Review Article

Application of MALDI-TOF mass spectrometry in clinical diagnostic microbiology

Elena De Carolis[1], Antonietta Vella[1], Luisa Vaccaro[1], Riccardo Torelli[1], Teresa Spanu[1], Barbara Fiori[1], Brunella Posteraro[2], Maurizio Sanguinetti[1]

[1] *Institute of Microbiology, Catholic University of the Sacred Hearth, Rome, Italy*
[2] *Institute of Hygiene, Catholic University of the Sacred Hearth, Rome, Italy*

Abstract

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has recently emerged as a powerful technique for identification of microorganisms, changing the workflow of well-established laboratories so that its impact on microbiological diagnostics has been unparalleled.

In comparison with conventional identification methods that rely on biochemical tests and require long incubation procedures, MALDI-TOF MS has the advantage of identifying bacteria and fungi directly from colonies grown on culture plates in a few minutes and with simple procedures.

Numerous studies on different systems available demonstrate the reliability and accuracy of the method, and new frontiers have been explored besides microbial species level identification, such as direct identification of pathogens from positive blood cultures, subtyping, and drug susceptibility detection.

Key words: MALDI-TOF MS; diagnostic microbiology; subtyping; antimicrobial resistance.

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Introduction

Mass spectrometry (MS) technology has been used for several decades in chemistry. In 1975, Anhalt and Fenselau suggested the use of this tool for bacterial characterization, as they observed that different and unique mass spectra were produced from bacterial extracts of different genera and species [1]. In the 1980s, desorption/ionization techniques (plasma desorption, laser desorption, and fast atom bombardment) that allow the generation of molecular biomarker ions from microorganisms were developed, opening the road for bacterial profiling [2,3]. The first experiments were based on a biomolecule ionization processes that allowed the generation of biomarker molecules of low molecular masses [4,5], mostly bacterial lipids. Only in the late 1980s, Tanaka and Fenn, thanks to the development of soft ionization techniques (matrix-assisted laser desorption/ionization [MALDI] and electrospray ionization), made possible the analysis of large biomolecules such as intact proteins [6,7]. For the first time, in 1996, MALDI-time of flight (TOF) spectral fingerprints could be obtained from whole bacterial cells by Holland *et al*., avoiding pretreatment before the MS analysis [8]. Nowadays, the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid, accurate, and sensitive tool for microbial characterization and identification of bacteria, fungi, and viruses [9], as demonstrated by the exponential number of publications about the issue.

This review provides an overview of the literature on the topic.

Technical description of MALDI-TOF MS

MALDI ionization is a soft ionization technique that allows ionization and vaporization of large nonvolatile biomolecules such as intact proteins [10]. It generates mostly single-charged ions ($z = 1$) so that the mass-to-charge ratio (m/z) of the analyte corresponds to its mass value.

Each MALDI-TOF mass spectrometer is composed of three principal units. The first is the ion source that makes ionization possible and transfers sample molecule ions into a gas phase. The second unit is the mass analyzer that allows ion separation

according to m/z. The last unit is a detection device for monitoring separated ions.

Samples are prepared by mixing the analyte with a matrix made of small acid molecules that possesses a strong optical absorption in the range of the wavelength used by the laser device; DHB 2,5-dihydroxybenzoic acid and CHCA α-cyano-4-hydroxycinnamic acid are optimal matrices for the detection of lower mass ions. After co-crystallization of the sample and matrix, the latter absorbs energy from the laser, leading to the desorption and ionization of the analytes in the gas phase. Ions are then accelerated through an electrostatic field (created by a potential of about 20 kV) into the high vacuum flight tube until they reach the detector, with smaller ions traveling faster than larger ones. Thus, the time of flight (TOF) required to reach the detector is dependent on the mass and charge of the bioanalyte, resulting in a spectral profile unique for a given species, composed of peaks ranging usually from 2 to 20 kDa.

