MALDI-TOF mass spectrometry technology for detecting biomarkers of antimicrobial resistance: current achievements and future perspectives

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Abstract: The laboratory diagnosis of infections is based on pathogen identification and antimicrobial susceptibility determination. The gold standard of cultivation, isolation and susceptibility testing is a time-consuming procedure and in some cases this can be threatening for patients' outcome. In the current review the applications of mass spectrometry in pathogen identification and especially in detecting biomarkers of antimicrobial resistance are analyzed. MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry is a new technology that has revolutionized pathogen identification and has also proven to accelerate detection of antimicrobial resistance compared to the traditional antibiotic susceptibility tests (AST) as well as DNA amplification methodologies. The technology has incorporated up to know four different methodologies: (I) the detection of differences of mass spectra of susceptible and resistant isolates of a given microorganism using the classical strain typing methodology; (II) the analysis of bacterial induced hydrolysis of β-lactam antibiotics; (III) the detection of stable (non-radioactive) isotope-labeled amino acids; and (IV) the analysis of bacterial growth in the presence and absence of antibiotics using an internal standard. The implementation of MALDI-TOF methodologies has improved detection of resistance in aerobic, Gram-positive and Gram-negative bacteria, mycobacteria, anaerobic bacteria, fungi and viruses. The MALDI-TOF is an easy to use, rapid, reliable, economical, and environmentally friendly methodology. However, this technology needs further development of research protocols that will be validated for routine application.

Keywords: Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF); mass spectrometry; resistance

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Introduction

The laboratory diagnostic management of patients with suspected infection includes the identification of the pathogen and the determination of the antimicrobial susceptibility. A common denominator in this management is the cultivation of the microorganisms which requires 36–48 hours. It is understood that the concept of "laboratory time" is a particular factor that becomes even more important, considering the most important problem in modern healthcare such as the increasing rates

of microorganisms' antibiotic-resistant (1). In addition, the management of septic patients, which requires the immediate launch of empirical treatment with broad spectrum antimicrobial agents, is shaped by the early identification of the pathogen which leads to more accurate and targeted treatment. It is understood that delays in final identification and appropriate antimicrobial therapy may affect survival of patients (2-4). The gold standard in the diagnosis of an infectious disease is the isolation of the microorganism and subsequently determination of its susceptibility, whenever possible, by detecting its growth or not in the presence of specific antibiotics.

The cultivation and isolation of bacterial or fungal agents requires 18–24 hours, and an additional 18–24 hours are required to identify them at genus and species level. This process may exceed 48 hours in the case of slow-growing or fastidious microorganisms or in cases where their susceptibility to antibiotics is not clear (5,6). Despite the high specificity and sensitivity of commonly used, standard methods and the fact that resistance can be detective irrespective of the mechanism, these methods need much time to produce results and in some cases this can be threatening for the patients.

In addition, the spread of broad antimicrobial resistances has become a serious public health problem that negatively affects the finding of appropriate antibiotic treatment. Antimicrobial stewardship strategies are the key to tackling increasing resistance. But the proper antibiotic treatment and the de-escalation of an initially applied broad-spectrum therapy require fast and reliable information about resistance status of the respective pathogen (7,8).

The introduction of molecular methods into the routine workflow of a clinical laboratory significantly reduced required time for microbiological results. However, most of these molecular methods rely on culture amplification as a precursor to diagnosis, are complicated, expensive, and require well-trained laboratory personnel for correct interpretation of results (9-13). The latest breakthrough in identification of pathogens and determination of susceptibility are the techniques of mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is an easy to use, rapid, reliable, economical, and environmentally friendly methodology that has revolutionized pathogen identification and detection of antimicrobial susceptibility/resistance in clinical settings (14).

What is MALDI-TOF technology?

MALDI-TOF is a mass spectrometry method that has been successfully introduced in clinical laboratories initially as an identification tool about ten years ago. Since then, MALDI-TOF has been widely used in routine laboratory practice due to its rapid (in about 2 min) and accurate identification of pathogens, especially bacteria and fungi, as well as its economical benefits. Although the primary cost of the platform is rather high, the cost of identifying one species is really low compared to any other identification technologies exist right now, especially compared to other molecular techniques, such as PCR or genome sequencing (15).

