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REVIEW

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for the Rapid Detection of Antimicrobial Resistance Mechanisms and Beyond

Marina Oviaño,[a,b] Germán Bou[a,b]

[a]Servicio de Microbiología, Complejo Hospitalario Universitario A Coruña, La Coruña, Spain
[b]Instituto Investigacion Biomedica A Coruña (INIBIC), La Coruña, Spain

SUMMARY Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been successfully applied in recent years for first-line identification of pathogens in clinical microbiology because it is simple to use, rapid, and accurate and has economic benefits in hospital management. The range of clinical applications of MALDI-TOF MS for bacterial isolates is increasing constantly, from species identification to the two most promising applications in the near future: detection of antimicrobial resistance and strain typing for epidemiological studies. The aim of this review is to outline the contribution of previous MALDI-TOF MS studies in relation to detection of antimicrobial resistance and to discuss potential future challenges in this field. Three main approaches are ready (or almost ready) for clinical use, including the detection of antibiotic modifications due to the enzymatic activity of bacteria, the detection of antimicrobial resistance by analysis of the peak patterns of bacteria or mass peak profiles, and the detection of resistance by semiquantification of bacterial growth in the presence of a given antibiotic. This review provides an expert guide for MALDI-TOF MS users to new approaches in the field of antimicrobial resistance detection, especially possible applications as a routine diagnostic tool in microbiology laboratories.

KEYWORDS antimicrobial resistance, MALDI-TOF

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Address correspondence to Germán Bou, german.bou.arevalo@sergas.es.

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INTRODUCTION

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has recently been introduced in clinical microbiology laboratories for identifying microorganisms (1–4). Although the initial investment in a mass spectrometer is relatively high, the cost of identifying a single isolate is lower than with previously used biochemical or molecular techniques (5, 6). In addition, the use of MALDI-TOF MS enables accurate identification at least 24 h earlier than phenotypic methods, even those that are automated (7–9). Among the advantages, the simplified workflow is one of the key points for the rapid acceptance of MALDI-TOF MS in laboratories and for constant growth in the range of applications.

Bacterial resistance to antibiotics has increased in recent years. In 2013, the Centers for Disease Control and Prevention (CDC) published a report outlining the top 18 drug-resistant threats to the United States: carbapenem-resistant *Enterobacteriaceae* are considered an urgent threat, and multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, fluconazole-resistant *Candida*, extended-spectrum-β-lactamase (ESBL)-producing *Enterobacteriaceae*, vancomycin-resistant enterococci, and methicillin-resistant *Staphylococcus aureus* are considered serious threats (https://www.cdc.gov/drugresistance/biggest_threats.html). Tests for the rapid diagnosis of infections produced by these microorganisms are therefore urgently needed. The time spent until microbiological results are provided is inversely proportional to the medical value (10), so the sooner information on antimicrobial resistance is obtained, the better the antimicrobial therapy that will be prescribed. The time to result is especially important to prevent unnecessary treatments and avoid the transmission of these bacteria of particular concern in hospital settings (11–13).

Five main approaches have been introduced as rapid means of detecting antimicrobial resistance (14–17) (Table 1). (i) Some of the techniques used are based on molecular genetics analysis. The sensitivity and specificity can be higher than those of previously used techniques: they deliver results in less than 1 h or within a few hours and have the advantage of being able to be applied directly to clinical samples. The disadvantages are the cost (usually higher); the lack of universal primers for detecting groups of resistance mechanisms, such as β-lactamases; the lack of proof of the biochemical activity of an enzyme, as detection of part of a gene sequence does not provide information regarding the phenotype associated with the enzyme; and the difficulty in performing the technique (by untrained or nonexperienced users) (18–20). One of these commercially available techniques is the GeneXpert system (Cepheid, France), which can detect carbapenemases like IMP, VIM, NDM, KPC, and OXA-48 with the Xpert Carba-R kit (21) and can also detect methicillin-resistant *Staphylococcus aureus* (MRSA) by the Xpert MRSA test (22). The Check-Direct CPE kit with the BD Max instrument detects KPC, VIM, NDM, and OXA-48 carbapenemases (23). (ii) Other methods for the rapid detection of antimicrobial resistance include biochemical methods such as the Carba NP test. These methods are based on the color change of the buffer in the presence of a pH indicator. For β-lactam antibiotics, the pH indicator changes its color when the hydrolysis of the antibiotic by bacterial resistance enzymes takes place. They are easy to perform, inexpensive, and universally applicable to different enzymes, and the results are delivered within 1 to 3 h. The disadvantages are the need for subjective interpretation of results, the low sensitivity relative to molecular methods, and the need to isolate the microorganism. It has shown good sensitivity with most carbapenemases but not with OXA-48-like enzymes, mainly because of the weak carbapenemase activity, or with OXA-23- and OXA-24-type enzymes in *Acinetobacter baumannii* (24). The Carba NP test has been commercialized through the Rapidec Carba NP system (bioMérieux, France) (25, 26), facilitating the procedure and hands-on time. However, false-positive and -negative results have been detected for OXA-48-like variants, in a more significant number than with the in-house Carba NP test (27). To date, the different variations of the Carba NP test has been used to detect not only β-lactamase resistance but also aminoglycoside and polymyxin resistance, all mediated

TABLE 1 Comparison of the different technologies for rapid detection of antimicrobial resistance

Technology	LOD (CFU/ml)[a]	Sensitivity (%)[b]	Specificity (%)[b]	TAT[c]	Price/sample ($)	References
Molecular genetics	10^2–2×10^4	96–99	96–99	1–3 h	5–50[d]	21–23
Biochemical methods	6×10^8	90–99	96–100	30 min–2 h	1–5[e]	24–29
Immunoassays	10^4–10^6	96–100	97–100	20 min	7–15[f]	30–33
Electrochemical assays	10^6–10^9	95–96	100	5–30 min	1 (plus $100 for the homemade reader)[g]	34–36
MALDI-TOF MS	10^5–10^6	98–100	92–100	30 min–3 h	1–10	44, 53–55, 60–66, 132

[a]LOD, limit of detection, expressed in CFU per milliliter, of the different technologies.
[b]Ranges of sensitivity and specificity of the different technologies.
[c]TAT, turnaround time of the different technologies.
[d]The price is lower if performing in-house methods and maximum if using commercial kits.
[e]The price is lower if performing in-house methods and maximum if using the Rapidec Carba NP system (bioMérieux).
[f]The price increases if resistance detection is focused on more than one cassette. The prices for OXA-48 K-SeT and KPC K-SeT are around $7/sample, the price for O.K.N. K-SeT is around $14/sample (detection of OXA-48, KPC, and NDM in the same test), and the price for O.K.N.V. K-SeT is around $15/sample (detection of OXA-48, KPC, NDM, and VIM in the same test).
[g]The price is around $1/sample, the electrodes are made in-house and cost around $2/electrode, and the reader is also produced in-house, at about $100.

by enzymes (28, 29). For the detection of aminoglycoside and polymyxin resistance, the principle of the assay is the detection of bacterial growth due to the fermentation process that acidifies the medium and makes the pH indicator change its color. (iii) Rapid immunoassays based on monoclonal antibodies have recently been developed for detecting carbapenemases (30). This technology has shown high sensitivity and specificity for detecting carbapenemase producers directly from bacterial colonies within 15 min. It has also shown promising results with biological samples such as positive blood cultures and urine samples (detection limit of 10^6 CFU/ml), as recently evaluated (31, 32). The main disadvantage of this method is that it does not yield quantitative results (33). This immunochromatographic assay could also be applied in the future to detect different types of resistance enzymes other than β-lactamases. (iv) The BYG Carba test is an innovative concept for the detection of carbapenemase-producing *Enterobacteriaceae* through an electrochemical assay that measures variations in conductivity in a polymer electrode due to the redox potential generated by imipenem hydrolysis by a carbapenemase enzyme. The measurement and analysis are reported in real time. The BYG Carba test has proven to be highly sensitive for discriminating between carbapenemase producers and nonproducers. It has the advantage of delivering results in 30 min without incubation at 37°C, and the sensitivity and specificity are higher than those of biochemical assays, although this requires further clinical validation (34–36). For the moment, the electrodes, the reader, and the software are made in-house and are not commercially available. (v) Finally, mass spectrometry analysis can be used to detect antimicrobial resistance mediated by all possible resistance mechanisms. Two main technologies are used: liquid chromatography-tandem mass spectrometry (LC-MS/MS) and MALDI-TOF MS. LC-MS/MS has been developed mainly to detect β-lactamases (37–40). The different procedures consist of either detecting the enzyme directly from bacteria or detecting the hydrolysis of the antibiotic by the β-lactamase enzyme. If detecting the enzyme, a first step of trypsin digestion and formic acid extraction should be performed. The main advantage compared to MALDI-TOF MS is the high sensitivity that can be achieved, and the main disadvantage of using this methodology is that microbiology laboratories do not usually have this technology. On the contrary, MALDI-TOF MS has several advantages: the use of solid samples; the low cost per test, despite the initial high investment in the equipment, as the savings for microbial identification are around 50% annually (5); the possibility of identifying microorganisms and detecting antimicrobial resistance with the same equipment; the simplicity of the procedure; and the automated interpretation of the spectra generated (41). The major disadvantages are the need for a high bacterial load, which precludes the use of the technique with clinical samples, except those with high burdens, such as positive blood cultures and urine samples (42–44), and the lack of commercially available kits with European Conformity (CE) regulatory clearance for *in vitro* diagnostics (IVD) or Food and Drug Administration

(FDA) clearance for IVD and developed software for the majority of techniques described in this document. In cases where no certified commercial kit is available, we recommend performing an internal validation of the procedure and using reagents with the mark of the European Pharmacopoeia (EP) reference standard or U.S. Pharmacopeia (USP) convention so that the maximum level of quality is guaranteed.