Very conserved proteins with housekeeping functions comprise the MALDI-TOF spectra. Detected biomolecules correspond mostly to ribosomal proteins that are abundant, basic, and of medium hydrophobicity [11], all biochemical traits that favor efficient ionization. Among the proteins ionized during the MALDI process are structural proteins, DNA or RNA binding proteins [12,13], ribosome modulation factors, carbon storage regulators, cold-shock proteins, and translation initiation factors. These biomarkers generate spectral fingerprints that vary between microorganisms and have peaks specific to genus, species, and subspecies.

For microorganism identification, protein mass patterns are compared within a few minutes with commercially available reference databases (Bruker Daltonics for Microflex LT spectrometer and Vitek MS for bioMérieux spectrometer, the latest developed by Shimadzu (Shimadzu-Biotech Corp., Kyoto, Japan), which include species-specific fingerprints of several bacterial and yeast isolates. Through a pattern matching procedure, mass peaks in the experimental spectra are matched with reference spectra included in the database; through this comparison, a numerical value is generated that allows accurate and rapid identification of the microorganisms to species level when score values obtained are in the range of the threshold values defined by the instrument manufacturer (score values higher than 2.0 for Bruker Biotyper software and higher than 90% for VITEK-MS, respectively). The identification process is performed in real time, as soon as the sample is analyzed by the spectrometer [14,15].

Bacterial identification

Routine laboratory techniques for microorganism identification in clinical diagnosis are mainly based on biochemical tests, microscopy, interpretation of bacteria phenotypic characteristics or, alternatively, on molecular biology. These analyses require expertise and are time-consuming.

MALDI-TOF technology allows accurate bacterial identification of a large variety of species in reduced time, from between 24 and 48 hours to a few minutes, with a small amount of microbial biomass required for the analysis (10^4 to 10^6 CFU). Bacterial isolate colonies are picked from agar medium with a sterile tip and smeared in a thin film onto a ground steel MALDI target plate, overlaid with CHCA (for the Bruker instrument) or DHB (for the Vitek MS instrument), and introduced in the mass spectrometer for data acquisition. Spectra are compared with those present in the reference database, with a short time to results. The two systems produce comparable results, and overall the MALDI-TOF identification performance is significantly better (93.2%) than that of BD Phoenix (Becton Dickinson Diagnostic Systems, France) (75.6%) and Vitek-2 (bioMérieux, Marcy L'Etoile, France) (75.2%) [16]. Species with a low rate of differences in their ribosomal protein sequences such as *Shigella* spp., *Escherichia coli*, some strains of *Stenotrophomonas maltophilia*, *Propionibacterium acnes* or *Streptococcus pneumoniae*, and members of the *S. oralis/mitis* group can be misidentified by MALDI-TOF MS. To date, the accuracy of the identification depends greatly on the number of database entries; an update of the reference database is needed to improve the identification performance of MALDI-TOF.

Improving database entries with multiple spectra of well-characterized species will allow an identification rate close to 100% for *Neisseria*, *Clostridia*, *Mycobacteria*, *Salmonella*, viridans group streptococci, *Helicobacter pylori*, and *Campylobacter* [17]. Reliable identification and classification of cultured *Mycobacteria* can also be obtained by MALDI-TOF MS using an adequate database [18].

Yeast and fungi identification

As yeasts possess a thick cell wall, its disruption requires an additional extraction step compared to bacterial protocol, with some differences between the two available systems. Briefly, for the Bruker system,

colonies are picked and inoculated in 70% ethanol, the suspension is pelleted, dried, and resuspended in 70% formic acid and acetonitrile. After a centrifugation step, one microliter of supernatant is applied on the MALDI target plate and dried, covered with matrix, and analyzed. For the Vitek MS system, colonies are directly smeared onto the plate and lysed with 25% formic acid before matrix deposition.

Bruker and Vitek MS commercially available databases contain reference spectra of the yeasts most commonly isolated in the clinical laboratory, including several *Candida* spp. and *Cryptococcus neoformans* strains.

