Evaluation of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry in Identification of Nontuberculous Mycobacteria

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Key Words

Bacterial infection · Respiratory tract infection · Respiratory tract pathogen · Nontuberculous mycobacteria · MALDI-TOF MS · Identification

Abstract

Background/Aims: Species-level identification of nontuberculous mycobacteria (NTM) is important in making decisions about the necessity and choice of antimicrobial treatment. The reason is predictable clinical significance and the susceptibility profile of specific NTM species. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is recognized as a diagnostic tool for routine identification of bacteria and yeasts in the clinical laboratory based on protein fingerprint analysis. The aim of the study was to evaluate MALDI-TOF MS in the identification of NTM. **Methods:** A total of 25 NTM isolates from liquid cultures were identified with both polymerase chain reaction (PCR)-based hybridization assay and MALDI-TOF MS at the University Hospital Center Zagreb. **Results:** PCR-based hybridization assay identified 96% (24/25) and MALDI-TOF MS 80% (20/25) of tested NTM isolates. Five isolates with no reliable MALDI-TOF MS identification belonged to the *Mycobacterium avium-intracellulare* complex. Seventy percent (14/20)

of NTM isolates successfully identified with MALDI-TOF MS had a score higher than 2.0, indicating reliable species identification. **Conclusion:** MALDI-TOF MS is a promising tool for the identification of NTM. With a further improvement of the protein extraction protocol, especially regarding the *M. avium-intracellulare* complex, MALDI-TOF MS could be an additional standard method for identification of NTM.

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Introduction

Nontuberculous mycobacteria (NTM) are attracting growing attention due to the increase in the isolation frequency, especially in the countries with a declining tuberculosis incidence [1]. Clinical diseases NTM can cause a range of problems from the most frequent pulmonary disease to lymphadenitis in children, skin disease and disseminated infections in immunocompromised patients [2]. Species-level identification of NTM is important because it can guide the clinician's decision about the necessity and choice of antimicrobial treatment due to predictable clinical significance and susceptibility profile of specific NTM species [3]. Biochemical tests previously used for identification are slow and surpassed by still techni-

Table 1. Identification of NTM isolates by MALDI-TOF MS and PCR-based hybridization assay

PCR-based hybridization assay identification	MALDI-TOF MS identification	
	species	score
<i>M. abscessus</i>	<i>M. abscessus</i>	2.208
<i>M. gordonae</i>	<i>M. gordonae</i>	1.887
<i>M. xenopi</i>	<i>M. xenopi</i>	1.852
<i>M. avium</i>	no reliable identification	–
<i>M. avium</i>	<i>M. avium</i>	2.009
<i>M. fortuitum</i>	<i>M. fortuitum</i>	2.370
<i>M. avium</i>	<i>M. avium</i>	2.036
<i>M. fortuitum</i>	<i>M. fortuitum</i>	2.465
<i>M. fortuitum</i>	<i>M. fortuitum</i>	2.308
<i>M. avium</i>	<i>M. avium</i>	1.906
<i>M. fortuitum</i>	<i>M. fortuitum</i>	2.256
<i>M. fortuitum</i>	<i>M. fortuitum</i>	2.411
<i>M. fortuitum</i>	<i>M. fortuitum</i>	2.384
<i>M. avium</i>	<i>M. avium</i>	1.978
<i>M. avium</i>	<i>M. avium</i>	2.252
Negative	<i>M. fortuitum</i>	2.365
<i>M. avium</i>	<i>M. avium</i>	2.059
<i>M. xenopi</i>	<i>M. xenopi</i>	2.187
<i>M. gordonae</i>	<i>M. gordonae</i>	1.466
<i>M. intracellulare</i>	no reliable identification	–
<i>M. avium</i>	<i>M. avium</i>	1.805
<i>M. intracellulare</i>	no reliable identification	–
<i>M. intracellulare</i>	no reliable identification	–
<i>M. gordonae</i>	<i>M. gordonae</i>	2.071
<i>M. intracellulare</i>	no reliable identification	–

cally cumbersome and relatively expensive molecular methods. Polymerase chain reaction (PCR)-based hybridization assays and gene sequencing are now routinely used in laboratories in developed countries [2, 4]. A new tool for species identification of NTM is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) based on unique spectral fingerprints produced by extracted proteins [4, 5]. The aim of this study was to evaluate the use of MALDI-TOF MS in the identification of NTM.