MALDI-TOF mass spectrometry as an analytical technique is based on the cellular proteome, which reflects gene products and metabolic products of the organisms. Especially, this method is based on the analysis of highly abundant, mainly ribosomal, proteins of microorganisms in the mass range of 2,000 to 20,000 Daltons (16). These proteins are ionized into charged molecules, by either addition or loss of one or more than one protons, in order to measure the mass to charge (m/z) ratio. An energy absorbent solution called "matrix" is mixed with the sample for analysis. When the matrix crystallizes on drying, the sample entrapped within it also co-crystallizes. A laser beam then ionizes the sample, generating singly protonated ions. These ions are then accelerated at a fixed potential and they separate from each other on the basis of their m/z ratio, which is measured by determining the time required for each ion to travel the length of the flight tube [time of flight (TOF)]. Based on the TOF information, a characteristic mass spectrum called "Peptide Mass Fingerprint" (PMF) is generated. This PMF, with peaks specific to genera and species unique to individual types of microorganisms, is then compared to a database. The PMF of unknown microbial isolates is compared with those of known microbial isolates contained in the database and thus the unknown organism is identified at the family, genus, and species level (1,10,16,17).

Different types of specimens are already used or can be used in the future for bacterial and fungal identification. The most usual type of specimen is bacterial colonies from fresh cultures. New protocols are published from time to time in an attempt to enhance the identification capability of MALDI-TOF. The ultimate goal, in any case, is to extract the maximum of the capabilities of the technique, which, with the implementation of some extra steps prior to identification, could provide data not only at the genus or species level, but also at the serogroup level or even at the targeting of a single protein (18). Recently, clinical samples such as blood, urine and cerebrospinal fluid (CNF) can be used for direct detection and identification of pathogens (19-24).

Application of MALDI-TOF in microorganisms' identification

The research of mass spectrometry use in bacterial species identification is dated of 70's. At the first years of MALDI-TOF technology only low molecular mass molecules were analyzed. Until the end of the century, the technic was evolved into the analysis of larger molecules and the mass spectral proteins (fingerprints) (25,26). The advantage of the modern technic is the small number of cells as a small part of the colony is necessary to be smeared on the target plate. In daily practice a solution of alpha-cyano-4-hydroxycinnamic acid (CHCA) in a mixture of organic solvents and water is used as the matrix. The spotted mixture is air-dried and then inserted into the mass spectrometer for automated measurement. The mass spectral signature is composed of peaks ranging from 1,000 to 30,000 m/z. The technique of MALDI-TOF produces singly charged ions. The measurement of the mass to charge ratio is compared with a data base of known spectra. The mass spectra obtained in the analysis of a microorganism are primarily assigned to ribosomal proteins. In addition to ribosomal proteins, nucleic acid-binding proteins and cold shock proteins were assigned (27-29). The bacterial identification is based upon the spectral fingerprints, the molecular masses (specified to the genus, species and subspecies), and the created spectra of proteins during the bacterial growth (30,31).

The microorganism identification by MADLI-TOF is based on four commercial systems and their databases: (I) the MALDI Biotyper (Bruker Daltonics, Bremen, Germany); (II) the Spectral ARchive And Microbial Identification System (SARAMIS™) (AnagnosTec, Potsdam, Germany); (III) the Andromas (Andromas, Paris, France) and (IV) the Vitek MS (bioMérieux, Marcy l'Etoile, France) (32,33). Each of the systems includes a MALDI-TOF instrument from either Bruker Daltonics or Shimadzu, and the most installed in routine laboratories systems are the MALDI Biotyper and the Vitek MS, which are the FDA-cleared platforms (34). The systems differ in databases, identification algorithms, and instrumentation (16).

In daily laboratory practice MALDI-TOF is used for bacterial or fungal identification from colonies grown on solid media. Up to now the procedure has been used for the identification of Gram-negative and positive rods, Gram-positive cocci, fastidious organisms, like mycobacteria, Nocardia and other actinomycetes, anaerobic bacteria, yeasts and filamentous fungi (10,35-37). The two systems have been compared in several research studies. It is seems the performance of the MALDI Biotyper and Vitek MS systems is similar. According to the data the identification rates of genus are extremely high (97–99%) and varies from 85% to 97% at the level of species (38-43).