Many studies have demonstrated that MALDI-TOF MS successfully differentiates yeast isolates [19-21], with a similar species identification rate for Bruker and Shimadzu systems – 97.6% and 96.1%, respectively [22] – with results comparable with those of biochemical analysis (96.9%). MALDI-TOF MS has the ability to distinguish within the *C. glabrata* clade (*C. glabrata*, *C. nivariensis*, and *C. bracarensis*) and between members of the *psilosis* complex (*C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*) – whose identification currently depends on molecular methods as biochemical ones do not allow the separation of these species [23] – as well as between *C. albicans* and *C. dubliniensis*. For *Cryptococcus neoformans*, an unreliable identification rate has been reported in comparison with biochemical methods [24], probably due to the problematic extraction and solubilization of this pathogen's proteins caused by its polysaccharide capsule and the insufficient database entries. Implementation of database entries, however, assures good identification performance also for *C. neoformans* and *C. gattii* species [25].

Fungi are difficult to identify because of their biological complexity and because of the continuous classification changes due to the introduction of nucleic acid-based methodology able to detect cryptic species [26]. Filamentous fungi exhibit different phenotypes depending on their growth conditions, secondary metabolite production, and the portion of mycelium or conidia taken for the analysis, making their protein extraction difficult and peak reproducibility low. In view of these considerations, it is not surprising that relatively few studies have been conducted regarding filamentous fungi and very few reference spectra have been included in commercially available mass spectrometer databases.

However, some recent publications have demonstrated the applicability of the MALDI-TOF MS technology to the differentiation of clinical fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, and dermatophytes [27] using reference databases built in-house and performing analysis on accurately pre-treated samples to enhance the performance of the identification.

The samples to be analyzed are suspended in 75% ethanol solution, centrifuged, and suspended in formic acid and acetonitrile. The supernatant is centrifuged and spotted on the MALDI target plate. Moreover, quick extraction protocols on intact cells have been developed to reduce analysis time, applying the fungal suspension directly onto the target plate [28] and overcoming the problem of the age of the colonies, which reflects different protein composition, by an accurate database update with spectra of young and mature colonies [29].

Direct identification in samples

The identification of microorganisms directly from biological samples is the next challenge of this technique, as the early establishment of an appropriate antimicrobial therapy will enhance patient survival rates and reduce costs of clinical patient management.

Molecular assays, such as the probe-based test (PNA-FISH) and nucleic acid amplification-based tests (PCR), have been applied to the identification of pathogens directly from blood cultures, but these are limited to the detection of some specific targets and are expensive or difficult to manage [30].

Until now, MALDI-TOF performance based on differentiation of pathogens directly from positive blood cultures has been investigated in a number of works [31]; there have been much fewer studies using direct urine samples.

As blood samples present high concentrations of host proteins that may interfere with the detection of specific bacterial and fungal proteins, cell lysis protocol or differential centrifugation steps have been developed to obtain reliable levels of identification [32-35] aside from the use of a commercial kit called Sepsityper with a limit of detection of 5.9×10^5 CFU Bruker Daltonics (Bruker, Bremen, Germany) [36].

Rapid identification has been achieved with greater success for Gram-negative organisms than Gram-positive ones [37], and, overall, the identification rate showed variable results ranging from 75% to 95% for monomicrobial blood cultures. The difference in Gram-negative and Gram-positive scores is probably due to the different composition in the peptidoglycan layer of the Gram-positive bacterial cell wall; some authors have proposed to treat bacteria with ultrasound [38] to improve Gram-positive

identification results or to lower criteria adopted for acceptable scores at the species level [39].

For yeasts identification directly from blood culture bottles, results are concordant with those of the conventional culture-based method for 95.9% of *Candida albicans* and 86.5% of non-*albicans Candida* species [34].

MALDI-TOF MS has also been successfully used for direct identification of bacteria from primary urine specimens, although an initial flow cytometer-based screening is needed to eliminate negative urine samples and perform MALDI-TOF analysis on samples reporting a bacterial load > 10^5 CFU/mL only. The protocol consist of a slow centrifugation step to pellet out leukocytes, and a faster centrifugation step to collect bacteria, followed by a washing in distilled water prior to analysis of samples with a MALDI-TOF MS. Rates of identification are high, reaching 91.8% for bacterial load higher than 10^5 CFU/mL [40,41].