The MALDI-TOF MS method is a rapid and simple procedure that combines the universal advantages of phenotypic assays with the rapidity and accuracy of molecular assays. Since the first studies reported in 2011 (45–47), further developments in the method, along with advances in the equipment and software, have led to the implementation of this new technology in clinical laboratories for the detection of antimicrobial resistance. In this review, we consider the different possible applications of MALDI-TOF MS in this respect.

Three main approaches are used to detect antimicrobial resistance by MALDI-TOF MS: the detection of antimicrobial resistance by measuring antibiotic modifications due to the enzymatic activity of bacteria, analysis of the peak patterns of bacteria, and semiquantification of bacterial growth in the presence of a given antibiotic. Each of these approaches has advantages and disadvantages that we consider further here. However, in our opinion, each has the potential to be automated and applied in a clinical setting. The first two applications are currently in use with the new MALDI Biotyper IVD (Bruker Daltonik, GmbH, Germany).

DETECTION OF ANTIMICROBIAL RESISTANCE MECHANISMS BY MEASURING THE ENZYMATIC ACTIVITY OF BACTERIA

One of the main resistance mechanisms is modification of the antibiotic structure by bacterial enzymes, which renders the antibiotic inactive (47–51) (Table 2). If the modification involves a change in the molecular mass of the native molecule, the reaction can be monitored by mass spectrometry. The first step in this process is the accurate determination of the structure of the antibiotic and the reaction metabolite(s). The antibiotic structure is studied by observing the mass peaks obtained in the MALDI-TOF MS spectrum and assigning a possible ionic form to each mass peak. Antibiotics and their degradation products are usually analyzed in the mass range between 100 and 1,000 Da (47). One of the mass peaks most likely to be present is that of the molecular ion peak that represents the exact mass of the antibiotic. In the ionization process, the matrix usually adds a proton to the antibiotic, but it can also provide sodium and potassium ions, thus increasing the mass of the antibiotic by 1, 23, and 39 Da, respectively (47). Other reactions that can take place during ionization of antibiotics include decarboxylation, decarbonylation, and loss of other groups of molecules. These reactions usually take place during hydrolysis of β-lactams, as the newly hydrolyzed molecule is rendered unstable by the opening of the β-lactam ring and tends to break into different fragments, yielding compounds of different molecular weights. The mass peak patterns are unique to each antibiotic and can be used to detect antibiotic resistance.

Detection of β-Lactamases

Direct detection of β-lactamase activity by MALDI-TOF MS by measuring the mass changes in the antibiotic is one of the fields in which the greatest advances have been achieved in recent years (52). A very similar method is used in all reported studies. A fresh bacterial culture is suspended in a buffer containing a β-lactam and incubated at 37°C. At the end of the reaction, the mixture is centrifuged; the supernatant is measured in a suitable matrix, usually α-cyano-4-hydroxy-cinnamic acid (HCCA); and the spectrum obtained is then analyzed for fragmentation of the compound.

Regarding the detection of cephalosporin resistance, Oviaño et al. validated a procedure for the direct detection and classification of ESBL and AmpC producers in positive blood cultures (53). The antibiotics cefotaxime and ceftazidime were used to detect resistance, and clavulanic acid was added for accurate differentiation between class A (ESBL) and class C β-lactamases. An incubation time of 90 min was established.

TABLE 2 Structural determination of β-lactam antibiotics (ampicillin, piperacillin, cefotaxime, ceftazidime, ceftriaxone, cefpodoxime, cefepime, ertapenem, meropenem, and imipenem) and their corresponding hydrolyzed forms, defining the susceptibility and resistance patterns[a] by MALDI-TOF MS[b]

		Susceptibility pattern (Da)									Resistance pattern (Da)									
Antibiotic	MW (g/mol)[c]	$[M + H]^+$	$[M + Na]^+$	$[M + 2Na]^+$	$[M + 3Na]^+$	$[M + K]^+$	$[M + Na + K]^+$	$[M + C^d + H]^+$	$[M - X + H]^+$	$[M - X^e - CO + H]^+$	$[M - X - CO - CO_2 + H]^+$	$[M_{hydrol} + H]^+$	$[M_{hydrol} + Na]^+$	$[M_{hydrol} + 2Na]^+$	$[M_{hydrol} + Na + K]^+$	$[M_{hydrol} - CO_2 + H]^+$	$[M_{hydrol} - CO_2 + Na]^+$	$[M_{hydrol} - CO_2 + K]^+$	$[M_{hydrol} - X + H]^+$	$[M_{hydrol} - X - CO_2 + H]^+$
Ampicillin	349.4	350.4	372.4	394.4								368.4	390.4	412.4		324.4				
Piperacillin	517.5	518.5	540.5	562.5								536.5	558.5	580.5						
Cefotaxime	455.5	456.5	478.5						396.5										414.5	370.5
Ceftazidime	546.6	547.6							468.6										486.6	442.6
Ceftriaxone	554.6	555.6	577.6				616.6		396.6	368.6	324.6								414.6	370.6
Cefpodoxime	427.5	428.5	450.5	473.5		466.5													414.5	370.5
Cefepime	480.6	481.6							296.6										414.6	370.6
Ertapenem	475.5	476.5	498.5	520.5	542.5	514.5	536.5					494.5	516.5	538.5	554.5	450.5	472.5	488.5		
Meropenem	383.4	384.5	406.5	428.5																
Imipenem	299.4	300.4						489.4												

[a]The terms susceptibility and resistance by MALDI-TOF MS refer to the characteristic mass peak pattern of the antibiotic without modifications and to its corresponding hydrolyzed form. M_{hydrol}, hydrolysis.

[b]See references 47 and 55.

[c]MW, molecular weight.

[d]Imipenem is complexed with the matrix (α-cyano-4-hydroxy-cinnamic acid), resulting in a peak that is the summation of their molecular masses.

[e]X, acetyl for cefotaxime, pyridine for ceftazidime, triazin-ythiol for ceftriaxone, methoxy for cefpodoxime, and pyrrolidine for cefepime.

A series of different enzymes were analyzed for optimization of the assay, those encoded by bla_{TEM-1}, bla_{SHV-12}, bla_{TEM-29}, $bla_{CTX-M-32}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, bla_{FOX-4}, bla_{CMY-2}, bla_{DHA-1}, bla_{DHA-6}, and bla_{DHA-7}. For clinical validation, 140 positive blood cultures were studied for detection of β-lactamases. The use of cefotaxime resulted in a shorter turnaround time and higher sensitivity, although ceftazidime is useful for detecting AmpC producers. This was the first study to report the detection of β-lactam hydrolysis activity by MALDI-TOF MS directly from positive blood cultures.

Jung et al. validated a procedure for detecting β-lactam resistance in 2.5 h in *Enterobacteriaceae* in positive blood cultures (54). The antibiotics cefotaxime and ampicillin were used, and data were analyzed using an in-house-developed program. The reproducibility of the measurements was calculated, and comparison between paired blood cultures and plated isolates was made. The results were expressed as logarithm of the quantification of resistance (logRQ) values using the formula logRQ = log(sum of hydrolyzed peak intensities)/(sum of nonhydrolyzed peak intensities) and displayed in a box plot diagram. Eighty-five positive blood cultures were used for validating the detection of cefotaxime resistance. The lowest logRQ values were obtained for chromosomal AmpC, SHV, and TEM producers, and the highest values were obtained for AmpC hyperproducers and ESBL isolates. Higher logRQ values were also observed for plated microorganisms than for blood cultures; nevertheless, the assay provides accurate classification of susceptible and resistant isolates from blood cultures. The sensitivity and specificity of this method were 100% and 91.5%, respectively. The findings of this study are of great interest, as they represent the first step toward automation of the hydrolysis procedure, which will overcome the need for visual inspection of the spectra of the antibiotics and subjective interpretation of the results.