It is understood that the main role in the performance of the systems is the completeness of the databases. The cases of problematic identifications at the early stages of MALDI evolution was the result of incomplete databases. The majority of the problematic identification cases involved the viridans streptococci group, pneumococci, anaerobic bacteria and the discrimination between the closely related members of Enterobacteriaceae family, *E. coli* and *Shigella* (10,44,45).

MALDI-TOF, antimicrobial susceptibility and antibiotic resistance

According to the international guidelines of medical boards, committees and scientific societies, the susceptibility testing must be performed in cases of serious life-threatening infections, relapses of infections, long term antibiotic therapies, and known resistance of microorganism against certain antibiotics or epidemiological surveillance (46-52). Routine antibiotic susceptibility tests (AST) are based on bacterial growth in the presence of various antimicrobial compounds. Such methods are: broth or agar dilution methods, gradient diffusion tests, like E-test (bioMérieux), disc diffusion methods, and automated or semi-automated approaches with the use of specific instruments, like Phoenix (BD Diagnostic Systems, Heidelberg, Germany), MicroScan (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) and Vitek 2 (bioMérieux) test systems. The above phenotypic ASTs require 18–24 h before resistance information is available and even the automated systems require at least 5h to report the first results on distinct antibiotics for fast growing pathogens. This is a disadvantage for timely targeted therapeutic intervention and application of infection control measures (53).

On the other hand DNA amplification methods are more rapid than the commonly used susceptibility testing methods, but they are based on the detection or absence of specific resistance genes, which does not always correlate with the phenotype. Moreover, molecular methods are available for only a few antibiotic resistances and novel resistance mechanisms may escape attention (54).

MALDI-TOF seems to have a value impact in the field of the antibiotic resistance by closing the gap between the

availability of species identification on the one, and the resistance status on the other hand. MALDI-TOF, similar to species-specific identification, significantly accelerates the detection of resistance compared to the commonly used AST methods using up to now four different methodologies (55,56):

(I) Analysis of MALDI-TOF mass spectrum of a given microorganism to detect a characteristic "resistance peak pattern". The principle of this procedure is to identify characteristic differences in the MALDI-TOF mass spectra of susceptible and resistant isolates of a given microorganism using the classical strain typing methodology;

(II) Analysis of bacterially induced hydrolysis of β-lactam antibiotics (MALDI Biotyper-Selective Testing of Antibiotic Resistance-Beta-Lactamase Assay, MBT-STAR-BL Assay). Hydrolysis is detected by the observation of specific mass shifts after a 30–180-minute incubation period of the pathogen with the tested β-lactam antibiotic (57-59);

(III) Detection of stable (non-radioactive) isotope-labeled amino acids, which have been incorporated into newly-synthesized bacterial proteins (MALDI Biotyper-Resistance Test with Stable Isotopes Assay, MBT-RESIST Assay). The amount of incorporated isotopically labeled amino acids into newly synthesized proteins in the presence of antibiotic is used to determine if a strain is susceptible or resistant (60);

(IV) Analysis of bacterial growth in the presence and in the absence of antibiotics using an internal standard (MALDI Biotyper-Antibiotic Susceptibility Test Rapid Assay, MBT-ASTRA). The tested bacteria are incubated for an adequate short incubation time that is species and antibiotic-dependent. Subsequently, bacterial cells are lysed according to the standard protocol used for the identification workflow and the acquired mass spectra are compared with an internal standard: lack of growth in the presence of an antibiotic (susceptibility) results in low peak intensities of bacterial biomass, normal growth (resistance) leads to intense bacterial protein peaks (61).

The first of the above methodologies is characterized as "MALDI-TOF equivalent to genotypic analyses". The rest, most recent ones [i.e., number (II), (III), (IV)] are defined as "MALDI-TOF equivalent to conventional, biochemical resistance tests" and their evaluation was made in the most commonly used Bruker platform (*Table 1*) (55). In contrast, relevant studies using the Vitek MS instrument are up to now very limited and focused on "analysis of bacterially induced hydrolysis of β-lactam antibiotics" and especially of the carbapenem hydrolysis by carbapenemase-producing *Enterobacteriaceae* (86,87).

