical isolates using the commercial system GenoType *Mycobacterium* CM/AS (HAIN Lifescience, Nehren, Germany). This technique comprises two kits: the CM kit, able to identify *Mycobacterium tuberculosis* complex and 13 common NTM, and the AS kit, which identifies 16 other NTM species. The assay was carried out in accordance with the manufacturer's recommendations. Partial sequencing of the 16S rRNA and/or *hsp65* and *rpoB* genes was performed in the strains that obtained discordant results between GenoType and MALDI-TOF MS. A sequence similarity of≥99% was used as the final identification.

MALDI-TOF MS Protein extraction protocol

The protein extraction protocol was performed by sonication as previously described (15, 16). Initially, from liquid media, 1 ml was centrifuged at 13,000 rpm for 2 min and the pellet was resuspended in 300 µl of HPLC water. From solid media, a 1 µl loopful of bacterial biomass was resuspended in 300 µl of HPLC water. Samples from both media were heat-inactivated in a dry water bath at 95°C for 30 min. Then, 900 µl of ethanol were added, the tubes were centrifuged at 13,000 rpm for 2 min and the supernatant discarded. The pellet was allowed to dry at room temperature. Then, the

tip of a small spatula of 0.5-mm-diameter silica/zirconia beads and 20 µl of acetonitrile were added. The tubes were vortexed for 5 s and sonicated for 15 min. After this step, 20 µl of formic acid were added and the tubes were vortexed again for 10 s. Then, the samples were centrifuged at 13,000 rpm for 2 min and 1 µl of the supernatant was deposited onto the MALDI target plate (Bruker Daltonics, Bremen, Germany) by duplicate and allowed to dry. Finally, the spots were covered with 1 µl of HCCA matrix and allowed to dry at room temperature before inserting the target plate with the samples into the MALDI-TOF instrument.

MALDI-TOF MS analysis

The mass spectrometer used was a MALDI-TOF Biotyper microflex LT (Bruker Daltonics). The software used was FlexControl v3.0 with the Mycobacteria Library v5.0. The spectra were obtained over a mass/charge (*m/z*) ratio of 2,000-20,000 Da. The accelerating voltage was 20 kV and the samples were measured in automatic mode with a total of 240 laser shots collected per spot.

RESULTS

Among the 693 clinical isolates analysed by MALDI-TOF MS from liquid media, 614 (88.6%) isolates obtained protein peaks and in 558 cases (80.5%) the score was ≥1.60. Among the 760 isolates analysed from solid media, protein peaks were obtained in 746 cases (98.2%) and the score was ≥1.60 in 712 (93.7%) isolates. Regarding the diversity of the species studied (n=67), MALDI-TOF MS was able to identify 64 (95.5%) different species, while the PCR-reverse hybridization (GenoType CM/AS) identified 24 (35.8%) species.

Species with 10 or more isolates included

Those mycobacterial species (n=19) with 10 or more isolates included in this study were selected to evaluate the score interval and median obtained by MALDI-TOF MS. *M. avium, M. abscessus, M. chelonae, M. fortuitum, M. mageritense, M. peregrinum* and *M. porcinum* obtained a median score≥2 .00 from both liquid and solid media. The species with a median score higher than 2.00 from one culture medium (solid or liquid) were *M. arupense, M. celatum, M. gordonae, M. intracellulare/M. chimaera, M. kansasii, M. kumamotonense, M. lentiflavum, M. marinum, M. mucogenicum, M. szulgai* and *M. xenopi*. In one species (*M. parascrofulaceum*) the median score was in the 1.60-1.99 range from both culture media (Figures 1 and 2).

Species with fewer than 10 isolates included

From the 67 different species analysed, 48 included fewer than 10 isolates. In 44 of them, MALDI-TOF MS obtained scores ≥1.60. One species (*M. conspicuum*) obtained a score <1.60 in its only isolate, with correct species level identification. Three species, *M. madagascariense, M. paraterrae* and *M. yongonense*, were not identified by MALDI-TOF MS.

Cut-off scores and misidentifications obtained with MALDI-TOF MS

Regarding the species identification obtained according to the logarithmic score of MALDI-TOF MS, all isolates with a score ≥2.00 were correctly identified to species level. By contrast, 248 out of 252 (98.4%) isolates from liquid media and 263 out of 267 (98.5%) isolates from solid media in the 1.60-1.99 range were correctly identified to species level. The isolates that obtained an identification different from that of the reference method (PCR-reverse hybridization and/or gene sequencing) are detailed in Table 2 and were the following: three strains of *M. scrofulaceum* were identified as *M. parascrofulaceum*; three strains of *M. setense* were identified as *M. peregrinum* or *M.*

porcinum, one isolate of *M. szulgai* was identified as *M. angelicum*, and one strain of *M. kansasii* was identified as *M. gastri* by MALDI-TOF MS. The reliability of the identification of the species included according to the score obtained by MALDI-TOF MS is shown in Table 3.