Oviaño et al. evaluated the sensitivities, specificities, and turnaround times for different antibiotics (cefotaxime, ceftazidime, ceftriaxone, cefpodoxime, and cefepime) and also the performance of the automated MALDI Biotyper (MBT) Selective Testing of β-Lactamase Activity (STAR-BL) module for Compass software (Bruker Daltonik) relative to qualitative interpretation of spectra for detecting β-lactamase resistance (ESBL and AmpC) by MALDI-TOF MS (Bruker Daltonik) (55). Regarding the antibiotics evaluated, ceftriaxone yielded 70% more positive results in relation to detecting resistance at the same incubation time than cefotaxime, 80% more than ceftazidime, and 20% more than cefpodoxime, with 100% specificity. Cefepime revealed 100% sensitivity but only 27% specificity. For the same incubation time, the automated software yielded on average 41% more positive results in relation to detecting resistance than qualitative interpretation of spectra. β-Lactamase resistance was detected after 30 min of incubation with ceftriaxone as the antibiotic marker in 100 genotypically characterized clinical isolates, with 100% sensitivity and specificity. This study reported the first structural determination of ceftriaxone by MALDI-TOF MS (Table 2).

The main advantage of measuring the activity of enzymes by MALDI-TOF MS is the potential power to detect all types of carbapenemases, even those that are considered rare (e.g., IMI, GES, FRI, and DIM, etc.) and are not included in commercially available molecular tests. Besides, with current clinical breakpoints defined by the EUCAST or the Clinical and Laboratory Standards Institute (CLSI), nearly 20% of carbapenemase-producing isolates can be missed, especially OXA-48-like enzyme producers (56). One of the major shortcomings of carbapenemase detection in previous papers was the lower sensitivity for detecting OXA-48 producers than for detecting other families of carbapenemases. This is due to the low catalytic efficiency of this family of enzymes in hydrolyzing carbapenems (57). Based on the structure of OXA-48 β-lactamases, carboxylation of the active site of lysine is important for enzyme activity (58). The addition of NH_4HCO_3 increased the k_{cat}/K_m values of the OXA-58 enzyme for imipenem by more than 5-fold (59). Applying this principle, Papagiannitsis et al. improved the detection of OXA-48-like enzyme producers from 76% to 98% by adding NH_4HCO_3 to the reaction buffer and using meropenem as an antibiotic marker of resistance, with an incubation time of 2.5 h (60). The assay was validated using 124 *Enterobacteriaceae* and 37

Pseudomonas aeruginosa isolates. A novelty introduced by the group led by Hrabák (60) is the use of 10 mg/ml 2,5-dihydroxybenzoic acid (DHB) as the matrix. This change in the procedure allows better identification of the hydrolysis products of meropenem. Automatic interpretation of the MALDI-TOF MS assay results by the MBT STAR-BL module (Bruker Daltonik) was generally consistent with the results obtained by manual analysis. The sensitivity of detection of bla_{OXA-48} by MALDI-TOF MS has since reached the same level as for other carbapenemase enzymes.

Another problematic aspect was the turnaround time for β-lactamase detection. In developing the method, Lasserre et al. used a 20-min incubation time with imipenem to detect carbapenemase producers and applied a cutoff MS ratio (mass peaks of the metabolite/imipenem plus the metabolite of $\geq$0.82) to classify 223 strains, 77 carbapenemase producers and 146 nonproducers, as isolates harboring carbapenemase enzymes, yielding 100% sensitivity and specificity (61). The peak of the imipenem metabolite was previously described as being at 254 Da by Kempf et al. (62) using an Ultraflex I instrument (Bruker Daltonik). However, several other authors seem hesitant to ensure the appearance of such a metabolite, as it cannot be visualized under standard MALDI-TOF MS conditions (47, 63–65). Lasserre et al. estimated the cost per test to be less than $0.10. To date, this is the fastest assay performed by MALDI-TOF MS and with imipenem as the standard antibiotic. Both groups used imipenem that contains cilastatin (Tienam; MSD, France) for MALDI-TOF MS measurements. Although the stability issue seems to be solved with the addition of cilastatin, we recommend using imipenem alone, as the complexation of imipenem with cilastatin and not with the matrix can make proper visualization of the mass peaks in the spectrum difficult.

In an attempt to improve the procedure, Monteferrante et al. developed a protocol involving cellular lysis and enzyme extraction from a defined number of bacterial cells, followed by the addition of an antibiotic (imipenem or ertapenem), in a series of 260 isolates (208 carbapenemase producers and 52 non-carbapenemase producers), yielding 100% sensitivity and specificity (64). Standardization was achieved by prior adjustment of the cells to a turbidity of a 3.0 McFarland standard. Comparison of the uses of imipenem and ertapenem revealed higher sensitivity and specificity and a higher hydrolysis rate for imipenem. However, imipenem is less stable in solution, and MS signals are about 5-fold lower than those for ertapenem. This group used the Ultraflex III instrument (Bruker Daltonik), which is a MALDI-TOF/TOF MS technology, so results may vary with respect to those obtained with the Microflex technology (MALDI-TOF MS) usually used in routine microbiology laboratories, especially regarding the detection of the imipenem metabolite. The longer flight tube used with the Ultraflex equipment provides a higher resolution. In this case, the authors described the appearance of the hydrolyzed decarboxylated form at 274 Da and the sodium adduct at 296 Da. The use of a standard number of cells and subsequent extraction was previously developed by Hrabák et al. (46). The main consequences are that it is a less technician-dependent technique but much more laborious and slower, with a possible hands-on time of 120 min relative to the 30 min required for the direct method using whole cells.

With the aim of establishing rapid and accurate procedures for detecting antimicrobial-resistant bacteria directly from positive blood cultures, Oviaño et al. developed a universal method for detecting carbapenemase producers among 119 Gram-negative bacilli, including *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp., in 30 min, adding imipenem to a buffer containing NH_4HCO_3, $ZnCl_2$, and sodium dodecyl sulfate (SDS) (66). NH_4HCO_3 proved useful for detecting OXA-48 producers, as previously demonstrated by Papagiannitsis et al. with meropenem (60). Zn^{2+} was essential for some metallo-β-lactamase-producing *Pseudomonas* species, and SDS was essential for satisfactory reactivity for *Acinetobacter* spp., as previously demonstrated by Hrabák et al. (65). The method of extraction from the blood culture using the Sepsityper kit (Bruker Daltonik) was also modified from that recommended by the manufacturer for identification purposes, reducing the amount of lysis and thus improving the yield. The overall sensitivity and specificity were 98% and 100%, respec-

tively. Analysis was performed using the STAR-BL module for MALDI-TOF Biotyper Compass software (Bruker Daltonik), which automatically provides a result for sensitivity or resistance, calculated as a logRQ value or a ratio of hydrolysis of the antibiotic. The logRQ value is the logarithm of the intensity of the area under the curve (AUC) of the internal standard and the AUC of imipenem. Reserpine was used as the internal standard, as it is a stable molecule in the mass range of study (608 Da). This compound is lyophilized into the MALDI matrix and then dissolved and finally added to the sample, in the same way as it is done for a conventional matrix. This method is ready to use in clinical practice. Until this work, OXA-type carbapenemases in *A. baumannii* from plate cultures required longer incubation times for detection and frequently remained undetected in blood cultures. In addition, this method unifies carbapenemase detection for all types of Gram-negative bacilli, and it does not require an initial identification step or any change in the procedure to detect resistance.

Oviaño et al. developed an interpretative automated MALDI-TOF MS-based method for the direct detection of carbapenemase-producing *Enterobacteriaceae* in clinical urine samples within 90 min of sample reception (44). A total of 3,041 urine samples were processed by flow cytometry, and a cutoff value of $\geq 1.5 \times 10^5$ bacteria/ml was used to select samples for the study. A novel protocol was developed for extracting bacteria from urine samples by using the Sepsityper kit (Bruker Daltonik). Carbapenem resistance was detected using imipenem, and the results were automatically interpreted with MALDI-TOF Biotyper Compass software (Bruker Daltonik). The assay yielded direct, reliable identification of 91% (503/553) of the samples and showed 100% sensitivity (30/30) and specificity (454/454) for detecting carbapenemase activity. The main advantage of the method is that the results are obtained 24 to 48 h earlier than with conventional methods (Fig. 1). However, it also has some disadvantages, such as the large volume (10 ml) of urine required, the need for bacterial counts higher than 1.5×10^5 bacteria/ml, and the exclusive identification of monomicrobial infections. This study demonstrated for the first time the potential detection of carbapenemase-producing Gram-negative bacilli directly from urine samples by using a standardized procedure and automated software.

All these previous studies were performed using MALDI-TOF MS (Bruker Daltonik). Carvalhaes et al. performed a study evaluating the detection of carbapenemase activity using ertapenem and Vitek MS (bioMérieux) for detection of antimicrobial resistance (67). Sensitivity was evaluated according to the incubation time, and it was found that it was dependent on the type of carbapenemase, as classes A and B were the fastest, achieving 62% sensitivity in 15 min and 87% in 60 min. Vitek MS proved useful for the detection of antimicrobial resistance; however, there is a need for more studies validating the procedure. No studies regarding automated interpretation of spectra have been performed using Vitek MS.

The findings of these studies enable us to conclude that the method shows great potential for use in all microbiology laboratories as a routine method for detecting β-lactamase activity.