Representative examples of the application of the all mentioned MALDI-TOF methodologies for the detection of resistance in aerobic, Gram-positive and -negative bacteria, mycobacteria, anaerobic bacteria, fungi and viruses will be mentioned below.

Aerobic Gram-positive bacteria

Staphylococcus aureus

At the dawn of the new century, the first evaluation of MALDI-TOF in determination of antibiotic resistance was the case of methicillin resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) (62). The rapid and accurate differentiation between MRSA and MSSA gives a therapeutic advantage in the selection of the appropriate antibiotic and the outcome of the patient. It seems that in MALDI-TOF the depiction of certain spectral peaks are unique and characteristic for MRSA and MSSA. Further studies provide us the data that the selection of the appropriate culture media in MALDI-TOF can improve the rapid determination of MRSA (63). Also, the mass spectra differences of MRSA isolates could be provide a discrimination of clonal complexes (88). Although the spectra are indicative for the isolates of MRSA, the reliability of MALDI-TOF in the sub-differentiation and typing of *S. aureus* in the level of distinct clones or clonal complexes is under investigation (89).

Moreover, the MBT-RESIST assay was performed by Sparbier *et al.* for the detection of MRSA using oxacillin and cefoxitin as antibiotics (60). In the presence of these antibiotics, only resistant strains (MRSA) are able to grow and to perform protein biosynthesis, while susceptible strains (MSSA) stop growing under conditions of antibiotic stress and thereby present different profile spectra compared to the setups without antibiotics. According to the results by Sparbier *et al.*, both antibiotics led to similar classifications of the strains tested (60).

In 2017 the MBT-ASTRA method was performed in strains of *S. aureus* and ciprofloxacin, oxacillin, cefepime and vancomycin with an overall accuracy rate of 95%. The same protocol was applied in spiked with *S. aureus* blood cultures and it was resulted in determination of species and

Table 1 MALDI-TOF-based resistance methodologies for routine laboratory use

Name	Principle of the method	Pathogens	Antimicrobial targets	References
Bacteria				
MALDI-TOF equivalent to genotypic analyses				
Specific resistant pattern	Analysis of MALDI-TOF mass spectrum of a given microorganism to detect a characteristic "resistance peak pattern"	Methicillin resistant S. aureus (MRSA) Methicillin-sensitive S. *aureus* (MSSA)	All antibiotics	(62-71)
		Vancomycin resistant *Enterococcus faecium*		
		Porins alteration in *Klebsiella*		
		Carbapenem resistant *Bacteroides fragilis*		
MALDI-TOF equivalent to conventional, biochemical resistance tests				
MALDI Biotyper-Selective Testing of Antibiotic Resistance-Beta-Lactamase Assay (MBT-STAR-BL Assay)	Analysis of bacterially induced hydrolysis of β-lactam antibiotics	*E. coli*	β-lactam antibiotics (ampicillin, piperacillin, cefotaxime, ceftazidime, ertapenem, imipenem, meropenem)	(55,57,72-76)
		K. pneumoniae		
		P. aeruginosa		
		A. baumannii		
		E. cloacae		
MALDI Biotyper-Resistance Test with Stable Isotopes Assay (MBT-RESIST Assay)	Detection of stable (non-radioactive) isotope-labeled amino acids, which have been incorporated into newly-synthesized bacterial proteins	Methicillin-resistant S. *aureus* (MRSA)	All antibiotics, but most suitable for protein synthesis blocking antibiotics (oxacillin, cefoxitin, ciprofloxacin, meropenem, tobramycin)	(55,60)
		P. aeruginosa		
MALDI Biotyper-Antibiotic Susceptibility Test Rapid Assay (MBT-ASTRA)	Analysis of bacterial growth in the presence and in the absence of antibiotics using an internal standard	*E. coli, K. pneumoniae*	All antibiotics: i.e., cefotaxime, meropenem, tobramycin	(56,61,77-80)
		P. aeruginosa, A. baumannii	*M. tuberculosis*: rifampin, isoniazid, linezolid, ethambutol. NTM: clarithromycin, rifabutin	
		S. aureus	Clindamycin, meropenem, metronidazole	
		Mycobacteria: M. tuberculosis, nontuberculous Mycobacteria (NTM)		
		Anaerobic bacteria (*Bacteroides fragilis*)		
Fungi				
MALDI-TOF Antifungal Susceptibility Testing	Exposure of the tested fungus in different dilutions of specific antifungal agent for several hours. Mass-spectrum profile change evaluation	*C. albicans, C. glabrata, C. tropicalis*	All antifungals: i.e., caspofungin, fluconazole, voriconazole, posaconazole	(81-85)
		A. fumigatus, A. flavus		

susceptibility, within three hours after the blood culture flagged positive (77).

