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Evaluation of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for Identification of Nontuberculous Mycobacteria from Clinical Isolates

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for the identification of nontuberculous mycobacterial (NTM) isolates was evaluated in this study. Overall, 125 NTM isolates were analyzed by MALDI-TOF and GenoType CM/AS. Identification by 16S rRNA/*hsp65* sequencing was considered the gold standard. Agreements between MALDI-TOF and GenoType CM/AS with the reference method were, respectively, 94.4% and 84.0%. In 17 cases (13.6%), results provided by GenoType and MALDI-TOF were discordant; however, the reference method agreed with MALDI-TOF in 16/17 cases (94.1%; $P = 0.002$).

Identification of nontuberculous mycobacteria (NTM) by conventional phenotypic tests requires long incubation periods that may take up to 12 weeks. The GenoType Mycobacterium CM/AS (Hain Lifescience GmbH, Nehren, Germany) is a test based on the amplification of a 23S rRNA gene region followed by reverse hybridization with specific probes that allows the identification of 40 of the most common NTM. However, the hybridization step requires high sequence homology, and even point mutations in the target regions may hamper the hybridization step, leading to unreliable results (1). Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) has been recently applied to the identification of a wide range of microorganisms (2), mainly clinically significant bacteria and fungi. Experience with MALDI-TOF for the rapid identification of NTM is very limited, mainly because of the number of identified species and because database information is scarce (3–5). In this study, a large collection of NTM clinical isolates and reference strains were analyzed using MALDI-TOF technology in comparison with the GenoType Mycobacterium CM/AS procedure and the reference procedure, 16S rRNA/*hsp65* gene sequencing.

From January 2010 to September 2014, all the NTM with purportedly clinical significance were grown on Lowenstein-Jensen agar and simultaneously identified by GenoType CM/AS assay (Hain Lifescience, GmbH, Nehren, Germany) and with MALDI-TOF MS technology (Bruker Daltonics, Bremen, Germany). Ten reference isolates were also included in this study as positive controls (Table 1). All the strains were also analyzed by 16S rRNA/*hsp65* sequencing as the reference method in order to resolve possible discrepancies.

Genotypic identification. Two gene regions were targeted for NTM identification: the 5′ end of the 16S rRNA gene with the universal primers E8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and E533R (5′-TTACCGCGGCTGCTGGCA-3′) (6) and a 439-bp fragment within the *hsp65* gene using the primers TB11 (5′-ACCAACGATGGTGTGTCCAT) and TB12 (5′-CTTGTCGA ACCGCATACCCT), as described by Telenti et al. (7). The amplification, purification, and sequencing process was carried out as previously described by Rodríguez-Sánchez et al. (8). Interpretation of sequencing results was performed considering CLSI recommendations for the genus *Mycobacterium* (9). Results obtained by 16S rRNA/*hsp65* sequencing were considered the final microorganism identification.

TABLE 1 Identification of the reference strains by the two methods analyzed

Mycobacterium ID by 16S rRNA/*hsp65*	Reference no.	Concordance with:	
		MALDI-TOF ID	GenoType CM/AS ID
M. avium	NC103034	*M. avium*	*M. avium*
M. haemophilum	DSM44634	*M. haemophilum*	*M. haemophilum*
M. malmoense	DSM 44163	*M. malmoense*	*M. malmoense*
M. palustre	DSM 44572	*M. palustre*	No ID
M. shimoidei	DSM 44152	*M. shimoidei*	*M. shimoidei*
M. simiae	DSM 44165	*M. simiae*	*M. simiae*
M. smegmatis	MC2 155	*M. smegmatis*	*M. smegmatis*
M. szulgai	DSM 44166	*M. szulgai*	*M. szulgai*
M. triplex	DSM 44626	*M. triplex*	No ID
M. xenopi	DSM 43995	*M. xenopi*	*M. xenopi*

GenoType CM/AS identification. The GenoType assay (Hain Lifescience, GmbH, Nehren, Germany) was applied to the NTM