Detection of antimicrobial resistance

MALDI-TOF MS technology opened new frontiers in diagnostic microbiology and resistant microorganism detection.

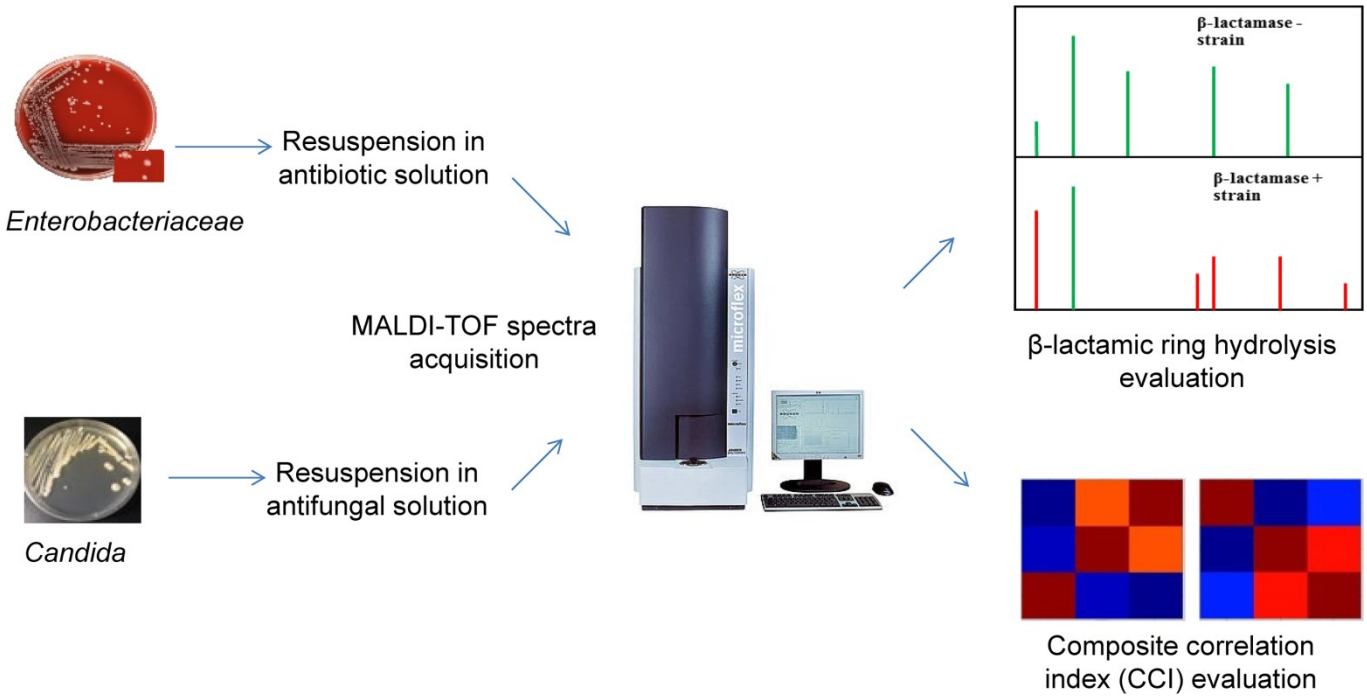
One of the more interesting topics is methicillin-resistant *Staphylococcus aureus* (MRSA). Although some studies showed the ability of mass spectrometry to distinguish between methicillin-susceptible and resistant *S. aureus* [42,43], conflicting data have been published on the issue [44,45].

Regarding the detection of β-lactamase production in *Enterobacteriaceae,* an interesting approach involves the detection of cleavage products produced by β-lactam rings hydrolysis. Hydrolysis is made by the bacterial enzymes eventually present and after an incubation (from one to three hours) of the bacteria with the antibiotic to be tested. A shift in the mass of the antibiotic compound is produced, which can be detected by the MALDI-TOF mass spectrometer, confirming the presence of a β-lactamase (Figure 1) [46-48].

