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ARTICLE

Implementation of MALDI-TOF MS technology for the identification of clinical isolates of *Mycobacterium* spp. in mycobacterial diagnosis

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Abstract A total of 243 clinical isolates of the *Mycobacterium* genus were studied, 143 and 100 using two protocols (Protocol v2 and Protocol v3, respectively) provided by the manufacturer. The overall correlation of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with the standard identification methods was 63.8 %. The rate of misidentification was 3.2 %, mainly affecting very close species. In Protocol v2, the correlation was 57.3 %, being greater in solid than in liquid media (71.7 % vs. 44.7 %, $p<0.05$). Albeit not significant, a trend to a greater correlation for *M. tuberculosis* complex compared to non-tuberculous mycobacteria (NTM) (63.6 % vs. 55.5 %) was observed. In Protocol v3, the correlation was 73 %, with no significant differences between solid and liquid media (70.8 % vs. 75 %). In conclusion, MALDI-TOF MS may play a role in identifying mycobacterial species isolated from clinical samples, being faster than sequencing and hybridization-based techniques.

Introduction

There are currently more than 150 species of the *Mycobacterium* genus [1]. The most important species is *M. tuberculosis* complex, which is responsible for 1.6 million deaths annually [2]. However, infections caused by non-tuberculous mycobacteria (NTM) have risen in recent years, particularly in immunosuppressed patients and those with underlying chronic pulmonary diseases [3, 4]. A collaborative study conducted by the Nontuberculous Mycobacteria Network European Trials Group (NTM-NET) [3] has described the diversity and the variable frequency of NTM in different countries. According to this study, the most frequent isolates in Spain were *M. avium* complex, followed by other slow- and rapid-growth mycobacteria.

Identification and differentiation of the species of the genus *Mycobacterium* is complex. Biochemical tests and growth characteristics were used for many years. However, hybridization-based techniques or polymerase chain reaction (PCR), together with the sequencing of the 16S and 23S rRNA genes, are currently the most widely used techniques [5], providing highly reliable results in 2–3 days. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique provides a rapid alternative for the identification of microorganisms based on differences in their protein profile [6–8].

The first advances in the identification of mycobacteria using the MALDI-TOF MS technique were developed in 1996 on analyzing an *M. smegmatis* strain [6]. Thereafter, several studies [9–12] have attempted to evaluate the utility of MALDI-TOF in the identification of mycobacteria.

The main objective of the present study was to evaluate the use of the MALDI-TOF MS technique to identify *Mycobacterium* spp. isolates compared to standard methods used in mycobacterial laboratories.

Materials and methods

Study samples

A prospective study was performed using *Mycobacterium* spp. isolates from clinical samples at the Microbiological

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Department of the Hospital Clínic of Barcelona from January 2013 to April 2014.

Isolation and standard methods for the identification of mycobacteria

The samples were processed following standard laboratory procedures [5]. According to the morphology of the bacilli [13], the identification was made using one or more of the following standard methods considered as gold standards: real-time PCR amplification IS*6110* [14] for *M. tuberculosis*; a commercial DNA test based on a single-stranded DNA probe with a chemiluminescent label (AccuProbe Test, Gen-Probe, San Diego, CA, USA) [15] for *M. avium*, *M. intracellulare*, and *M. gordonae*; and sequencing a 500-bp fragment of the 16S rRNA mycobacterial gene [16] for the remaining species.

MALDI-TOF MS methodology

The strains were processed for MALDI-TOF identification 1–3 days after the culture was positive. Samples were prepared according to the manufacturer's instructions (Bruker Daltonics Inc., Bremen, Germany). From January 2013 to January 2014, the samples were processed using the recommended protocol at that time (Protocol v2) [17]. In January 2014, a new, more accurate version of Protocol v2 was provided by the manufacturer, hereafter known as Protocol v3 [18].

Sample preparation for Protocol v2 and Protocol v3

From mycobacteria grown in solid media, several colonies were harvested and suspended in 300 µl of CHROMASOLV® grade water (Sigma-Aldrich). From mycobacteria grown in liquid medium, 1.2 ml from the culture was centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and 300 µl of CHROMASOLV® water were added.