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TABLE 2 *Mycobacterium* isolates identified by 16S rRNA/*hsp65* sequencing and their correlation with MALDI-TOF, grouped by score and GenoType results

Mycobacterium species ID by 16S rRNA/*hsp65*	No. (%) of isolates						
	With MALDI-TOF ID at species level with a score of:				With discordant or no MALDI-TOF ID	With concordance with GenoType CM/AS ID	Total
	≥2.0	≥1.9	≥1.7	<1.7			
M. abscessus	7	7	9	0	0	7	9
M. arupense	0	1	1	0	0	0	1
M. avium	6	9	14	3	0	16	17
M. bohemicum	1	1	1	0	0	0	1
M. chelonae	5	5	6	0	0	6	6
M. fortuitum	10	14	17	0	0	16	17
M. gastri	1	1	1	0	0	1	1
M. gordonae	4	8	13	2	3	17	18
M. haemophilum	0	1	1	0	0	1	1
M. intracellulare	2	3	4	1	0	4	5
M. kansasii	3	5	10	0	0	10	10
M. lentiflavum	6	8	8	0	0	7	8
M. mageritense	5	5	6	0	0	5	6
M. malmoense	1	1	1	0	0	1	1
M. marinum	1	1	1	0	0	1	1
M. massiliense	0	0	0	0	4	0	4
M. mucogenicum	2	2	2	0	0	2	2
M. palustre	0	0	1	0	0	0	1
M. peregrinum	3	3	3	0	0	1	3
M. porcinum	1	1	1	0	0	0	1
M. shimoidei	1	1	1	0	0	1	1
M. simiae	1	2	2	0	0	1	2
M. smegmatis	1	1	1	0	0	1	1
M. szulgai	1	1	1	0	0	1	1
M. terrae	1	1	1	0	0	1	1
M. triplex	0	1	1	0	0	0	1
M. xenopi	1	3	4	1	0	5	5
Total	64 (51.2)	86 (68.8)	111 (88.8)	7 (5.6)	7 (5.6)	105 (84)	125

TABLE 3 Resolution of discrepancies between MALDI-TOF and the GenoType CM/AS ID according to 16S rRNA/*hsp65* ID[a]

No. of isolates (*n* = 17)	MALDI-TOF ID	GenoType CM/AS ID	Mycobacterium ID by 16S rRNA/*hsp65*
2	*M. abscessus*	*M. chelonae*	*M. abscessus*
1	***M. abscessus***	***M. chelonae***	***M. massiliense***
1	*M. arupense*	*Mycobacterium* spp.	*M. arupense*
1	*M. avium*	*M. kansasii*	*M. avium*
1	*M. bohemicum*	*M. scrofulaceum*	*M. bohemicum*
1	*M. fortuitum*	*Mycobacterium* spp.	*M. fortuitum*
1	*M. gordonae*	*M. szulgai*	*M. gordonae*
1	*M. intracellulare*	*M. avium* complex	*M. intracellulare*
1	*M. lentiflavum*	*Mycobacterium* spp.	*M. lentiflavum*
1	*M. mageritense*	*M. fortuitum*	*M. mageritense*
1	*M. palustre*	*M. avium*	*M. palustre*
2	*M. peregrinum*	*M. fortuitum*	*M. peregrinum*
1	*M. porcinum*	*M. fortuitum*	*M. porcinum*
1	*M. simiae*	*M. gordonae*	*M. simiae*
1	*M. triplex*	*M. avium*	*M. triplex*

[a] The line in bold shows isolates with discordant ID by MALDI-TOF and the reference method.

isolates following the manufacturer's instructions. The test consists of PCR amplification of a 23S rRNA gene region, a reverse hybridization with specific probes immobilized on nitrocellulose strips, and detection of the banding patterns. The turnaround time for this technique is approximately 8 h.