Methods

A total of 25 NTM isolates collected between January and October 2014 at the Department of Clinical and Molecular Microbiology, University Hospital Center Zagreb, Zagreb, Croatia, were tested. The NTM strains were primarily isolated on solid medium (Löwenstein-Jensen). For each isolate a fresh subculture in Mycobacterium Growth Indicator Tube (MGIT) culture bottles incubated in BACTEC® MGIT™ 960 System (Becton, Dickinson and Company, Sparks, Nev., USA) was used and identification was

done with both, PCR-based hybridization assay and MALDI-TOF MS. For MALDI-TOF MS analysis a protein extraction was performed according to the manufacturer's instructions as follows. A 1.5-ml aliquot was removed from the MGIT culture bottle and centrifuged for 2 min at 14,000 rpm. The supernatant was removed from the pellet and 500 µl of deionized water was added to the pellet and centrifuged for 2 min at 14,000 rpm. The supernatant was then removed and the pellet was suspended in 300 µl of deionized water before 900 µl of 100% ethanol was added and mixed thoroughly. For inactivation and reducing the number of viable cells after adding of ethanol the sample was incubated for 10 min. The ethanol suspension was centrifuged for 2 min at 14,000 rpm. The supernatant was then removed and the pellet was suspended in 500 µl of deionized water and centrifuged for 2 min at 14,000 rpm. The supernatant was removed and the pellet was suspended in 50 µl of deionized water and then put in a heat block at 95°C for 30 min to inactivate the organisms. After heat inactivation, the suspension was allowed to cool to room temperature before adding 1,200 µl of 100% ethanol precooled in a freezer at –18°C. The suspension was then vortexed and centrifuged for 2 min at 14,000 rpm. The supernatant was removed, and the pellet was centrifuged again and the residual ethanol was carefully removed by pipetting. After the pellet was completely dry, 25 µl of acetonitrile and 0.5 mm zirconia/silica beads on the tip of a small spatula were added. The sample was then vortexed for 1 min to disrupt any aggregates, and 25 µl of 70% formic acid was added and mixed by vortexing for 5 s and centrifuged for 2 min at 14,000 rpm. A 1-µl portion of supernatant was added on an MALDI target and overlaid with 1 µl of matrix solution after drying. All samples with no peaks found were submitted to re-extraction. In addition, each extracted sample was deposited twice on the steel target plate and the better score was used for the study purpose.

The extract was analyzed with MALDI Biotyper 3.1 (Bruker Daltonik GmbH, Bremen, Germany) and the spectra obtained were compared to the Mycobacteria Library 1.0 that contains 173 spectra representing 94 mycobacterial species. The Bruker Bacterial Test Standard was used for calibration. Protein profiles with a mass-to-charge ratio (m/z) of 2,000–20,000 Da were produced based on a laser frequency of 60 Hz, 240 laser shots of each spectrum and 40 shot steps used.

Confidence scores greater than 2.0 were considered secure species identification, scores of 1.7–2.0 were considered intermediate identification, and scores of less than 1.7 were considered unreliable identification. For PCR-based hybridization assay (GenoType Mycobacterium CM; Hain Lifescience, Nehren, Germany) DNA extraction protocol with heat inactivation and ultrasonic bath according to the manufacturer's instructions was used.

Results and Discussion

PCR-based hybridization assay identified 96% (24/25) of tested NTM isolates which included *Mycobacterium avium* (n = 8), *M. fortuitum* (n = 6), *M. intracellulare* (n = 4), *M. gordonae* (n = 3), *M. xenopi* (n = 2), and *M. abscessus* (n = 1) (table 1). Analysis of the same isolates by MALDI-TOF MS identified 80% (20/25) of tested NTM

isolates. Five isolates in which identification was not successful included *M. intracellulare* (n = 4) and *M. avium* (n = 1). Seventy percent (14/20) of isolates successfully identified with MALDI-TOF MS had a score higher than 2.0, 25% (5/20) a score ranging from 1.7 to 2.0, and only 5% (1/20) had a score less than 1.7 (table 2). Out of 24 isolates identified with the PCR hybridization method, 79.2% (19/24) were also identified with the MALDI-TOF MS as a same mycobacterial species.

At the moment MALDI-TOF MS is recognized as a very simple and fast method with significantly lower consumable costs suitable for routine identification of bacteria and yeasts in the clinical laboratory [6, 7]. However, the critical point for accurate MALDI-TOF MS identification of NTM isolates is a protein extraction protocol [8]. Direct smearing and simple extraction methods used for other bacteria and yeasts are not sufficient for NTM because of the genus's hardy cell envelope and tendency to form clumps which have to be dispersed during the extraction procedure [9]. Therefore, the majority of successful mycobacteria protein extraction protocols use glass or zirconia-silica beads which yield high-quality spectra and lead to reproducible identifications at the species level. Furthermore, the use of heat in the protocol inactivates mycobacteria and minimizes the exposure of laboratory workers to viable mycobacteria [10].