Extraction Protocol v2 [17]

The culture was inactivated at 95 °C for 30 min and centrifuged at 13,000 rpm for 2 min. The pellet was resuspended in 300 µl of CHROMASOLV® water, followed by 900 µl of absolute ethanol and centrifugation at 13,000 rpm for 2 min. Fifty microliters of CHROMASOLV® water were added to the pellet and centrifuged at 13,000 rpm for 2 min, discarding the supernatant. Afterwards, 50 µl of CHROMASOLV® water were added and intensively vortexed for 1 min. Samples were incubated at 95 °C for 10 min, and then 1,200 µl of cold absolute ethanol (−20 °C) were added, and the samples centrifuged at 13,000 rpm for 2 min, discarding the supernatant. The residual ethanol was evaporated at room temperature. According to the pellet volume, around 100–200 mg of zirconia/silica beads (0.5-mm diameter-beads, BioSpec Products) were added, as well as around 10–50 µl of acetonitrile CHROMASOLV® grade (Sigma-Aldrich) and intensely vortexed for 1 min. Formic acid 70 % (*v/v*) LC-grade (Sigma-Aldrich) was added, and samples were thoroughly vortexed for 5 s, and centrifuged at 13,000 rpm for 2 min.

Extraction Protocol v3 [18]

Briefly, the culture was inactivated for 30 min at 100 °C. Afterwards, 900 µl of absolute ethanol were added, with centrifugation at 13,000 rpm for 2 min. The supernatant was removed. The residual ethanol was evaporated at room temperature. According to the pellet obtained, 100–200 mg of zirconia/silica beads were added, as well as around 10–50 µl of acetonitrile. Samples were intensely shaken for 1 min with the tissue homogenizer Minilys (Bertin Technologies, France). Formic acid 70 % (*v/v*) was added. The samples were shaken using the Minilys for 5 s, and centrifuged at 13,000 rpm for 2 min.

Mass spectra acquisition

One microliter of the final supernatant was spotted onto an MSP 96-spot plate (Bruker Daltonics) and 1 µl of a saturated solution of MALDI-TOF matrix HCCA (Bruker Daltonics) was added and left to dry. Each sample was analyzed in triplicate.

The spectra acquisition was performed with a Microflex™ mass spectrometer (Bruker Daltonics), using the FlexControl™ software (v.3.0). The calibration of the spectrometer was performed according to the manufacturer's specifications. The analysis was carried out in automatic mode and each isolate was submitted to 240 laser shots. The spectrum obtained was compared with the 173 patterns available in the Mycobacteria Library v.3.1 (Bruker Daltonics). According to the MALDI-TOF MS equipment, the spectra were classified into category A with a score ≥2, category B with a score 1.700–1.999, and category C with a score <1.700. Categories A and B reported identification considered as reliable. Category C was considered as not reliable, requiring reanalysis. "No peaks" results also required reanalysis.

Interpretation of results and statistical analysis

The identification obtained with both protocols was compared to the gold-standard methods. Final identification, differences between liquid and solid media, and differences between slow- and rapid-growth NTM for each protocol were analyzed using the Chi-square test. Statistical analyses were performed using SPSS 16.0.2 (SPSS Inc., Chicago, IL, USA).

Results

A total of 243 positive mycobacterial cultures were isolated during the study period (Table 1): 25.9, 56.7, and 21.4 % corresponded to *M. tuberculosis* complex, slow-, and rapid-growth NTM, respectively. Among the slow-growth NTM, the most frequent species identified were *M. avium*, *M. intracellulare*, and *M. kansasii*, and *M. abscessus* was found among rapid-growth NTM (Table 1). The overall correlation between MALDI-TOF and the standard methods was 63.8 % (155/243). According to the type of culture medium in which the strains were isolated, the correlation achieved was 71.3 % (82/115) in solid media and 57.8 % (74/128) in liquid medium ($p<0.05$) (Tables 2 and 3). Eight MALDI-TOF spectrums (3.2 %) presented discordant identification compared to the standard methods: five and three in Protocol v2 and Protocol v3, respectively. The misidentifications were: *M. intracellulare* instead of *M. avium* in two isolates; *M. fortuitum* instead of *M. abscessus*; *M. fortuitum* instead of *M. chelonae*; *M. fortuitum* instead of *M. intracellulare*; *M. intracellulare* instead of *M. abscessus*; *M. abscessus* instead of *M. tuberculosis*; and *M. malmoense* instead of *M. tuberculosis*.