Regarding carbapenemases, MALDI-TOF MS was found to be suitable for differentiating *B. fragilis* strains, which harbor the *cfi*A gene, from those that do not [49].

Regarding yeasts changes in the proteome profile have been detected by MALDI-TOF MS after *C. albicans* exposure to different antifungal fluconazole drug concentrations (corresponding to its fluconazole MIC, measured with conventional

Figure 1. Schematic representation of antimicrobial resistance detection workflow by MALDI-TOF MS approach for *Enterobacteriaceae* and *Candida*. In the upper part of the figure, a mass shift in the spectra profile of the antibiotic tested reveals the presence of carbapenemases in the β-lactamase-positive strain. In the lower part of the figure, two possible matrices obtained from *Candida* susceptible and resistant strains by composite correlation index analysis of the spectra profiles acquired from isolates exposed at high concentration, no drug and breakpoint test concentration of antifungals, are shown.



methods) [50], so that this technology is promising for future application, especially for the detection of emerging resistant pathogenic fungi. Moreover, comparison of spectra profiles of *Candida* and *Aspergillus* strains exposed to serial dilution of caspofungin by composite correlation index analysis allowed detection of fungal isolates with reduced caspofungin susceptibility, leading to a correct categorization of FKS mutant and wild-type isolates of *C. albicans* after an incubation time of only three hours, thus confirming the reliability of MALDI-TOF MS for antifungal susceptibility testing (Figure 1) [51,52].

Subtyping

Differentiation of isolates below the species level is important for epidemiological analysis. Most subtyping methods are based on the use of a restriction enzyme (ribotyping, PFGE, AFLP) or a PCR-based technique (RAPD, REP and ERIC elements-based PCR, MLST); all these techniques possess different discriminatory powers, and their use depends on the final objective to be achieved.

Overall, these applications are highly expensive, time-consuming, and sometimes present a low level of reproducibility.

Differences between protein spectra can be used for individual strain typing by MALDI-TOF MS, as reported in recent studies on *Salmonellae* [12], *Francisella tularensis* [53], *Bacterioides fragilis* [49], *Streptococcus agalactiae* [54], *Acinetobacter baumannii* [55,56], *Yersinia enterocolitica* [57], *Staphylococci* [58], and *Cryptococcus* [25]. Each spectrum is compared with another through a matrix of cross-wise identification values that are used to calculate the distance values for each spectra pair. On the basis of these values, a mass spectrometry-based dendrogram is generated, in which closely related strains can be separated hierarchically according to their mass signals and intensities.

Because of its speed, ease of use, and low costs, MALDI-TOF MS potential in microorganism typing has to be further explored to determine the level of its discriminatory power.

Concluding remarks

Recently, the availability of simple-to-use MALDI-TOF MS devices in clinical microbiology laboratories has changed routine identification workflows of pathogenic bacteria, yeasts, and, to a lesser extent, fungi. Although all prospective studies show that MALDI-TOF MS correctly identifies the great majority of isolates processed routinely, discrepancies between MALDI-TOF MS-based identification results and biochemical or molecular tests have been observed for the identification of *Shigella* species, pneumococci, and viridans streptococci. To increase MALDI-TOF MS power, database upgrades and sample enrichment are important. For yeasts, an extraction step is mandatory on the day prior to analysis. Because of the low consumable costs and the speed of MALDI-TOF MS, the technique can improve laboratory performance for clinical sample management; otherwise, the high cost of the equipment makes mass spectrometry suitable only for big hospitals. Further prospective studies are warranted to increase MALDI-TOF MS reliability to directly identify pathogens in biological fluids, such as urine samples and blood cultures. However, this technique has the potential to identify clinical samples at the serotype or strain level, and is a good candidate to open the road for antibiotic resistance profiling within minutes in the years to come.

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Corresponding author
Prof. Maurizio Sanguinetti
Institute of Microbiology, Università Cattolica del Sacro Cuore
Largo F. Vito 1, 00168 Rome, Italy
Phone: +39-630154218
Fax: +39-63051152
Email: msanguinetti@rm.unicatt.it

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