This field of work is progressing very rapidly, and since the last review, written by Hrabák et al. (52) in 2013, some notable methodological advances have been made. (i) Incubation times have been reduced from around 3 h to 30 min by using ceftriaxone for β-lactamase detection (ESBL and AmpC) and by using imipenem for carbapenemase detection (55, 61, 66). (ii) A larger number of antibiotics have been tested, and ceftriaxone, cefpodoxime, and cefepime can now be analyzed (55). (iii) Visual interpretation has been replaced by automatic acquisition and interpretation of spectra, thereby eliminating subjective measurement of mass peaks and making previous expertise in mass spectrometry unnecessary, thus facilitating the implementation of MALDI-TOF MS in routine in clinical microbiology laboratories (66). New antibiotics not previously evaluated by MS must first be properly analyzed by structural determination, and once the mass peaks of the antibiotic and the hydrolysis products are established, automated interpretation can be performed so that the logRQ value of the antibiotic is provided. To establish a threshold for positivity, hydrolysis peaks must have an AUC of

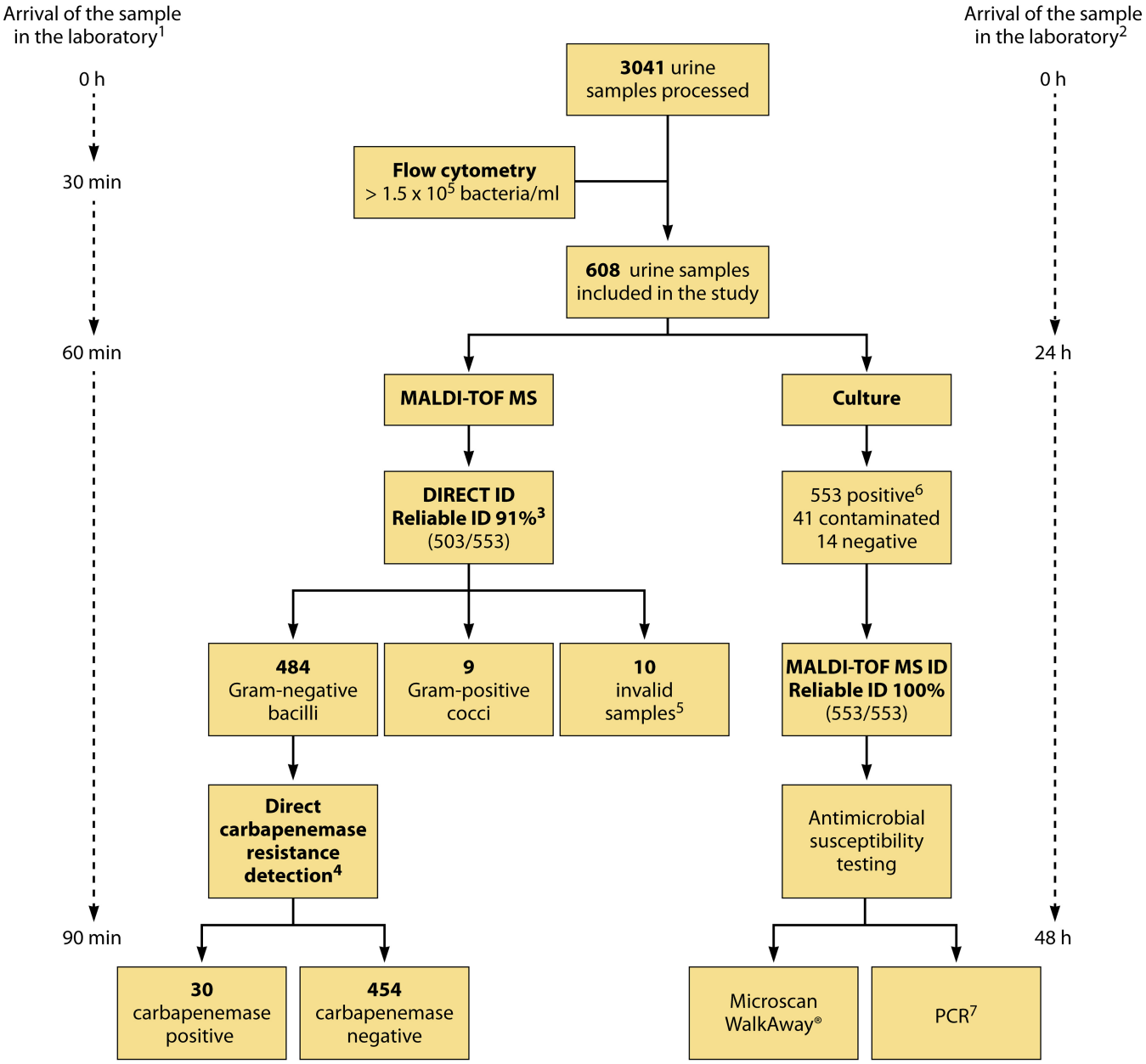

FIG 1 Workflow diagram showing the detection of carbapenemase activity directly from clinical urine samples. (Republished from reference 44 with permission of the British Society for Antimicrobial Chemotherapy.) [1], expected time for direct identification of bacteria and detection of carbapenemase susceptibility in urine samples by MALDI-TOF MS. [2], expected time for identification of bacteria and detection of carbapenemase susceptibility in urine samples by routine microbiological processing. [3], according to the manufacturer, a score of <1.7 indicates unreliable identification, a score of between 1.7 and 2.0 indicates genus identification, and a score of ≥ 2.0 indicates species identification. [4], carbapenemase activity was detected directly in urine samples and was measured by MALDI-TOF MS and automated spectrum interpretation. LogRQ values below 0.2 represent negative strains, values above 0.4 indicate carbapenemase activity, and values between 0.2 and 0.4 represent an ambiguous hydrolysis pattern. [5], invalid samples were those in which the amount of pellet obtained was not sufficient to carry out direct identification of bacteria and the carbapenem resistance assay. [6], urine samples were considered positive with $\geq 10^4$ CFU/ml of one microorganism, whereas samples with two or more microorganisms were considered contaminated. [7], Gram-negative bacilli in positive urine samples were characterized by PCR and further sequencing of carbapenemase genes.

at least 40%, and positive and negative controls must be used in every assay (66). Negative controls are necessary so that spontaneous hydrolysis is not interpreted as a positive reaction (especially for unstable compounds like imipenem). Besides, positive and negative controls can be used for normalization of the hydrolysis values so that intra-assay variability is minimized. This is especially important for assays in which the amount of cells added has not been previously standardized and positivity is related to the value of the positive control and not to the absolute value of the sample. (iv) Finally,

detection of carbapenemase activity directly from clinical samples without enrichment of the bacteria in specific cultivation medium is already being performed with urine samples. Although large amounts of bacteria are used in the analysis (44), similar to those required for identification (42), this test could potentially be used with other types of clinical samples in the future (68).

Detection of the AAC(6′)-Ib-cr Enzyme

MALDI-TOF MS has proven useful for detecting β-lactamase activity, either narrow-spectrum, ESBL, AmpC-like, or carbapenemase-type enzymes, as we note above. Using the same concept of detecting antimicrobial resistance mechanisms by measuring the enzymatic activity of bacteria, any type of resistance that produces a change in the mass of the antibiotic can be detected. Detection of the AAC(6′)-Ib-cr enzyme highlights the ability of MALDI-TOF MS to detect resistance determinants other than β-lactamases, by monitoring the acetylation reaction in the substrate, in this case the fluoroquinolones ciprofloxacin and norfloxacin (69).

The AAC(6′)-Ib-cr enzyme is the most prevalent plasmid-mediated quinolone resistance mechanism in *Enterobacteriaceae* (70, 71). This resistance mechanism provides by itself low MICs that are not included in the clinical category of resistance; however, it facilitates the selection of higher-level resistance during treatment and results in limited therapeutic options for patients with bacteria containing the AAC(6′)-Ib-cr enzyme (51). In addition, detection is important from an epidemiological point of view and as a surrogate marker for specific bacterial clones. At present, this antimicrobial resistance mechanism is detected mainly by a PCR-based assay.

Pardo et al. developed a method of detecting the presence of the AAC(6′)-Ib-cr enzyme by measuring acetyltransferase activity in a series of 113 strains of *Enterobacteriaceae*, 64 of which harbored the AAC(6′)-Ib-cr enzyme (72). MALDI-TOF MS measurements were performed after 4 h of incubation with norfloxacin. Results were analyzed by considering the ratio of the sum of areas under the curve of the different forms of acetylated norfloxacin and of those of native norfloxacin by using Vitek MS research-use-only (RUO) software (bioMérieux).

Oviaño et al. studied the presence of the AAC(6′)-Ib-cr enzyme in a collection of 81 isogenic *Escherichia coli* control strains [10 carrying the AAC(6′)-Ib-cr enzyme] during exposure to ciprofloxacin, norfloxacin, and levofloxacin and a further 36 clinical isolates [25 carrying the AAC(6′)-Ib-cr enzyme in addition to different combinations of quinolone resistance mechanisms] (73). Acetylation yields increases of 43 Da in the masses of ciprofloxacin and norfloxacin, but not of levofloxacin, which can be observed by visual inspection of the mass peaks in the spectra (Fig. 2). On the basis of the characteristic peak patterns of the acetylated and nonacetylated forms of ciprofloxacin and norfloxacin, AAC(6′)-Ib-cr-producing isolates and nonproducing isolates were clearly differentiated after an incubation time of 30 min. The presence of other determinants of quinolone resistance had no impact on the acetylation reaction or on the results obtained by MALDI-TOF MS. Total disappearance of the nonacetylated forms did not occur in any of the isolates tested, and both acetylated and nonacetylated forms coexisted in the same spectrum, in contrast to what usually happens when β-lactam antibiotics are observed in the presence of a β-lactamase (47). Those authors confirmed that the AAC(6′)-Ib-cr acetyltransferase enzyme confers only a low level of resistance, as previously reported, and described the structural determination of compounds other than β-lactam antibiotics with great success.

