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Review

How to: identify non-tuberculous *Mycobacterium* species using MALDI-TOF mass spectrometry

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ABSTRACT

Background: The implementation of MALDI-TOF MS for microorganism identification has changed the routine of the microbiology laboratories as we knew it. Most microorganisms can now be reliably identified within minutes using this inexpensive, user-friendly methodology. However, its application in the identification of mycobacteria isolates has been hampered by the structure of their cell wall. Improvements in the sample processing method and in the available database have proved key factors for the rapid and reliable identification of non-tuberculous mycobacteria isolates using MALDI-TOF MS.
Aims: The main objective is to provide information about the proceedings for the identification of non-tuberculous isolates using MALDI-TOF MS and to review different sample processing methods, available databases, and the interpretation of the results.

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Sources: Results from relevant studies on the use of the available MALDI-TOF MS instruments, the implementation of innovative sample processing methods, or the implementation of improved databases are discussed.
Content: Insight about the methodology required for reliable identification of non-tuberculous mycobacteria and its implementation in the microbiology laboratory routine is provided.
Implications: Microbiology laboratories where MALDI-TOF MS is available can benefit from its capacity to identify most clinically interesting non-tuberculous mycobacteria in a rapid, reliable, and inexpensive manner. **F. Alcaide, Clin Microbiol Infect 2018;24:599**

Background

The genus *Mycobacterium* comprises *Mycobacterium tuberculosis*, a major worldwide public health threat [1] and an increasing number of non-tuberculous mycobacteria (NTM) species which are commonly found in water and other environmental sources, [2–4]. Their common presence in man-made water systems results mainly from their co-occurrence with free-living amoebae that are used as a replicative niche and widespread reservoir [5]. Almost one-third of the 186 NTM species described today (http://www.bacterio.net/) have been associated with infections in humans, such as pulmonary infections, lymphadenitis in children, skin diseases, and disseminated infections in immunocompromised patients [6–9]. Since 2015, several cases of infections after cardiosurgical intervention in Western countries have been associated with *Mycobacterium chimaera* present in heater/cooler devices used for extracorporeal cardiopulmonary support [10,11].

As a consequence, the American Thoracic Society and Infectious Diseases Society of America (ATS/IDSA) recommend the assignment of species-level identification of NTM isolates from clinical specimens to establish clinical significance [12].

In the last decade we have witnessed how MALDI-TOF MS has become a reliable tool for NTM identification [13–24] because of (i) the development of several extraction methods that enhance the amount of bacterial proteins available for MALDI-TOF MS identification, and (ii) the increasing number of mycobacterial spectra in commercial databases. On the Bruker Daltonics platform (Bremen, Germany), the database version 3.0 has been a great advance (regarding its predecessor) and it included as many as 853 spectra from 149 different *Mycobacterium* species [21,23]. The latest released version (Mycobacteria Library v5.0) represents a further slight improvement with a total of 912 spectra from 159 *Mycobacterium* species. The NTM species still lacking in the most updated versions of the Mycobacteria Library (Bruker Daltonics) are listed in Table 1.

Similarly, the Saramis v4.12 RUO library (bioMérieux, Marcy-l'Étoile, France) contains 1286 spectra from 45 *Mycobacterium* species. Both systems use different algorithms for the identification of microbial protein spectra but have been shown to perform similarly for NTM identification [13,14]. Recently, the IVD 3.0 version (VITEK MS, bioMérieux, Marcy-l'Étoile, France) has also been released, providing robust identification of *Mycobacterium* isolates both from solid and liquid cultures [25].

As a result of these important improvements, MALDI-TOF MS technology has been implemented for routine identification of NTM in many laboratories and has been shown to perform similarly to conventional methods (DNA amplification-hybridization with species-specific probes) [13–24]. This technique has achieved up to 98% agreement with 16S rRNA, *hsp65* and *rpoB* genes sequencing.

It is important to remark that MALDI-TOF MS performance for species belonging to the *Mycobacterium tuberculosis* complex (MTBC) allows only complex-level identification.

Biosafety issues

The recommendation of global advisory bodies such as the Centers for Disease Control and Prevention (CDC) is to apply Biosafety Level 2 (BSL2) measures for the manipulation of NTM isolates (https://www.cdc.gov/biosafety/publications/bmbl5/bmbl5_sect_viii.pdf). However, BSL3 is recommended when the simultaneous presence of NTM and MTBC isolates in the same sample cannot be ruled out. Samples are to be handled in BSL3 during the first steps of the sample processing protocol, until they are heat-inactivated. Incubation of a small amount of bacteria in 70% ethanol has also been demonstrated as a safe way to render both MTB and NTM isolates nonviable [19].