MALDI-TOF mass spectrometry. Isolates were treated prior to MALDI-TOF analysis following the manufacturer's instructions. Under biosafety level 3 (BSL3) conditions, samples were inactivated for 30 min at 95°C, resuspended in 300 μl of deionized water plus 900 μl of ethanol, and centrifuged at 13,000 rpm, and the supernatants were discarded. Afterwards, the pellet was taken to a BSL2 laboratory in order to conclude the preprocessing steps (10). The MALDI-TOF settings used have already been described (11). All isolates were analyzed by MALDI-TOF MS, using a Microflex LT benchtop mass spectrometer (Bruker Daltonics, Bremen, Germany). FlexControl 3.3 and Maldi Biotyper 3.1 software (Bruker Daltonics, Bremen, Germany), respectively, were used for the control of the instrument and for spectrum analysis and comparison with the database (updated with 5,627 species plus the specific Mycobacteria Library v2.0 with 313 isolates from 127 species; Bruker Daltonics). When this method was performed, the total turnaround time until MALDI-TOF identification was obtained was 90 min.

Sensitivity and specificity values were compared using the McNemar test for paired samples, with two tails. Validity values were calculated with a 95% confidence interval (CI) following an exact binomial distribution. Data were analyzed using SPSS software package 18.0 (IBM, Chicago, IL, USA).

Identification of the 125 NTM isolates by MALDI-TOF and 16S rRNA/*hsp65* sequencing was compared, and agreement between methods at the species level was shown in 118/125 cases (94.4%) (Table 2). A score of >1.7 and/or consistent top four matches were considered reliable MALDI-TOF identification at the species level; none of the isolates analyzed was identified with this technique at the complex level. Discordant identifications (IDs) consisted of 4 minor errors (4 isolates of *Mycobacterium massiliense*, a species not represented in the database, identified as *Mycobacterium abscessus*). In addition, three *Mycobacterium gordonae* isolates could not be identified: two achieved no peaks and one yielded no reliable results.

Agreement between GenoType CM/AS and the reference method at the species level was shown in 105 cases (84.0%). Discrepancies were mainly due to the lack of identification of the less common *Mycobacterium* species (*Mycobacterium arupense*, *M. bohemicum*, *M. massiliense*, *M. palustre*, *M. porcinum*, and *M. triplex*) with GenoType CM/AS. Excluding the isolates from these species, agreement with the reference method was 90.5% (105/116) and 97.4% (113/116) for GenoType CM/AS and MALDI-TOF, respectively. This is actually one of the limitations of GenoType CM/AS, while the MALDI-TOF database is constantly expanding. In fact, the Mycobacteria Library v2.0 used in the present study includes 313 isolates from 127 species. To our knowledge, this is the first time this database has been evaluated.

MALDI-TOF MS results were also compared with those obtained by GenoType CM/AS. Both methods provided consistent identification in 108/125 cases (86.4%). In 16/17 of the remaining cases (94.1%; $P = 0.002$) the gold standard was consistent with MALDI-TOF results (Table 3). Besides, the reference method provided an ID different than MALDI-TOF and the GenoType CM/AS in one case: a *M. massiliense* identified as *M. abscessus* by MALDI-TOF and as *M. chelonae* by GenoType CM/AS. According to Fangous et al. (12), the correct identification of *M. massiliense* can be performed with MALDI-TOF using peak analysis.

MALDI-TOF identification obtained a score ≥2.0 (valid at the species level) in 64 cases (51.2%). However, with a cutoff ≥1.9 a total of 68.8% of the isolates were correctly identified at the species level and 88.8% with a score between 1.9 and 1.7 (valid at the genus level). Moreover, 7 isolates (5.6%) with a score below 1.7 (not reliable according to the manufacturer) were identically identified by MALDI-TOF and 16S rRNA/*hsp65* sequencing at the species level (Table 2). Thus, a score ≥1.7 and/or consistent top four identifications can be considered a reliable NTM identification with MALDI-TOF without losing accuracy.