Vancomycin-resistant enterococci

Another health care issue is the restriction of vancomycin-resistant enterococci (VRE). The laboratory techniques of detection are time-consuming, the phenotypic and the molecular methods are costly. The resistance to vancomycin and teicoplanin is mediated by proteins coded by genes sush as vanA and vanB. Organisms with vanA gene have resistance to both vancomycin and teicoplanin, whereas vanB positive organisms are resistant to vancomycin only (49). According to recent data, in cases of vanB-positive Enterococcus faecium, MALDI-TOF is capable to differentiate vancomycin resistant enterococci from vancomycin susceptible based on the presence or absence of some specific peaks. The results demonstrated a sensitivity of 96.7% and a specificity of 98.1% compared to the control method (PCR) (64,65). In 2015 was tested the vancomycin susceptible and vancomycin resistant strains of enterococci and he resistant genes were detected by gene sequencing. The VRE method resulted in a sensitivity of 80%, a specificity of 90% proving that MALDI-TOF is a quite accurate tool for clinical practice, against VRE pathogens (66).

Aerobic Gram-negative bacteria

Detection of β-lactamase activity

The group of β-lactam antibiotics includes different antibiotics such as penicillin derivatives, cephalosporins, monobactams and carbapenems. They all share the β-lactam ring in their structure and the most common mechanism of resistance is the expression of β-lactamase which destroys the β-lactam ring and makes the antibiotic inactive. In some cases MALDI-TOF was effective in detecting the susceptibility against β-lactams, by detecting the hydrolyzed antibiotic that remains after the β-lactamase activity (55). The applicability of this methodology has been demonstrated for different Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacter cloacae*) and different β-lactam antibiotics (ampicillin, piperacillin, cefotaxime, ceftazidime, ertapenem, imipenem, and meropenem).

The detection of resistance against carbapenems using MALDI-TOF is one the most studied topics right now. Hrabák *et al.* described an innovative method that detects degradation products of carbapenems from the activity of carbapenem resistant pathogens (57). They used cultures of enterobacteria and *Pseudomonas aeruginosa*. The positive cultures were mixed with solution of different concentrations of meropenem for three hours and then the analysis by MALDI-TOF followed. They tested 124 strains. Thirty of them produced carbapenemases, 55 were resistant and thirty nine susceptible to carbapenems. A comparison of the different peaks produced by the MALDI-TOF assay was performed using specialized software. They managed to distinct the carbapenemase producing bacteria from those that did not. No false positives or false negatives results came up from the Enterobacteriaceae. There were two false positive results from the 25 *P. aeruginosa* strains that did not produce carbapenemases though. These results could be explained by the presence of other resistance mechanisms. Their method had a sensitivity of 96.67% and a specificity of 97.87%.

In 2012 Hrabák *et al.* published a modified assay (72). Meropenem degradation products seemed to be problematic in some cases, probably because the molecules bind in cell lysate components. The modification they applied was successful as they managed to detect carbapenemase activity in all carbapenemase positive isolates they used and there were neither false negative nor false positive results.

Another modified technique was developed by Vogne *et al.* and their target was to identify the degradation of ertapenem (73). Their approach produced results in about 1 hour and 15 minutes and had 100% sensitivity and 100% specificity on the strains they used.

Recently, a phenotypic method which allows rapid susceptibility testing, independently of resistance mechanism, was proposed (78). In this study 24 isolates of *Klebsiella pneumoniae* and 24 isolates of *P. aeruginosa* were used. Twelve isolates of each pathogen were susceptible to meropenem and 12 were resistant. Bacterial suspension for each isolate was added to the same volume of broth containing meropenem and the microdroplets produced were test by MALDI-TOF. They tested several microdroplets volumes to determine the suitable one, along with several hours of incubation. With their method, they managed to correctly categorize all isolates, after 18 hours of incubation. Moreover, for *K. pneumoniae* specificity and sensitivity reached 100% for all valid tests after only 4 hours of incubation, whereas for *P. aeruginosa* specificity and sensitivity of 100% was achieved after 5 hours for 83.3% of valid tests.