Sample preparation

The main goal of the procedure is to break the cell wall using chemical and mechanical methods and to extract the bacterial proteins using formic acid and acetonitrile. Physical disruption of the cells is achieved using sonication, bead beating, or vortexing in the presence of silica beads (Fig. 1).

The protocol starts from a 1 μL loopful of bacterial biomass which is gently resuspended in 300 μL distilled water. Bacteria are heat-inactivated in a dry water bath at 95°C for 30 minutes and

Table 1
List of NTM species lacking in the most updated Mycobacteria databases (Mycobacteria Library 4.0 and 5.0) provided by Bruker Daltonics

NTM species not present in the Mycobacteria Library 4.0	NTM species not present in the Mycobacteria Library 5.0
M. alsense	*M. arcueilense*
M. angelicum	*M. bouchedurhonense*[a]
M. anyangense	*M. helvum*
M. arcueilense	*M. lutetiense*
M. bouchedurhonense[a]	*M. montmartrense*
M. helvum	*M. oryzae*
M. lutetiense	*M. paraintracellulare*
M. montmartrense	*M. paraterrae*
M. oryzae	*M. sarraceniae*
M. paraintracellulare	*M. timonense*[a]
M. paraterrae	*M. ulcerans*[b]
M. saopaulense	*M. yongonense*[c]
M. sarraceniae	
M. saskatchewanense	
M. timonense[a]	
M. ulcerans[b]	
M. yongonense[c]	

According to the Release Notes from these libraries.
[a] *M. bouchedurhonense* and *M. timonense* are members of the *M. avium* complex and show high confidence matches with *M. avium* reference entries.
[b] *M. ulcerans* has not been analysed at the time of publishing these libraries and it is therefore possible to obtain an *M. marium* match for *M. ulcerans* isolates. The photo-chromogenic behaviour of these species can be used for differentiation purposes.
[c] *M. yongonense* has been closely related to *M. intracellulare* and shows high-confidence matches with these reference entries.