In order to improve this procedure and for the purpose of automation, Oviaño et al. established an algorithm that enables the quantification of acetylation in relation to enzyme activity applied on a series of 122 clinical isolates, 15 of which harbored the AAC(6′)-Ib-cr enzyme (74). The MALDI Biotyper PeakShift prototype (Bruker Daltonik) was used for automation of the interpretation of spectra and semiquantification of the ratio of acetylation by logarithm intensity quantification (logIQ), the same principle that was used for measuring the hydrolysis of β-lactam antibiotics by β-lactamases. This method was 100% sensitive and specific for the detection of the AAC(6′)-Ib-cr enzyme.

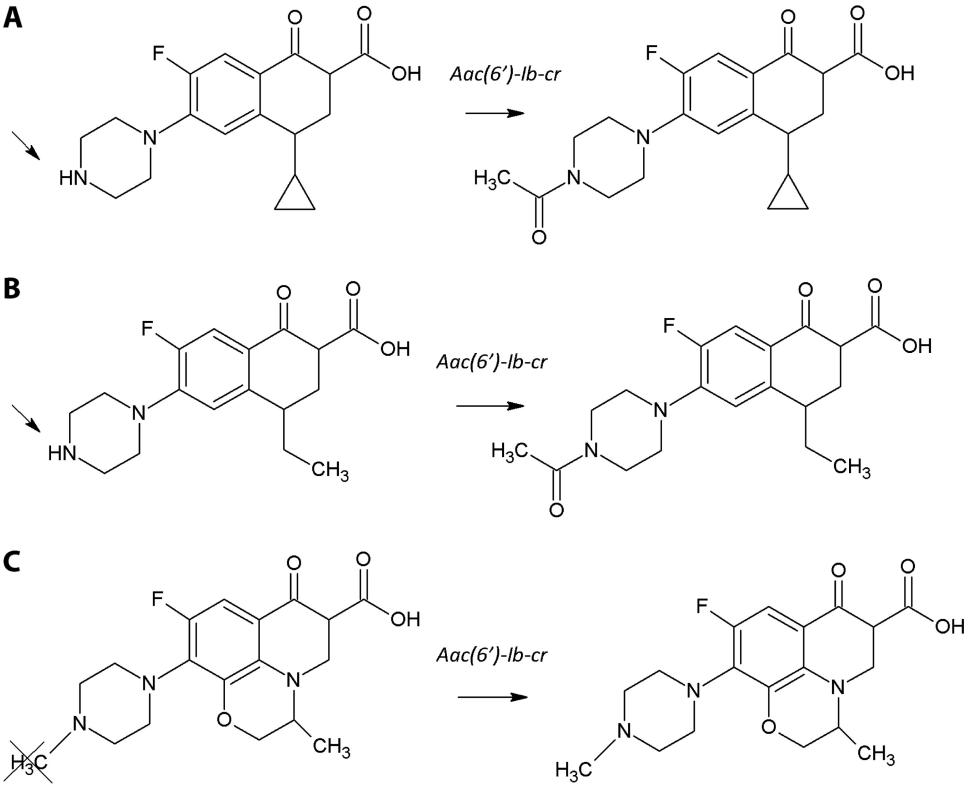

FIG 2 Acetylation reaction in fluoroquinolones. (Republished from reference 73 with permission of the British Society for Antimicrobial Chemotherapy.) Shown are effects of the *aac(6′)-Ib-cr* gene on the chemical structures of ciprofloxacin (A), norfloxacin (B), and levofloxacin (C). Acetylation occurs at the amino nitrogen on the piperazinyl substituent of fluoroquinolones, a structural feature shared by ciprofloxacin and norfloxacin but not levofloxacin. Acetylation yields an increase of 43 Da in the mass of the antibiotic, which can be measured by MS.

The automated interpretation of spectra led us to conclude that the use of norfloxacin as a resistance marker in MS will enhance detection as more-complete acetylation of the isolates is achieved, as the MICs are also higher for norfloxacin than for ciprofloxacin.

The findings of these previous studies indicate the potential use of MALDI-TOF MS to detect other mechanisms of resistance that are not β-lactam related, based on structural modification of antibiotics.

Detection of Aminoglycoside-Modifying Enzymes

Aminoglycoside resistance based on enzymatic modification of the antibiotic is one of the most common mechanisms of resistance in this group of antibiotics and is highly prevalent in both Gram-positive and Gram-negative microorganisms (75–77). Aminoglycoside-modifying enzymes display acetyltransferase, phosphotransferase, and nucleotidyltransferase activities. In addition, the substrate is not always of the same functional group, as acylation can affect hydroxyl and/or and amino groups, giving rise to more-complex reactions (78, 79). Zimmermann developed a rapid (<3-h) method of detecting *N*-acetyltransferase-mediated aminoglycoside resistance in Gram-negative bacilli by using a liquid chromatography-electrospray ionization-time of flight (LC-ESI-TOF) method (80). Isolates were characterized as AAC-6′ carriers. However, the mass shifts on the acetylated aminoglycoside molecules were not detected by MALDI-TOF MS. We had the same experience (data not shown). Further studies are required to finally rule out the possibility of detection of aminoglycoside resistance by MALDI-TOF MS. We acknowledge that some molecules will not be detected by MALDI-TOF MS, as negatively charged molecules can be detected only in a negative-ion mode of acquisition that is not available with conventional instruments like Microflex (Bruker Daltonik) or Vitek MS (bioMérieux). Additional research will confirm whether we will finally

be able to detect these resistance mechanisms by MALDI-TOF MS, or we will have to rule out the use of this technology for detecting aminoglycoside-modifying enzymes.

DETECTION OF ANTIMICROBIAL RESISTANCE BY ANALYSIS OF THE MASS PEAK PROFILES OF THE BACTERIA

In the application of MALDI-TOF MS for the detection of antimicrobial resistance by analysis of the mass peak profiles of bacteria, susceptible and resistant microorganisms of the same species are differentiated on the basis of their spectra. Some mass peaks are associated with a resistance pattern of an isolate due to the expression of a specific protein involved in the directed or associated resistance phenotype and may be useful for differentiating between susceptible and resistant isolates. Although this technique has been successfully applied in some studies that are explained further below (81–83), it must be validated in clinical settings to ensure the reproducibility of the results. The main advantage of this methodology is its simplicity, as the sample preparation procedure is the same as or quite similar to that used for identification. The mass peaks (biomarkers) identified can also be automatically analyzed in real-time workflows in laboratories, at no additional expense.

Detection of MRSA

Many studies that have investigated how best to discriminate between methicillin-susceptible *S. aureus* (MSSA) and MRSA have obtained different sensitivity values as well as variable findings in regard to the mass peaks used to differentiate these two groups (84–87). Other studies have stressed the lack of correlation between any peak profiles and MRSA or MSSA (88, 89). The possible clinical application of this methodology was in dispute until Josten et al. detected a small peptide called phenol-soluble modulin (PSM-*mec*) that is encoded on the type II, III, and VIII staphylococcal cassette chromosome *mec* element (SCC*mec*) cassettes present in the genomes of health care-associated MRSA strains by MALDI-TOF MS (81). This peptide is excreted by *agr*-positive strains, and the presence of the peptide can be detected in the MALDI-TOF MS spectra of whole cells as a peak at 2,415 Da (Fig. 3). PSM-*mec* is detected by MALDI-TOF MS with high sensitivity and specificity values of 0.95 and 1, respectively. Rhoads et al. reported that the sensitivities of the 2,415-Da peak for *mecA* carriage in *S. aureus* and *Staphylococcus epidermidis* were low (37% and 6%, respectively), whereas the specificities were high (≥98%) (90).

This method could be used to establish a sound approach to distinguishing between MRSA and MSSA with higher specificity. However, sensitivity is in this case dependent on the prevalence of the cassettes in each geographical area, thus limiting the application to those areas with a high prevalence of cassettes II, III, and VIII. Besides, negative results should be confirmed by molecular methods. In areas with a low prevalence of the above-mentioned cassettes, phenotypic or molecular methods should not be replaced by MALDI-TOF MS given the worse accuracy of this method (91).