DISCUSSION

Overall, one of the most striking features of MALDI-TOF MS is its ability to identify almost the entire diversity of the mycobacterial species included in this study (64 of 67 species). The three species not identified were the following: *M. madagascariense*, which obtained protein peaks but no coincidence in the identification list results (even though it is included in the current database); and *M. paraterrae* and *M. yongonense*, which are not included in the database.

The species with more than 10 isolates included in this study were selected in order to evaluate the score range that they can obtain with MALDI-TOF MS and to see which of them had the highest scores (Figures 1 and 2). Among the species selected, those with the best identification results were mainly rapid growing mycobacteria, such as *M. abscessus, M. chelonae, M. fortuitum, M. mageritense, M. peregrinum* and *M. porcinum*, and the slow growing mycobacterium *M. avium*. No misidentifications were found among these species. Previous studies have reported a confusion between *M. abscessus* and *M. chelonae* when using MALDI-TOF MS, using the Mycobacterial Library v1.0 (17), due to the fact that they are related species and may be included in the same mycobacterial complex. However, in this study, 63 isolates of *M. abscessus* and 81 *M. chelonae* were tested and no misidentification was found between them. Another species with a high representation in this study was *M. intracellulare-chimaera*, identified as a group by MALDI-TOF MS. Recently, new software called "the subtyping module" has been developed to offer the possibility of distinction

between these two species (18). However, this new application is not yet available in all clinical microbiology laboratories, and to date only an evaluative analysis has been performed (19).

Most of the species with fewer than 10 isolates included in this study were successfully identified by MALDI-TOF MS with a score $\geq$1.60. Only one species obtained a score below 1.60 in all isolates tested, but the identification provided by MALDI-TOF MS was correct: *M. conspicuum* (n=1). Currently only two spectra references are included for *M. conspicuum* in the database used (v5.0). Therefore, the addition of more spectra of these species in future databases may probably help to obtain reliable results.

Regarding MALDI-TOF MS's accuracy, some discrepancies were found in this study. Although many isolates of *M. szulgai* were tested (n=11), only one misidentification was found in an isolate from liquid media, which was incorrectly identified as *M. angelicum* with a score of 1.83 (Table 2). Surprisingly, the same isolate from solid media was identified correctly. Therefore, when a *M. angelicum* is identified by MALDI-TOF MS, a misidentification might be suspected, due to the close-relatedness of this species with *M. szulgai* (20). The second challenging species was *M. parascrofulaceum*, which is closely related to *M. scrofulaceum* (21). The isolates reported as *M. parascrofulaceum* by MALDI-TOF MS with a score $\geq$2.00 were correctly identified to species level. However, several isolates in the score range of 1.60-1.99 were in fact shown to be *M. scrofulaceum* (Table 2) by gene sequencing; this was the reference identification method in this case, since these two species showed the same pattern by PCR-reverse hybridization. This misidentification by MALDI-TOF MS has also been observed in previous studies in which the Mycobacteria Library v1.0 was applied (13, 17, 22). In the present study, the Mycobacteria Library v5.0 was used, but

the discordance persisted. In addition, another misidentification was found in some *M. setense* isolates which were identified as *M. peregrinum* or *M. porcinum* by MALDI-TOF MS, both in the score range of 1.60-1.99 (Table 2). There are several possible explanations for this. First, these three species are grouped in the *M. fortuitum* complex, so they are phylogenetically close to each other. Second, only one reference spectrum for *M. setense* is included in the current database. As mentioned above, the addition of several new reference spectra to a database can greatly improve the identification reliability for those species (7). Lastly, one isolate of *M. kansasii* was identified as *M. gastri* with a score of 1.64, in another case of strong phylogenetic closeness.

Although a large number of strains and a great diversity of the most frequent mycobacterial species were analysed, one limitation of this study is that not all the mycobacteria described were tested, and that few isolates from several species were included. In addition, the analysis was not performed in parallel from liquid and solid media. The results are nonetheless, interesting, since they provide a better reflection of routine microbiological practice.

All in all, MALDI-TOF MS has proved extremely useful for the identification of a large amount of different species. However, it is necessary to establish general interpretation criteria in order to obtain the highest accuracy of mycobacterial identification using mass spectrometry. Table 3 shows a proposal for score interpretation based on the findings of this study. Thus, a score ≥2.00 should be taken as indicating high confidence for mycobacteria identification, instead of 1.80. Interestingly, the score range of 1.60-1.99 was valid for almost all the species analysed, with the exception of *M. angelicum*, *M. parascrofulaceum*, *M. peregrinum*, *M. porcinum* and *M. gastri* which required higher scores (≥2.00).