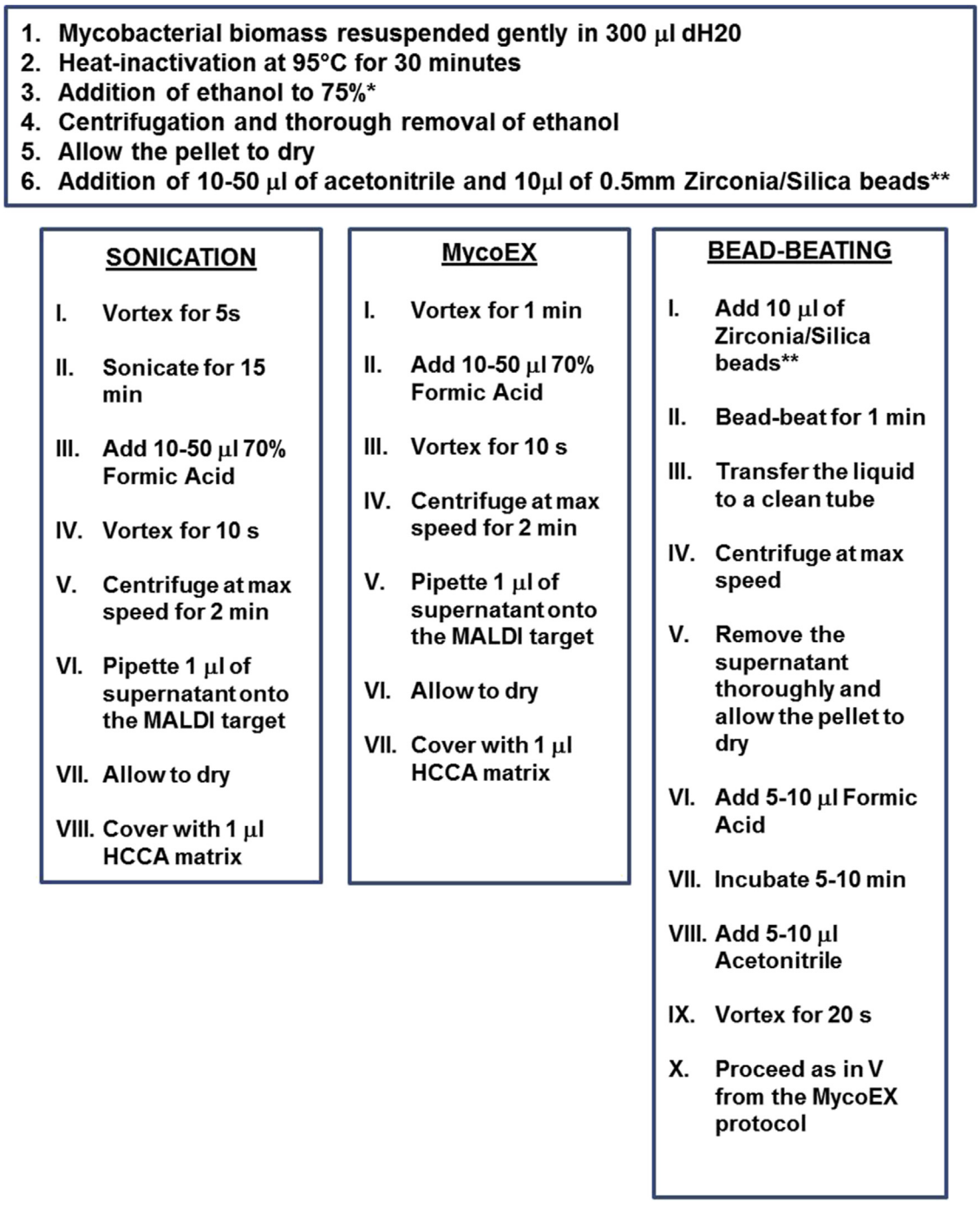

Fig. 1. Schematic NTM sample processing for MALDI-TOF MS identification. Modified from O'Connor et al., 2016 [22]. *The bead-beating protocol continues from this step. **Zirconia/silica can be replaced by glass beads.

submerged in ethanol to a final concentration of 75%. At this point, many research groups report minor variations for cell disruption and protein extraction (Fig. 1).

Sonication protocol

The protocol based on sonication continues by centrifuging the sample at maximal speed and removing ethanol thoroughly, including a second centrifugation step if required. The pellet must be then dried to evaporate the rest of the ethanol. Total removal of ethanol is important to obtain high-quality protein spectra. Acetonitrile and 0.5 mm zirconia/silica or glass beads are added (Biospec -www.biospec.com- and Thistle Scientific, Glasgow, UK) and the mix is vortexed for 5 seconds followed by a 15-minute sonication step in a simple water bath sonicator with only one sonication intensity (Mechanical Ultrasonic Cleaning Bath, Thermo Fisher, Waltham, USA or equivalent). Next, 70% formic acid is added at 1:1 vol/vol. Tubes are vortexed again for 10 seconds and centrifuged for 2 minutes at maximal speed. One microlitre of supernatant is spotted on the MALDI target microplate, allowed to dry and covered with 1µL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker Daltonics, Bremen, Germany) prepared following

the manufacturer's instructions. Once dried, the sample is ready to be analysed by MALDI-TOF MS [22].

Bead-beating protocol

Alternatively, the bead-beating protocol [24] may be used to improve the mechanical rupture of the bacterial cell walls. Practically, the bacteria are beaten with zirconia/silica beads suspended in 70% ethanol. Afterwards, the suspension is transferred to a clean tube where a protein extraction step with formic acid and acetonitrile is performed (Fig. 1).

MycoEX protocol

The procedures proposed by MALDI-TOF MS manufacturers are similar, with the MycoEX protocol [17] consisting of a 1-minute vortex in the presence of acetonitrile and zirconia/silica beads. For the Vitek MS system (bioMérieux), similar sample processing has been reported (suspension in ethanol followed by mechanical disruption using silica beads and a protein extraction step) [25,26]. Once the samples have been inactivated, the turnaround time for the sonication, bead-beating, and MycoEX protocol is 40, 45 and 30 minutes, respectively.

It is important to note that bioMérieux have standardized the pretreatment of *Mycobacterium/Nocardia* and moulds through reagent kits (bioMérieux). An interlaboratory evaluation of these sample preparation methods was recently performed and is described in the following section.