Detection of β-Lactamases

In a study reporting a method of real-time analysis of outbreaks of infections caused by carbapenemase-producing *Enterobacteriaceae*, Lau et al. observed a mass peak at 11,109 Da corresponding to a specific protein (p019) carried on the pKpQIL plasmid after a simple formic acid extraction step (82). This mass peak was used as a biomarker to identify KPC-producing *Klebsiella pneumoniae*, with excellent results. The insertion containing p019 has been found only in plasmids carrying bla_{KPC} to date (92). In a later study, Youn et al. applied the above-described method of identifying KPC-producing *Enterobacteriaceae* along with different extraction methods (plate extraction and full formic acid extraction) and different acquisition methods (manual and automated), demonstrating 96% sensitivity and 99% specificity under the best conditions, for plate extraction and interpretative automation analysis, respectively (93). Gaibani et al. demonstrated that the 11,109-Da peak was detected in 88.2% of the KPC producers, confirming previous results (94).

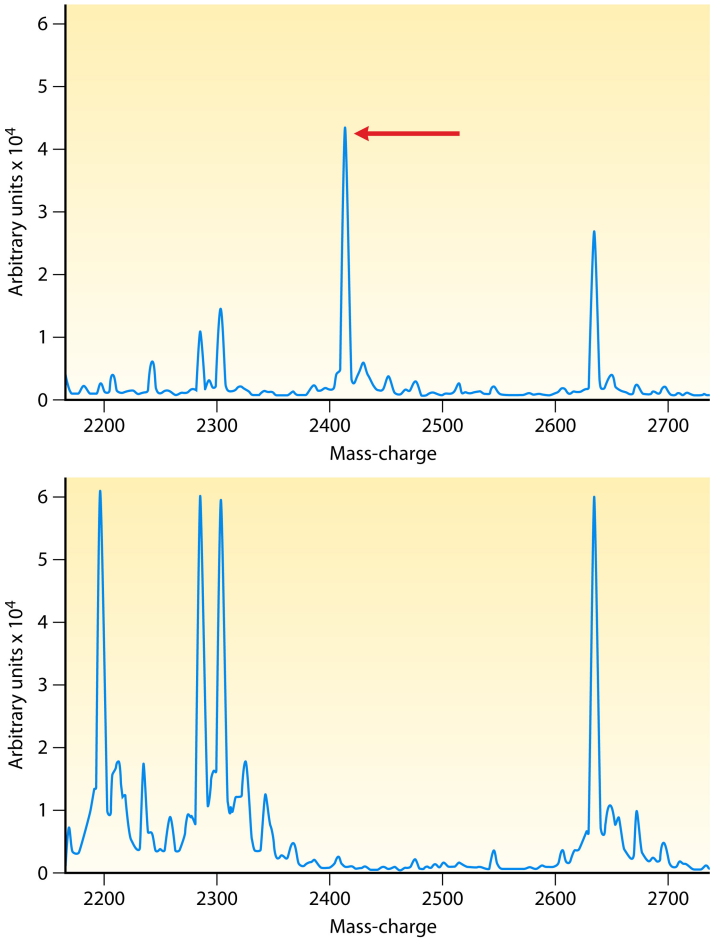

FIG 3 MALDI-TOF spectra showing the 2,415-Da mass peak representing PSM-*mec* of methicillin-resistant *S. aureus*. (Republished from reference 90 with permission of the publisher.) Shown are MALDI-TOF mass spectra for 2 MRSA strains. One strain is a USA 100 strain (top), carrying the *mecA* gene, and the other strain is a USA 300 strain (bottom), which lacks the *mecA* gene. The *x* axis depicts mass/charge ratios, and the *y* axis is arbitrary units.

Papagiannitsis et al. described the identification of CMY-2 β-lactamases by detection of the mass peak at 39.850 Da by MALDI-TOF MS (95). The preparation of the sample involved a labor-intensive method with extraction of the periplasmic proteins performed by a modified sucrose method that lasts around 22 h. This group also performed some preliminary analyses for detection of ACC and DHA enzymes. However, these methods could hardly be used by routine microbiology laboratories.

In any case, caution should be applied when using this type of approach, as protein expression may depend on specific clones or growth terms, and the data cannot be extrapolated to all β-lactamases.

Detection of *cfiA*-Positive *Bacteroides fragilis*

Carbapenemase production encoded by the *cfiA* gene in *Bacteroides fragilis* is increasingly recognized in clinical isolates (96–98). The detection and control of *B. fragilis* and resistance of the species to carbapenems are of great concern in relation to prescribing the most appropriate antibiotic therapy, as this is one of the first-choice treatments.

Wybo et al. differentiated *cfiA*-positive *Bacteroides fragilis* in 248 clinical samples by grouping the samples into 2 clusters (one with the *cfiA* gene, encoding carbapenemase production, and the other without the gene) in a matrix calculated from the mass peak profiles (83). The relatedness of the spectra was determined by using the composite

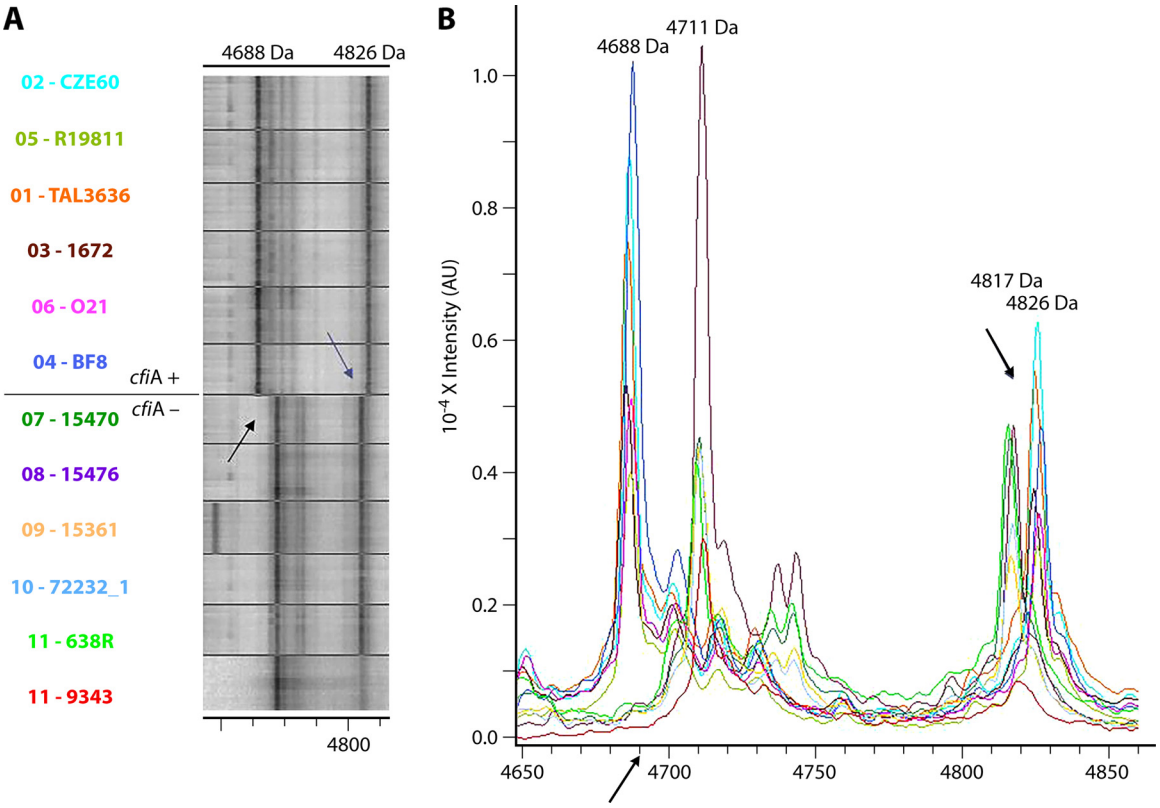

FIG 4 Representative spectra of *cfiA*-positive and *cfiA*-negative *Bacteroides fragilis* strains. (Republished from reference 99 with permission of the publisher.) Shown is an example of the clear differences in the MS peaks in the interval from 4,650 to 4,850 Da between *cfiA*-positive and -negative strains. (A) Visualization by a gel/stack view. (B) Visualization by mass spectra. Peaks are shifted from 4,711 and 4,817 Da (*cfiA* negative) to 4,688 and 4,826 Da (*cfiA* positive), respectively. AU, arbitrary units.

correlation index (CCI) tool of the MALDI Biotyper (Bruker Daltonik). The CCI is a mathematical algorithm used to compare and distinguish MALDI mass spectra, where CCI values of around 1 represent a close relationship between spectra, matching an intense red color in the matrix, whereas a value close to zero represents a distant relationship, with a blue color in the matrix. Nagy et al. analyzed 28 isolates (9 *cfiA* producers and 19 nonproducers) to validate a similar method based on the presence or absence of a series of mass peaks in the range of 4,000 to 5,500 Da observed in MALDI-TOF mass spectra, with 100% accuracy (99). The ability to compare spectra by using the MALDI-TOF MS graphical matrix was successfully applied by these authors to detect antimicrobial resistance (Fig. 4).