In conclusion, applying general identification criteria, MALDI-TOF MS can be implemented as a first line identification method from pure cultures for almost all mycobacteria isolated in clinical microbiology laboratories.

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FIGURE LEGENDS

FIGURE 1 Ranges and median scores obtained by MALDI-TOF MS from liquid media for species with more than 10 isolates included

FIGURE 2 Ranges and median scores obtained by MALDI-TOF MS from solid media for species with more than 10 isolates included

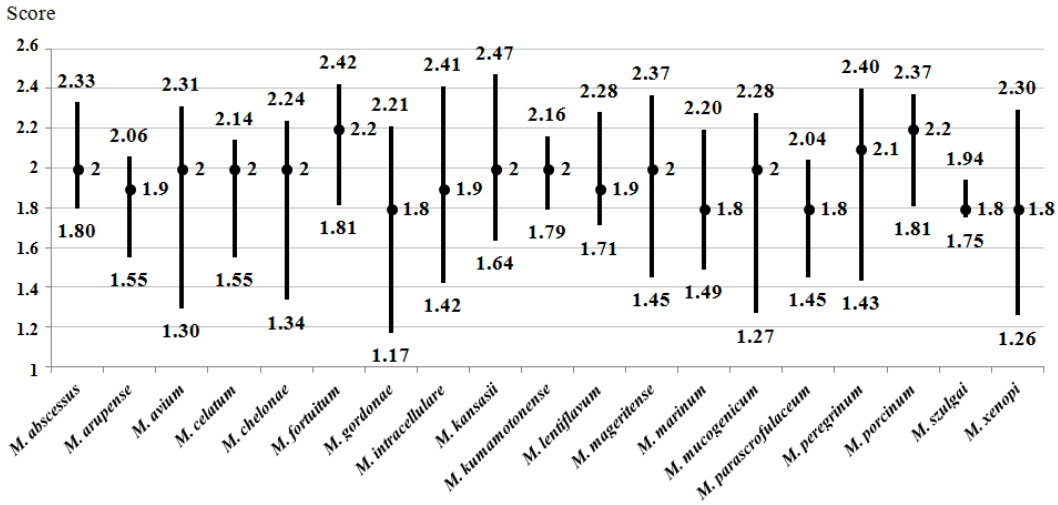

FIGURE 1 Ranges and median scores obtained by MALDI-TOF MS from liquid media for species with more than 10 isolates included

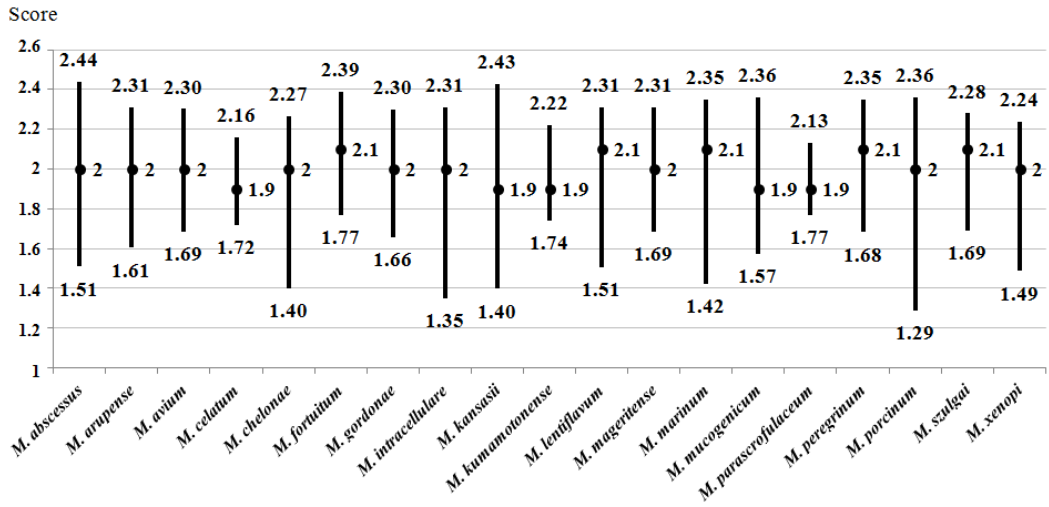

FIGURE 2 Ranges and median scores obtained by MALDI-TOF MS from solid media for species with more than 10 isolates included

TABLE 1 Mycobacterial species and the number of strains analysed from liquid and solid media.