The analysis by species showed that *M. avium*, with 3 isolates identified with a score below 1.7, and *M. gordonae*, with 3 isolates not identified by MALDI-TOF and 2 isolates with a score below 1.7, were the most challenging NTM species for MALDI-TOF ID. We hypothesize that this could be due to the extraction method limitations or to the low number of isolates from these two species in the database. Both factors will require further analysis.

MALDI-TOF had already shown to be a reliable tool for the identification of mycobacterial isolates (3–5, 13, 14). However, both the improvement of the sample pretreatment and the update

TABLE 4 Identification of NTM isolates grouped by growth rate

Mycobacterium group	MALDI-TOF ID at the species level with a score of:						GenoType CM/AS ID at the species level		No. of isolates
	≥2.0		≥1.9		≥1.7				
	% sensitivity (95% CI)	% specificity (95% CI)	% sensitivity (95% CI)	% specificity (95% CI)	% sensitivity (95% CI)	% specificity (95% CI)	% sensitivity (95% CI)	% specificity (95% CI)	
Rapid growers[a]	68.0 (53.3–80.5)	100 (95.2–100)	78.0 (64.0–88.5)	100 (95.2–100)	92.0 (80.7–97.8)	100 (95.2–100)	76 (61.8–86.9)	93.3 (85.1–97.8)	50
Slow growers, nonchromogens[b]	41.4 (23.5–61.0)	100 (96.2–100)	62.1 (42.3–79.3)	100 (96.2–100)	86.2 (68.3–96.1)	100 (96.2–100)	86.2 (68.3–96.1)	97.9 (92.7–99.7)	29
Slow growers, scotochromogens[c]	39.4 (22.9–57.8)	100 (96.1–100)	63.6 (45.1–79.6)	100 (96.1–100)	81.8 (64.5–93.0)	100 (96.1–100)	90.9 (75.7–98.1)	97.8 (92.4–99.7)	33
Slow growers, photochromogens[d]	38.5 (13.8–68.4)	100 (96.7–100)	61.5 (31.6–86.1)	100 (96.7–100)	100 (75.2–100)	100 (96.7–100)	92.3 (64.0–99.8)	99.1 (95.1–100)	13

[a] *M. abscessus*, *M. arupense*, *M. chelonae*, *M. fortuitum*, *M. mageritense*, *M. massiliense*, *M. mucogenicum*, *M. peregrinum*, *M porcinum*, and *M. smegmatis*.
[b] *M. avium*, *M. gastri*, *M. haemophilum*, *M. intracellulare*, *M. malmoense*, *M. palustre*, *M. shimoidei*, *M. terrae*, and *M. triplex*.
[c] *M. bohemicum*, *M. gordonae*, *M. lentiflavum*, *M. szulgai*, and *M. xenopi*.
[d] *M. kansasii*, *M. marinum*, and *M. simiae*.

of the specific mycobacterial database allowed the correct identification of 118 isolates from 26 different NTM species. The agreement with the reference method was 94.4% at the species level. Similar results have been reported, but the studies included fewer mycobacterial species (15, 16). Our results remain below the 97.2% reported by Balada-Llasat et al. (5), although their study included 13 different NTM species, half the number identified in this study.

In summary, MALDI-TOF identification of NTM species proved decisively superior to GenoType CM/AS both in accuracy (94.4% versus 84.0% according to the genomic sequencing) and in turnaround time (90 min versus 8 h). The high agreement of MALDI-TOF with 16S rRNA/*hsp65* sequencing allows its implementation in the microbiology laboratory for NTM identification, especially for rapidly growing NTM (Table 4), where MALDI-TOF has shown a very low rate of discrepant results (3 isolates [0.6%] in this study). MALDI-TOF is also cheaper than GenoType CM/AS (1.5€ versus 42€ per sample). Thus, our recommendation is to perform MALDI-TOF, when it is available, for all the samples suspected of being NTM and sequence only the isolates with unreliable or no MALDI-TOF ID, which may represent only 5.6% of isolates subjected to MALDI-TOF, according to our results.

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