Interestingly, a well-organized study for rapid detection

of β-lactam resistance in Enterobacteriaceae was performed directly in positive blood cultures using MALDI-TOF assay. Every blood culture that developed Gram-negative roads was then processed with MALDI-TOF for identification. The products of the blood cultures were incubated with antibiotic solution (cefotaxime based or ampicillin based) and then the processed product was analyzed spectrophotometric (74). At the same time conventional antibiograms were used as controls for products of the same blood cultures. All culture flasks containing *E. coli* were tested for ampicillin susceptibility and those which contained Enterobacteriaceae were tested for cefotaxime susceptibility. No false-negative results were obtained which is very important for patient safety, since the sensitivity of the test is more important than specificity. It seems that the effectiveness of β-lactam therapy was available only after 2 to 2.5 hours after the cultures were flagged positive. They managed to detect resistance against antibiotics of this group 24 to 48 hours earlier than the other conventional methods.

Another study for the rapid detection of carbapenemase-producing *K. pneumoniae* from blood cultures by MALDI-TOF was lately performed be Sakarikou *et al.* (75). They showed once more that MALDI-TOF is capable to detect the hydrolysis of β-lactams after incubation for a short time of the Gram-negatives isolates with the antibiotic, directly from positive blood cultures. Strains of *K. pneumoniae* from blood cultures were tested using MALDI-TOF. For the detection of carbapenemase production they collected data every 30 minutes. The identification of the strains that produced carbapenemases were easily detected compared to strains that did not produce it. Even from the first 30 minutes of incubation there were some positive results and after only 3 hours the detection was completed for all carbapenemase producing strains.

A highly sensitive method was developed for detection of Enterobacteriaceae producing extended spectrum beta-lactamases directly from positive blood cultures using MALDI-TOF by Oviaño *et al.* In this study, β-lactamase activity was determined through the profile of peaks associated with the antibiotics cefotaxime and ceftazidime and its hydrolyzed forms. Clavulanic acid was added to rule out the presence of non-ESBL mechanisms. The overall sensitivity was determined at 99% and results were obtained in a mean time of 90 min (76).

Detection of rRNA methyltransferase

The rRNA methylation is the main cause of resistance in antibiotics that target protein synthesis of the pathogens. Antibiotics of this group are macrolides, streptogramins, chloramphenicol, tetracyclines and aminoglycosides. Douthwaite *et al.* published one of the first protocols of studying rRNA methylation using MALDI-TOF mass spectrometry (67). At first they isolated the group of methyltransferase enzymes, then proceeded in the transcription of RNA methylation substrate and the methylation of RNA by the enzymes. After this they performed analysis by MALDI-TOF to detect the methylation of the molecule (which increases the mass by 14 Da). Great limitation of the method was the need to use purified ribosomes and enzymes.

In 2016, Stojković *et al.* used a similar method to test a group of methyltransferase enzymes. First, they isolated rRNA from two different strains of *E. coli*, then they incubated it with specific enzymes in order to methylate, and finally they lyophilized the mixture and analyzed it with MALDI-TOF (68). Until now there is not a standard protocol for detection of rRNA methylation resistance against antibiotics, but the work of many researchers makes the future promising.

Porin detection

Another rather common mechanism which leads to resistance against antibiotics is the differentiation in the structure of porins. Cai *et al.* managed to detect porins alteration in *Klebsiella* spp. using MALDI-TOF method (69). They used 5 carbapenem susceptible isolates of *K. pneumoniae* and 7 carbapenem resistant in their study. They extracted and analyzed the proteins of the outer membrane. They managed to show the loss of specific porins in the resistant strains as a result of MALDI-TOF analysis. In 2015 Hu *et al.* performed a similar essay, using *E. coli* and *K. pneumoniae* strains (90). One *E. coli* was carbapenem susceptible, eight were resistant or with reduced susceptibility against carbapenem, one strain of *K. pneumoniae* was carbapenem susceptible and six similarly, were resistant or with reduced susceptibility. The proteins of the outer membrane were extracted and analyzed with SDS-PAGE technique and MALDI-TOF. The essay showed that the latter method was able to detect porin alterations rapidly, with better sensitivity and lower cost.