It is still not clear which medium, liquid or solid, it is better select as a source of NTM isolate for MALDI-TOF MS identification. Some authors had a higher identification rate from solid medium compared to MGIT liquid medium, a possible explanation being the lower number of bacteria obtained in the liquid medium [11]. The authors achieving a better identification rate on liquid media employed another type of liquid media than MGIT in combination with an optimized extraction protocol using formic acid and acetonitrile in addition to physical disruption using silica beads, and, finally, they inoculated liquid medium with subculture from solid medium in order to have a more reproducible biomass [8]. In the present study, MALDI-TOF MS identification was also done from subcultures, although in MGIT culture bottles, an identical extraction procedure was used as in the study mentioned. A high identification rate in our study indicates that the use of a subculture in liquid medium in combination with optimized extraction procedure can make an MGIT liquid medium a favorable source of NTM isolates for identification by MALDI-TOF MS.

Incubation time may also influence the quality of the extract, with some authors suggesting better results for older cultures [11]. In our study, 8 out of a total of 25 iso-

Table 2. Scores achieved with MALDI-TOF MS identification of NTM

NTM isolate	MALDI-TOF MS score, n			No reliable identification with MALDI-TOF MS, n
	<1.7	1.7–2.0	>2.0	
<i>M. avium</i> (n = 8)	0	3	4	1
<i>M. fortuitum</i> (n = 7)	0	0	7	0
<i>M. intracellulare</i> (n = 4)	0	0	0	4
<i>M. gordonae</i> (n = 3)	1	1	1	0
<i>M. xenopi</i> (n = 2)	0	1	1	0
<i>M. abscessus</i> (n = 1)	0	0	1	0

lates (32.0%) were rapid growers represented by *M. fortuitum* and *M. abscessus*. All of them gain the identification score greater than 2.0 showing excellent species identification. On the other hand, all the NTM isolates with an identification score less than 2.0 were slow growers.

There was a high level of agreement between MALDI-TOF MS identification and the PCR-based hybridization assay in terms of NTM species identified. In other studies, misidentification and lack of correlation with standard methods were mainly attributed to phylogenetically close species such as *M. avium* and *M. intracellulare* and rapid-growth NTM [8, 11].

If we consider the PCR-hybridization method as one of the standards for the identification of NTM, the correlation between the standard method and MALDI-TOF in our study was 79.2%. Similar results were described in other studies using a similar extraction protocol [8, 12–15]. It should be emphasized that our study is limited by the small number of NTM isolates. Furthermore, only six of the most frequent clinical NTM species were analyzed and several studies revealed that limitations of MALDI-TOF MS for NTM species identification are associated with less frequent species [8, 12, 14]. Furthermore, 70% of isolates successfully identified with MALDI-TOF MS had a score higher than 2.0 indicating secure species identification.

For one isolate of *M. gordonae* the identification score was only 1.466. Although the criteria categorize this score as not a reliable identification, due to the concordant PCR-based hybridization method, this identification was considered correct. Some authors suggest decreasing MALDI-TOF MS species-level identification score thresholds in order to resolve more mycobacterial identifications [16–18]. By lowering the MALDI score for the species level from 2 to 1.9, some of the authors were able

to resolve 97.2% mycobacterial identifications to the species level [16]. Furthermore, decreasing the species-level threshold score, the performance of solid and liquid media was similar [16]. However, the optimizing of extraction protocol to improve identification should still be a cornerstone for more successful MALDI-TOF MS identification of NTM.

However, *M. intracellulare* isolates in our study were successfully identified only with PCR-based hybridization methods while MALDI-TOF MS gave no reliable identification. Whether *M. intracellulare* has some characteristics which make this species difficult to identify with MALDI-TOF MS remains unclear. One of the possible explanations proposed in recent studies is the presence of glyco-

peptidolipids in the cell wall of several mycobacterial species including the *M. avium-intracellulare* complex [19]. Glycopeptidolipids may be responsible for some characteristics in mycobacteria such as biofilm formation. This could prevent protein extraction reagents from penetrating the cell wall in these NTM species and resulting in no reliable MALDI-TOF MS identification [9].

In conclusion, MALDI-TOF MS is a promising diagnostic tool for identification of NTM isolates. Its simplicity, speed and further improvement of protein extraction protocol could make this method the future standard method for the identification of NTM in routine microbiological diagnostics.

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