Preliminary studies that evaluated MALDI-TOF MS for NTM identification showed discrepancies according to the type of culture medium. Whereas the performances were reliable for NTM isolates cultured on solid medium, deficiencies in the method were observed for liquid medium culture [27,28]. Updated protocols for sample preparation from liquid medium describe taking 1-2 mL from the bottom of the tube, where the liquid medium is turbid and centrifuged at maximal speed in an Eppendorf tube. Where the biomass appears to be less than 10μL, a second aliquot of the mycobacterial biomass is extracted and centrifuged. The pellet is then processed as explained above for solid cultures.

These improvements in sample processing methods and in the availability of updated databases have allowed the identification of NTM with a similar level of confidence to that obtained from solid cultures [14,26,29]. These results support the feasibility of implementing the MALDI-TOF MS in the workflow for NTM identification in routine.

MALDI-TOF analysis

Several features of the *Mycobacterium* genus, such as the thickness of their cell wall, the lower number of ribosomes and ribosomal proteins compared with other bacteria, or their low growth rate have rendered these microorganisms particularly challenging for MALDI-TOF MS. Therefore, for the Bruker system the score value accepted for 'high confidence identification' has been established at ≥1.8 instead of ≥2.0 and the cut-off value for 'low confidence identification' at ≥1.6 instead of ≥1.7 [21]. For the Vitek MS system, the cut-off for reliable species-level identification has been established at >90%, although score values between 80% and 90% are accepted. Results between 60% and 80% are considered as 'low confidence' results and might be consistent only at genus level, while those below 60% are considered as 'not reliable' identifications [30].

For both systems, analysis of NTM isolates in duplicates or triplicates is recommended. When the results from the different spots are consistent and display a score ≥1.8 for the Bruker system or >85% with the Vitek MS instrument, they can be considered as 'high confidence' results and can be reliably transmitted to the clinicians. However, when the results from different spots are only consistent at the genus level, that is when the score value is <1.6 for the Bruker system or below 60% for the Vitek MS system, the results should be handled with caution, although a high correlation with DNA sequencing identification has been reported [14,17]. In such situations, the recommended way to proceed is to spot again the protein extraction from the NTM isolate in two to three positions and repeat MALDI-TOF MS analysis or inactivate a fresh loopful of biomass and repeat the whole procedure from scratch. Successful identification rates at the species level are reported to range between 80% and 98% in most studies [13–26]. Besides, the high quality of the peaks obtained allows the discrimination of closely related species. For this purpose, it is also important to mention that in-house libraries can be built with local and reference strains using a simple proceeding developed by the manufacturers. Thus, the number of isolates that require other molecular methods for a final identification is continuously decreasing.

Recently, a multicentre study has been carried out [31] for comparison of the three sample processing protocols described in this article. Results from 12 NTM isolates tested in 14 different laboratories showed that the three protocols yielded above 89% correct species level identifications with log(score) values above 2.0. The sonication protocol provided accurate species assignment of 98% of the analysed isolates and no misidentifications. It is worth mentioning that the misidentifications detected with the other protocols always involved closely related species and they would have not led to a different therapy for the patient.

Finally, a recent paper from Pranada et al. [32] has reported the differentiation of closely related NTM species such as *M. chimaera* and *Mycobacterium intracellulare* by detecting the presence of species-specific protein peaks. This shows the high capacity of MALDI-TOF MS for NTM species discrimination and its value to differentiate between invasive infection and environmental colonization, along with the clinical evaluation of the patient.

Despite all the above-mentioned advantages of MALDI-TOF MS, the (sub)species comprised within the MTBC currently cannot be differentiated and are only identified at complex level. Besides, further studies are required using clinical NTM isolates to assess the performance of MALDI-TOF MS in routine samples.

Quality control

Internal quality control for MALDI-TOF MS in general bacteriology is in part achieved using Bacterial Test Standard (BTS, Bruker Daltonics), consisting of an extract of *Escherichia coli* proteins for the calibration of the instrument. For the NTM, given their specific extraction procedure, a control strain to assess the performance of the extraction is important but has not been yet standardized. One option would be to use a strain also in use in quality controls of NTM susceptibility testing (*Mycobacterium peregrinum* ATCC 700686 and/or *Mycobacterium avium* ATCC 700898), as these strains are already present in many mycobacteriology reference laboratories. Otherwise, international agreement on, and exchange of, control strains might also be considered as an option.

External quality control, that is blinded interlaboratory exchange of well-identified isolates, is important to benchmark performance of individual laboratories using this technique. International organizations such as ESCMID/ESGMYC or NTM-NET (www.ntm-net.org) are good candidates to organize and coordinate external quality assurance.

Transparency declaration

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