Porin Detection

The interaction between β-lactamase (ESBL, AmpC, or carbapenemase) production and porin loss in bacteria is important in carbapenem resistance (100, 101). *Klebsiella pneumoniae* produces two main porins (OmpK35 and OmpK36) (102). While most clinical isolates of *K. pneumoniae* express both the OmpK35 and OmpK36 porins, most ESBL-producing *K. pneumoniae* clinical isolates produce only OmpK36 (103). Loss of porins is one of the factors contributing to antimicrobial resistance and may favor the selection of additional mechanisms of resistance. OmpK35 and OmpK36 are especially related to resistance to β-lactams and quinolones (104).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most common method used to analyze porins. However, SDS-PAGE is laborious, time-consuming, and not available in most clinical laboratories. MALDI-TOF MS has arisen as a new technique to detect the presence and absence of porins and to help in the detection of antimicrobial resistance. Disappearance of the mass peak related to the porin would

imply resistance to the antibiotics that penetrate the porin. On the contrary, the presence of the mass peak related to the porin would not strictly mean susceptibility, as quantification of the expression level cannot be performed yet, and we cannot ensure that the porin has a natural behavior or has reduced expression.

Cai et al. described a new method for the rapid detection of porins in *K. pneumoniae*. MALDI-TOF MS was performed with a Microflex LT mass spectrometer (Bruker Daltonik) operating in positive-linear-ion mode between 15,000 Da and 80,000 Da. MALDI-TOF MS analysis showed that mass peaks at 38,000 Da and 19,000 Da could be used as indicators of the OmpK36 porin (105). In a similar study, Hu et al. identified mass peaks at 35,000, 37,000, and 38,000 Da, corresponding to OmpA, OmpC, and OmpF, respectively, in the mass spectrum of *E. coli* ATCC 25922 (106). The OmpC and OmpF porins were easily differentiated by MALDI-TOF MS but were indistinguishable by SDS-PAGE. In *K. pneumoniae* isolates, mass peaks were observed at 36,000 and 38,600 Da, corresponding to the OmpA and OmpK36 porins. Porin OmpK35 was not detected by the SDS-PAGE method, but it was apparently detected by MALDI-TOF MS. Further studies must be conducted to check the reproducibility of the technique and to expand this novel approach to other microorganisms. However, these studies demonstrate the usefulness of MALDI-TOF MS in saving time and reducing the analytical effort involved in microbiology laboratories, opening the possibility for a future application of these techniques in clinical practice.

Detection of Polymyxin Resistance

As carbapenemase-producing *Enterobacteriaceae* have become more prevalent, the use of polymyxin has gradually increased, and the resistance of these bacteria has subsequently also increased (107, 108). Acquired resistance to polymyxins in *Enterobacteriaceae* is due mainly to modifications in the polymyxin target, the lipopolysaccharide. These modifications may be brought about by chromosome-encoded mechanisms or by the acquisition of the plasmid-borne phosphoethanolamine transferase gene *mcr-1*. Dortet et al. developed a novel MALDI-TOF MS method for detecting polymyxin resistance in *Enterobacteriaceae* within 15 min (109). This process is similar to that used for identifying bacteria but with a modified atypical matrix, which enables observation of the peaks corresponding to intact lipid A and lipid A modified by either chromosome-encoded or plasmid-mediated mechanisms. Independently of the resistance mechanism involved, a mass peak at 1,919 Da corresponding to the addition of a phosphoethanolamine group to the 1,796-Da nonmodified lipid A has been detected for all polymyxin-resistant isolates, including *E. coli* and *K. pneumoniae*. In the case of resistance due to the *mcr-1* gene, an additional mass peak was observed at 1,821 Da. Although no information has been given regarding the nature of this mass peak, it appears to be specific to the MCR-1-producing isolates, independently of the species considered. As far as we are aware, no other studies on polymyxin resistance have been carried out by direct detection of lipid A by MALDI-TOF MS, and further research is needed to confirm the validity of this novel approach and broaden the number of *Enterobacteriaceae* and nonfermenter microorganisms, such as *Acinetobacter baumannii*, with which the method can be used. This application is potentially of great interest because of the rapidity and simplicity of the procedure relative to previous methods used to identify lipid A that needed a laborious extraction procedure (110). The atypical matrix must be fully characterized so that it does not complicate the identification process and to enable further automation of the process.

DETECTION OF ANTIMICROBIAL RESISTANCE BY MEASUREMENT OF THE EFFECTS OF THE ANTIBIOTIC ON MICROORGANISM GROWTH

For the application of MALDI-TOF MS for the detection of antimicrobial resistance by measurement of the effects of the antibiotic on microorganism growth, susceptible and resistant microorganisms of the same species are differentiated on the basis of the relative growth of the isolate in the presence of a particular antibiotic, as in other nonproteomic approaches (111, 112).

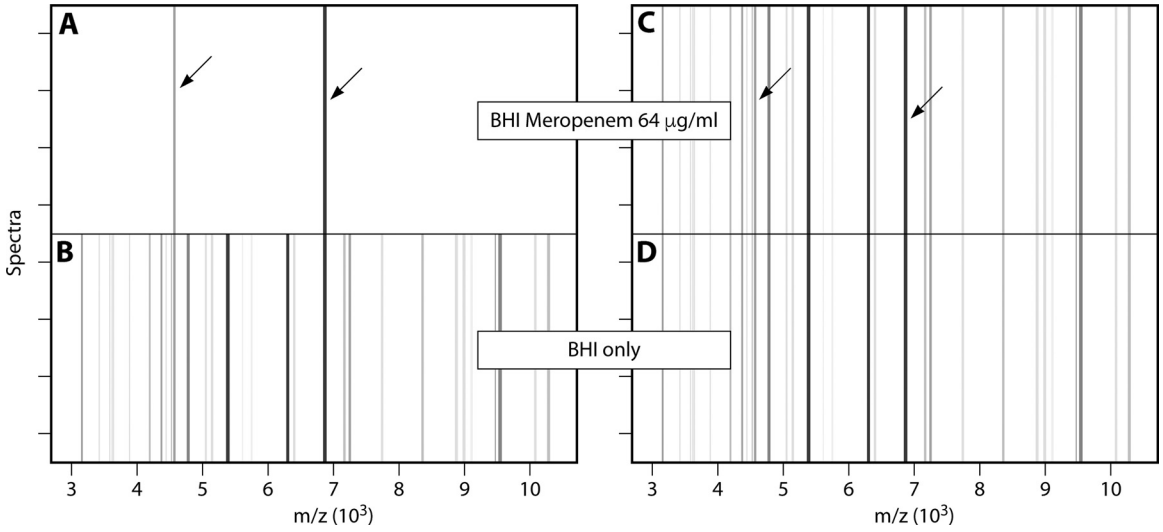

FIG 5 Pseudogel view of the mass spectra of susceptible and resistant *K. pneumoniae* strains after growth in the presence of meropenem. (Republished from reference 114 with permission.) Shown are pseudogel views of the mass range between 3 and 10 kDa of susceptible (A and B) and resistant (C and D) *K. pneumoniae* strains after incubation in the absence (bottom panels) or presence (top panels) of meropenem (64 μg/ml) for 1 h. For each incubation, four spectra acquired from two different spots are shown. Internal-standard peaks are marked by arrows.

Quantitative Resistance Profile

First, Sparbier et al. measured the protein synthesis of bacteria growing in an isotope-labeled amino acid medium (113). The incorporation of heavy amino acids increases the molecular weight of the newly synthesized proteins, causing peak shifts in the mass spectral profiles that can be automatically detected by a software algorithm. This assay is called MBT-Resist (MALDI Biotyper resistance test), with stable isotope-labeled amino acids. Bacteria are incubated with the antibiotic in an isotope-labeled medium, lysed, and spiked with an internal standard for quantification of bacterial growth. In this case, lysine was the amino acid that was isotope labeled. Ten MRSA and 10 MSSA isolates were analyzed for the detection of methicillin resistance by calculating the ratio between the sum of areas of the "heavy" peaks and that of the areas of the "normal" peaks. The assay provides results in 3 h, being able to differentiate between MRSA and MSSA.

Next, Lange et al. developed the MBT-ASTRA (MALDI Biotyper antibiotic susceptibility test rapid assay), which facilitates measurement of the quantity of peptides and small proteins within a spectrum (114). These quantities are correlated with the number of microorganisms and, therefore, with the growth of a microorganism. If the microorganism is unable to grow in the presence of a certain antibiotic, it is susceptible to that antibiotic, and the spectrum obtained will be almost flat, with no mass peaks, while resistant isolates will grow, and the corresponding mass peaks will be visible in the spectrum.

The bacterial growth ratio is calculated by measuring the whole mass peak profile of a microorganism with and without the antibiotic analyzed. The use of an internal standard enables quantification as the ratio of the AUCs of a microorganism incubated with and without the antibiotic: relative growth = $AUC_{BHI + meropenem}/AUC_{BHI}$. In this case, meropenem was used for the evaluation, and brain heart infusion (BHI) broth was used to culture the microorganisms. The results were analyzed by box plots and pseudogels. The use of meropenem at a concentration of 8 μg/ml to detect carbapenem resistance in 108 *K. pneumoniae* isolates yielded a sensitivity of 97.3% and a specificity of 93.5%. As illustrated in Fig. 5, for susceptible bacteria, no visible mass peaks were observed (Fig. 5A and B) (the internal standard is shown for reference purposes), whereas for resistant bacteria, the whole mass spectrum was observed (Fig. 5C and D).