Slow-growing species (n=36)	**Liquid media**	**Solid media**	**Rapid-growing species (n=31)**	**Liquid media**	**Solid media**
M. arupense	10	16	*M. abscessus*	30	67
M. avium	92	59	*M. algericum*	4	6
M. bohemicum	1	4	*M. aubagnense*	3	3
M. branderi	1	1	*M. brumae*	3	4
M. celatum	7	12	*M. canariasense*	4	4
M. colombiense	2	3	*M. chelonae*	46	81
M. conspicuum	1	1	*M. cosmeticum*	2	3
M. doricum	1	1	*M. elephantis*	5	5
M. europaeum	1	1	*M. fortuitum*	32	64
M. gastri	0	1	*M. frederiksbergense*	5	5
M. gordonae	39	23	*M. goodii*	3	4
M. haemophilum	0	2	*M. hassiacum*	1	1
M. heraklionense	4	4	*M. holsaticum*	1	1
M. interjectum	3	3	*M. insubricum*	0	1
M. intracellulare/chimaera	155	78	*M. iranicum*	1	2
M. kansasii	41	45	*M. madagascariense*	1	1
M. kumamotonense	10	10	*M. mageritense*	19	29
M. lentiflavum	10	13	*M. monacense*	2	2
M. longobardum	1	1	*M. moriokaense*	1	1
M. malmoense	3	7	*M. mucogenicum*	27	32
M. marinum	11	24	*M. neoaurum*	1	3
M. marseillense	0	3	*M. novocastrense*	1	1
M. palustre	0	5	*M. peregrinum*	10	13
M. parascrofulaceum	17	15	*M. phlei*	1	1
M. paraterrae	1	1	*M. porcinum*	10	12
M. scrofulaceum	4	2	*M. senegalense*	1	1
M. senuense	2	2	*M. septicum*	1	1
M. sherrisii	0	2	*M. setense*	4	4
M. shimoidei	4	6	*M. smegmatis*	2	4
M. simiae	0	5	*M. thermoresistibile*	3	4
M. szulgai	8	11	*M. wolinskyi*	1	1
M. terrae	1	2			
M. triplex	0	2			
M. vulneris	0	1			
M. xenopi	37	32			
M. yongonense	1	1			

TABLE 2 Misidentifications obtained by MALDI-TOF MS with score ≥1.60.

Strain	Culture media	MALDI-TOF MS top identification	Score
M. scrofulaceum 53447	Solid	*M. parascrofulaceum*	1.98
M. scrofulaceum 163633	Solid	*M. parascrofulaceum*	1.78
M. scrofulaceum 62886	Liquid	*M. parascrofulaceum*	1.81
M. setense 26612	Liquid	*M. porcinum*	1.76
M. setense 26824	Solid	*M. peregrinum*	1.81
M. setense 27376	Liquid	*M. porcinum*	1.82
M. szulgai 65533	Liquid	*M. angelicum*	1.83
M. kansasii 315208	Solid	*M. gastri*	1.64

TABLE 3 Proposal of score criteria for the species analysed in this study.

Score	Reliable species			Unreliable species
≥ 2.00	All species tested			-
1.60-1.99	*M. abscessus*	*M. haemophilum*	*M. novocastrense*	*M. angelicum*
	M. algericum	*M. hassiacum*	*M. palustre*	*M. parascrofulaceum*
	M. arupense	*M. heraklionense*	*M. paraterrae*	*M. peregrinum*
	M. aubagnense	*M. holsaticum*	*M. phlei*	*M. porcinum*
	M. avium	*M. insubricum*	*M. scrofulaceum*	*M. gastri*
	M. bohemicum	*M. interjectum*	*M. senegalense*	
	M. branderi	*M. intracellulare/chimaera*	*M. senuense*	
	M. brumae	*M. iranicum*	*M. septicum*	
	M. canariasense	*M. kansasii*	*M. setense*	
	M. celatum	*M. kumamotonense*	*M. sherrisii*	
	M. chelonae	*M. lentiflavum*	*M. shimoidei*	
	M. colombiense	*M. longobardum*	*M. simiae*	
	M. conspicuum	*M. madagascariense*	*M. smegmatis*	
	M. cosmeticum	*M. mageritense*	*M. szulgai*	
	M. doricum	*M. malmoense*	*M. terrae*	
	M. elephantis	*M. marinum*	*M. thermoresistibile*	
	M. europaeum	*M. marseillense*	*M. triplex*	
	M. fortuitum	*M. monacense*	*M. vulneris*	
	M. frederiksbergense	*M. moriokaense*	*M. wolinskyi*	
	M. goodii	*M. mucogenicum*	*M. xenopi*	
	M. gordonae	*M. neoaurum*	*M. yongonense*	
< 1.60	Unreliable			