Protocol v2

We studied 143 isolates, 114 (79.7 %) and 29 (20.2 %) classified as slow- and rapid-growth NTM, respectively. Thirty-three isolates (23.07 %) were *M. tuberculosis* complex (Table 1). The overall correlation between MALDI-TOF and the standard identification was 57.3 %, being 57.9 and 55.2 % for isolates of slow and rapid growth, respectively ($p>0.05$). For *M. tuberculosis* complex, the correlation was 63.6 % (Table 2). The correlation was 71.7 % (48/67) for strains isolated from solid medium and 46 % (35/76) for those isolated in liquid medium ($p<0.05$) (Table 2). The distribution of the isolates according to the categories defined previously showed that 58.7 % were classified into categories A and B, 23.8 % into category C, and 17.5 % were reported as “no peaks”.

Table 1 Mycobacterial species studied ($n=243$)

		Protocol v2		Protocol v3	
Species	N[a]	No. of isolates	%	No. of isolates	%
M. tuberculosis	63	33	23.1	30	30
Slow-growth NTM	**128**	**81**	**56.6**	**47**	**47**
M. arupense	1	1	0.7	–	–
M. avium	53	26	18.2	27	27
M. celatum	2	2	1.4	–	–
M. gastri	1	–	–	1	1
M. gordonae	6	5	3.5	1	1
M. intermedium	2	2	1.4	–	–
M. intracellulare	30	20	14	10	10
M. kansasii	13	12	8.4	1	1
M. malmoense	1	1	0.7	–	–
M. marinum	4	2	1.4	2	2
M. parascrofulaceum	2	2	1.4	–	–
M. silvaticum	1	1	0.7	–	–
M. simiae	1	–	–	1	1
M. szulgai	3	3	2.1	–	–
M. terrae	1	–	–	1	1
M. xenopi	7	4	2.8	3	3
Rapid-growth NTM	**52**	**29**	**20.3**	**23**	**23**
M. abscessus	31	16	11.2	15	15
M. chelonae	6	4	2.8	2	2
M. fortuitum	8	6	4.2	2	2
M. mageritense	2	–	–	2	4
M. peregrinum	3	3	2.1	–	–
Total	**243**	**143**	**100**	**100**	**100**

[a] Total number of each species studied

Table 2 Mycobacterial species identified using Protocol v2 (n=143)

Species	ID[a]	ID MALDI-TOF[b]	%	Solid media[c]	%	Liquid medium[c]	%
M. tuberculosis	33	21	63.6	5	62.5	16	64
Slow-growth NTM	**81**	**45**	**55.6**	**33**	**75****	**12**	**32.4****
M. arupense	1	0	0	0	0	–	–
M. avium	26	11	42.3	4	57.1	6	31.6
M. celatum	2	2	100	2	100	–	–
M. gordonae	5	4	80	2	100	2	66.7
M. intermedium	2	1	50	1	50	–	–
M. intracellulare	20	11	55	10	90.9	1	11.1
M. kansasii	12	8	66.7	6	75	2	50
M. malmoense	1	1	100	1	100	–	–
M. marinum	2	2	100	2	100	–	–
M. parascrofulaceum	2	0	0	1	50	–	–
M. silvaticum	1	1	100	1	100	–	–
M. szulgai	3	1	33.3	1	50	0	0
M. xenopi	4	3	75	2	66.7	1	100
Rapid-growth NTM	**29**	**16**	**55.2**	**10**	**66.7****	**6**	**42.8****
M. abscessus	16	6	37.5	2	28.6	4	44.5
M. chelonae	4	1	25	–	–	1	25
M. fortuitum	6	6	100	5	100	1	100
M. peregrinum	3	3	100	3	100	–	–
Total	**143**	**82**	**57.3**	**48**	**71.7***	**34**	**44.7***

[a] Identification with gold-standard methods

[b] Number of isolates correctly identified with the MALDI-TOF MS technique

[c] Number of species identified with respect to the culture media in which the strains were isolated

*$p<0.05$; **$p>0.05$

Protocol v3

One hundred isolates were analyzed, 77 and 23 % of slow and rapid growth, respectively. Thirty isolates (30 %) were *M. tuberculosis* complex (Table 1). The overall correlation reached 73 % (73/100). For *M. tuberculosis* complex, the correlation was 80 % (Table 3). In solid and liquid medium, the correlation was 70.8 and 75 %, respectively ($p>0.05$) (Table 3). Seventy-eight percent of the isolates were classified as categories A and B, 12 % as category C, and 10 % as "no peaks".