Resistance to different antibiotic classes

It is possible the detection of resistance to different antibiotic classes (i.e., penicillin, cephalosporins, carbapenems, fluoroquinolones, aminoglycosides) in different Gram-

negative bacteria species with the performance of MBT-ASTRA method, evaluated by Sparbier *et al.* (56). The studied combinations of species/antibiotics were: *E. coli*/cefotaxime, piperacillin, ciprofloxacin; *P. aeruginosa*/ceftazidime, meropenem, tobramycin; *K. pneumoniae*/tobramycin; and *A. baumannii*/tobramycin. Optimal species-specific incubation times and species-specific breakpoint concentrations were established, thus resulting in a protocol that generated reliable resistance/susceptibility information for all the species/antibiotic combinations, except two that need further optimization: *E. coli*/piperacillin and *P. aeruginosa*/ceftazidime. But, despite rapid, valuable results, thorough validation is necessary for the application of this MALDI-TOF MS-based assay in routine AST use (56). The main disadvantage of the MBT-ASTRA assay is that the concentration of antibiotic used and the incubation time must be optimized for each species and antibiotic combination (56).

Mycobacteria

Identification and drug susceptibility testing (DST) of mycobacteria are time consuming and traditionally reserved for reference laboratories. Recently MALDI-TOF assay has started to facilitate routine mycobacterial identifications in clinical laboratories. In 2017 a promising study performed MBT-ASTRA methodology in randomly selected clinical *Mycobacterium tuberculosis* and nontuberculous mycobacterial (NTM) strains. Drug susceptibility was tested for rifampin, isoniazid, linezolid, and ethambutol in *M. tuberculosis*, and clarithromycin and rifabutin in NTM. By semi-quantitatively measuring the alteration in bacterial biomass caused by the addition of antibiotics, MBT-ASTRA methodology correctly detected 156/156 *M. tuberculosis* and 65/66 NTM drug resistance profiles. Unfortunately, turnaround times were not significantly different in *M. tuberculosis*, but results for resistance in NTM were delivered a week faster than routine susceptibility tests. However, further validations are needed before routine implementation can be envisioned (79).

Anaerobic bacteria

As it is well known, the susceptibility testing of anaerobic bacteria is not only time-consuming, but also requires experienced laboratory staff. Different experimental studies were conducted to test the potential future applications of MALDI-TOF in the detection of various resistance determinants in anaerobes. In these studies, *Bacteroides fragilis* was predominantly used as a test pathogen for the detection of carbapenem resistance, because this resistance is one of the most worrying trends, mainly from standard laboratory strains and later from clinical samples, such as blood cultures (46,91,92). Nagy at al. showed that MALDI-TOF is a suitable tool for differentiating *B. fragilis* strains which harbour the cfiA gene, the gene encodes the unique carbapenemases found in *Bacteroides* strains. More in details, *B. fragilis* cfiA-positive strains were differentiated from cfiA-negative ones according to a set of peak shifts in the MALDI-TOF mass spectra (70). Recently MALDI-TOF ASTRA methodology was used for the detection of resistance of two reference *B. fragilis* strains in clindamycin, meropenem and metronidazole with promising results (80).

Fungi

The identification of fungi using the MALDI-TOF technique hasn't progressed as fast as the identification of bacteria. Fungi are more complex organisms, they may have different phenotypes at different times (conidia or hyphae for example). In addition to this, the fungal cell wall which is consisted of chitin and other polysaccharides demands a special treatment in order to lyse it.

Recently, the emergence of fungal species and strains with reduced susceptibility or resistance against the commonly used antifungal agents in clinical practice has reinforced (I) the importance of performing AST on isolates recovered from infected patients, and (II) the need for rapid and clinically relevant AST methods (81). Various studies provide an alternative phenotypic approach to reference by CLSI or EUCAST AST techniques, with the use of MALDI-TOF methodology (82-84). In particular, exposure of the tested fungus (i.e., *Candida*, *Aspergillus*) in different dilutions of specific antifungal agent (i.e., fluconazole, caspofungin) for 15 hours results in the production, at a certain antifungal concentration, of a significant change in the mass spectrum profile. The lowest concentration of antifungal agent that induced the spectrum change was called the minimal profile change concentration (MPCC), analogous to the classical MIC endpoint (84). According to the results of the study by Marinach *et al.*, the MPCC did not differ more than one dilution step from the MIC determined using the CLSI reference for all *C. albicans* isolates studied (84).

With slight modifications, the MALDI-TOF based AST assay was applied to other *Candida* species, as well as *Aspergillus* species, by incubating the fungal with the

presence of different concentrations of caspofungin, with excellent results: MPCC values were in full essential agreement with the MIC values for 100% of the isolates tested (82). However, because the above method requires an overnight incubation step (i.e., 15 hours) before the endpoint readings, a more rapid and simplified version of the MALDI-TOF-based AST assay was proposed for the detection of caspofungin resistance in *C. albicans* (83). In details, fungal cells exposed to three concentrations of caspofungin for only three hours of incubation with excellent results: the categorical agreement was 98.4% with only one major error, for an isolate harboring a D648Y FKS1 genotype—this mutation is known to confer a lower level of echinocandin resistance (83).

A recent evaluation of the MALDI-TOF assay by Saracli *et al.* confirms the need for further assessment of the method (85). In that study, investigators used three-point antifungal concentrations (null, midrange, and highest) to detect triazole (fluconazole, voriconazole, and posaconazole) resistance in *Candida* species isolates (*C. albicans*, *C. glabrata*, and *C. tropicalis*). The overall essential agreement between the MALDI-TOF and the CLSI AST methods ranged from 54% to 97%, whereas the reproducibility of the MALDI-TOF -based AST assay varied between 54% and 83%. Because very major and major errors were noticed, the poor categorical agreement in this study emphasizes the necessity of establishing a quality control procedure and defining a gray zone for the MALDI-TOF MS-based AST method (85).

Viruses

According detection of viral resistance using MALDI-TOF methodology up to now little advance has been made. The fact that viruses are characterized by low protein content, but with a higher molecular weight, might be the main reason for this. Most researchers studying the use of MALDI-TOF in virology analyze products that have been amplified by PCR. Although identifying viruses by mass spectrometry techniques may not be something that will dominate in the future, these techniques can be used in genotyping or determining mutations of known viruses (17). As for the prediction of drug resistance, Zürcher *et al.* did manage to detect it in cases of cytomegaloviruses infections which resist to ganciclovir using a novel primer extension/MALDI-TOF(PEX/MALDI-TOF) based assay (71). A limitation of this PEX/MALDI-TOF method is that only known mutations can be detected.

Future perspectives—conclusions

The MALDI-TOF technology has decreased significantly the time needed for identification of pathogens. Significant steps have also been taken towards sensitivity determination in antibiotics from fresh cultures and blood cultures, as shown before. A great advantage of this technique is that antibiotics and their degradation products can be tested at the same time without the need of separate tests. Identification of pathogens and susceptibility testing directly from clinical samples would be a great addition in healthcare, as it can minimize even more the testing time. Especially, as regard the performance of different methodologies for antimicrobial resistance, these assays should be extended to more pathogens and more antibiotics. Studies are run in this area with successful results, for example the performance of MALDI-TOF ASTRA methodology in anaerobic bacteria (80), but more investigations are needed.

MALDI-TOF can rapidly characterize microorganisms by generating mass spectra of proteins that are compared to the known databases. If those proteins could be identified as well, the pathogenesis of the bacteria and the mechanisms of resistance could be understood too. Moreover the detection of toxins has been achieved in some cases using MALDI-TOF (93). In the future MALDI-TOF has helped to achieve much in identification of pathogens, susceptibility to drugs, has already narrowed down the time of results which is very important in clinical practice and has lowered significantly the costs in medical laboratory practice. Particularly for the detection of resistance in various pathogens, this promising technology needs further assay optimization to establish robust protocols. The combination of MALDI-TOF with powerful surface protein isolation methodologies, such as the use of detonation nanodiamonds for concentration and extraction of *A. baumannii* carbapenemase-associated proteins prior to mass spectrometry analysis, gives promising results (94). A thorough validation will be necessary to prepare this fast assay for routine application. The benefit would be accelerated information about bacterial resistance on the same day as identification is available.

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Footnote

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