Discussion

The MALDI-TOF MS technology provides rapid, effective identification of the different species of mycobacteria from positive cultures [9–12, 19–22].

We analyzed 243 mycobacterial isolates of positive cultures from consecutive clinical samples. The overall correlation between the gold-standard methods and MALDI-TOF identification was 63.8 %. However, on distinguishing between the two extraction protocols, Protocol v3 had a correlation of 73 %, being statistically higher than Protocol v2 and closer to the results of other studies [9, 19, 20]. The improvement in Protocol v3 is probably due to a simplification of the protein extraction process, avoiding loss of protein material. Other studies have reported improvements in the extraction protocol, such as vortexing with glass beads and resuspension with formic acid and acetonitrile [7, 10, 11], which have been adopted by other authors and by the manufacturer. A modification substituting the vortex with an automatic shaker has recently been described [12]. The effect of the tissue homogenizer used in the present study is probably due to a non-homogeneous movement in the suspension, producing more efficient rupture of the bacteria.

Interestingly, another difference we found between the two protocols was the distribution in the categories in which MALDI-TOF classified the spectrums analyzed. Protocol v3 classified 78 % of isolates in categories A and B, compared to 58.7 % classified in Protocol v2, significantly reducing the percentage of isolates to be retested.

Several authors have observed differences between liquid and solid media, with the latter providing a better yield [19,

Table 3 Mycobacterial species identified using Protocol v3 (n=100)

Species	ID[a]	ID MALDI-TOF[b]	%	Solid media[c]	%	Liquid medium[c]	%
M. tuberculosis	30	24	80	5	71.4	19	82.6
Slow-growth NTM	**47**	**31**	**65.9**	**22**	**73***	**9**	**53***
M. avium	27	16	59.3	13	72.2	3	30
M. gastri	1	1	100	1	100	–	–
M. gordonae	1	1	100	1	100	–	–
M. intracellulare	10	8	80	4	66.7	4	100
M. kansasii	1	0	0	–	–	0	–
M. marinum	2	2	100	2	100	–	–
M. simiae	1	1	100	1	100	–	–
M. terrae	1	0	0	0	0	–	–
M. xenopi	3	2	66.7	–	–	2	66.7
Rapid-growth NTM	**23**	**18**	**78.2**	**7**	**63.7***	**11**	**91.7***
M. abscessus	15	11	73.3	5	62.5	6	85.7
M. chelonae	2	1	50	0	0	1	100
M. fortuitum	2	2	100	1	100	1	100
M. mageritense	4	4	100	1	100	3	100
Total	**100**	**73**	**73**	**34**	**70.8***	**39**	**75***

[a] Identification with gold-standard methods

[b] Number of isolates correctly identified with the MALDI-TOF MS technique

[c] Number of species identified with respect to the culture media in which the strains were isolated

*p>0.05

21]. It has been suggested that supplements added to the liquid media could interfere with the extraction process [21], although this was not verified by other authors [10, 22]. Since the same supplements are used in solid media, a more plausible explanation could be the lower number of bacteria obtained in the liquid medium [11]. Some authors have suggested that the period of incubation may influence the quality of the extract, with better results for older cultures [21]. In the present study, the differences observed between solid and liquid cultures were not significant in Protocol v3 but were significant in Protocol v2, suggesting that the differences between media could be due to the bacterial load or that the efficiency of the extraction may reduce the differences between media described by other authors [21, 22].

Misidentification and lack of correlation with the standard methods has mainly been reported in phylogenetically close species [9, 11, 21, 22]. In the present study, 3.2 % of isolates were misidentified by MALDI-TOF. Half of these isolates were closely related species, such as *M. avium*–*M. intracellulare* or rapid-growth NTM. These differences have also been observed previously, reflecting the complexity of the differentiation between phylogenetically close species, which has not yet been completely solved by standard methods [9, 11, 21, 23].

The results of this study suggest that MALDI-TOF MS may be used in mycobacteriological diagnosis. From a practical point of view, this technique is rapid, providing results within a few hours, and allowing the identification of a broad spectrum of species. Possible limitations of this study may include the need to further improve the extraction protocol in order to achieve a higher rate of identification. The high cost of the MALDI-TOF MS equipment should also be considered, although it may be used to identify many other microorganisms.

In conclusion, MALDI-TOF MS may play a role in identifying mycobacterial species isolated from clinical samples, being faster than sequencing and hybridization-based techniques.

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Conflict of interest The authors declare no conflict of interests.

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