Jung et al. also evaluated this method for detecting resistance against cefotaxime, piperacillin-tazobactam, gentamicin, and ciprofloxacin (115), by analyzing a series of 30 spiked blood cultures and 99 patient-derived blood cultures. Bacterial cells extracted from positive blood culture bottles were used to prepare an inoculum of 5×10^6 CFU/ml. The isolates were evaluated using a concentration of antibiotic that was 1 dilution higher than the EUCAST susceptibility breakpoint for *Enterobacteriaceae*. This is referred to as the MBT-ASTRA breakpoint concentration. The assay yielded almost 100% sensitivity and specificity for all antibiotics evaluated, with poorer results for piperacillin-tazobactam. Identification of bacteria and analysis of susceptibility are possible within 4 h. In another study, Maxson et al. applied this method for the first time to study the susceptibilities of 35 *S. aureus* isolates to several antibiotics, such as ciprofloxacin, oxacillin, cefepime, and vancomycin (116). The incubation time was established as 2 h, and the resistance breakpoints for classifying the isolates were based on current guidelines from the CLSI (117). Interpretative automation was performed with MBT-ASTRA software (Bruker Daltonik), and results were displayed in box-and-whisker plots. In this case, the assay showed an overall accuracy of 95%.

Idelevich et al. developed an interesting method based on the MBT-ASTRA but with microdroplet incubation carried out directly on the MALDI-TOF MS target (118). The isolates (24 *K. pneumoniae* and 24 *P. aeruginosa* isolates) were incubated in a wet chamber, and the broth was supplemented or not supplemented with the antibiotic (meropenem) evaluated. Using 6-μl microdroplets and incubation times of 4 h for *K. pneumoniae* and 5 h for *P. aeruginosa*, the rate of efficiency was maximum, with almost 100% sensitivity. This study is of the utmost importance because it has simplified the procedure and reduced the time required for processing. However, the overall time needed to deliver the results is still too long for the method to be considered a rapid technique for clinical application.

The main advantage of the MBT-ASTRA is the universality of the method for detecting resistance to different antibiotics and different resistance mechanisms. The main disadvantage is the incubation time (2 to 4 h), which is much longer than those of the other MALDI-TOF MS-based approaches used to detect resistance. The incubation time cannot be reduced, as the microorganisms must grow before the susceptibility or resistance of the microorganism can be evaluated. These advantages and disadvantages are also shared by the MBT-Resist method. The difference in the analysis is that the MBT-Resist assay compares single peak shifts derived from the incorporation of heavy labeled amino acids during bacterial protein synthesis with the usual mass peak profile of the bacteria analyzed. This analysis requires a previous species-specific test that is not necessary for the MBT-ASTRA, as it analyzes the whole mass profile of the bacteria for evaluation of susceptibility or resistance.

Quantitative Resistance Profiles Applied to Mycobacteria and Yeast

Ceyssens et al. used the MBT-ASTRA to detect susceptibility to rifampin, isoniazid, linezolid, and ethambutol in *Mycobacterium tuberculosis* and to detect susceptibility to clarithromycin and rifabutin in nontuberculous mycobacteria (119). Although molecular techniques can rapidly detect resistance genes in *M. tuberculosis*, they have not yet replaced the need for culture-based susceptibility assays to provide information about the full spectrum of available antituberculous drugs. Molecular techniques have not been widely developed for the detection of resistance in nontuberculous mycobacteria (120, 121), and broth microdilution is recommended as the gold-standard method (122). However, Cesyssens et al. correctly identified 156/156 *M. tuberculosis* and 65/66 nontuberculous mycobacterium drug resistance profiles by using the MBT-ASTRA. Although this novel application of the MBT-ASTRA yielded excellent results for *M. tuberculosis*, routine use of the technique in clinical settings is unlikely. First, MALDI-TOF MS requires a larger biomass of sample than molecular techniques, which leads to longer turnaround times, especially for slow-growing mycobacteria. Second, turnaround times were not significantly different for *M. tuberculosis* testing, although the MBT-ASTRA method delivered results a week earlier than routine susceptibility meth-

ods for nontuberculous mycobacteria. On average, with the MALDI-TOF MS method, an incubation time of 6 days was needed for both *M. tuberculosis* and nontuberculous mycobacteria. Thus, the method still needs prolonged incubation and a sustained processing time, and there is a greater risk of exposure. Moreover, the technique has not yet been standardized.

Regarding the study of fungi, Marinach et al. used MALDI-TOF MS to detect fluconazole resistance in *Candida albicans* based on monitoring modifications in the mass peak profile of the yeast in the presence or absence of different concentrations of the antifungal agent (123). The yeast cells were incubated with the antifungal agent for 15 h. Protein extraction was performed prior to acquisition of the spectra. The spectra were acquired for 12-fold dilutions starting at 128 μg/ml. The minimal profile change concentration (MPCC), corresponding to the lowest fluconazole concentration at which a mass spectrum profile change can be detected, was established. When relating the value of the MPCC obtained by MALDI-TOF MS with the susceptibility category assigned by using CLSI criteria, only 1 out of 16 isolates was incorrectly classified. Although excellent as a proof of concept, routine application of this method in clinical practice is unlikely due to the long processing time (as the samples must be incubated overnight). De Carolis et al. applied the protocol to study the susceptibilities of *Candida* and *Aspergillus* to caspofungin (124). These researchers used the CCI matrix tool of the MALDI Biotyper to analyze the relationships between the spectra generated at different concentrations of the antifungal. The MPCC was obtained from the matrix, and an agreement of 94.1% was reached in establishing the clinical categories of susceptibility in the *Candida* isolates tested. Finally, Vella et al. used a simplified version of the above-described approach, with an incubation time of 3 h and only three breakpoint concentrations of caspofungin: 0 μg/ml (null), 0.03 μg/ml (intermediate), and 32 μg/ml (maximal). The overall agreement was 98% for comparison of the MALDI-TOF MS methodology with CLSI-established categories of susceptibility (125). The latter approach shows very promising results for further clinical application.

AUTOMATION IN ROUTINE DIAGNOSIS

Although technological advances have been introduced in microbiology laboratories, there is still a real or perceived idea that microbiological assays cannot be automated. In comparison with clinical biochemistry, which has traditionally been highly automated, clinical microbiological analysis still requires expert staff and prolonged processing times (126, 127). Microbiological analysis is often considered too complex to automate because of the various types of samples involved, and automation is considered too expensive for microbiology laboratories. Although complex molecular tests are frequently used in reference laboratories or in virology laboratories, bacteriology is increasingly benefiting from the introduction of these techniques in routine laboratories (128, 129). As a result of the growing demand for microbiological analysis of samples, increased workloads, the shortage of trained personnel, the need for quality standards, and reductions in financial resources, automation has become a real possibility in recent years in clinical microbiology laboratories (130). The emergence of MALDI-TOF MS technology is a major factor enabling automation in microbiology laboratories (131). MALDI-TOF MS is easy to perform and highly automated because it is technically very simple. The ability to perform more tests, with minimal staffing, with less expertise, with high-quality standards, and with guaranteed traceability throughout the process, can be achieved by the use of MALDI-TOF MS.

Ready-To-Use Techniques in Clinical Practice

Throughout this review, we have exposed the different available software packages for analysis of spectra for the detection of antimicrobial resistance. However, not all these software packages have been approved for clinical use. Here, we review the different available products for the various principles that have been described for MALDI-TOF MS and their regulatory status (Table 3).

TABLE 3 Summary of the different MALDI-TOF MS principles for detection of antimicrobial resistance and available analysis software

MALDI-TOF MS principle	Application	Software	Target(s)	Commercially available kit	Regulatory status[b]	Advantage(s)	Disadvantage(s)
Detection of enzymatic activity	Detection of β-lactamases (ESBL and AmpC, etc.)	MBT-STAR BL module for Compass software[a]	Ratio between the sum of areas of the hydrolyzed antibiotic and the intact form for ampicillin, piperacillin, ceftazidime, cefotaxime, ertapenem, and meropenem	No	RUO	Easy and fast technique (~30 min); detects resistance directly from the clinical sample; does not require further interpretation of spectra	No identification of the β-lactamase type; does not provide information regarding the MIC
	Detection of carbapenemases		Ratio between the area under the peak of the internal standard and intact imipenem	MBT STAR-Carba kit	CE-IVD		
	Detection of the AAC(6′)-Ib-cr enzyme	MBT PeakShift prototype[c]	Ratio between the sum of areas of the acetylated antibiotic and the intact form for ciprofloxacin and norfloxacin	No	RUO	Easy and fast technique (~30 min); more simple than the reference method (molecular technique)	Does not provide information regarding the MIC; developed skills for interpretation of spectra needed
Measurement of growth in the presence of an antibiotic	Detection of resistance by measuring protein synthesis in the presence of isotope-labeled amino acids	MBT-Resist[c]	Ratio between the sum of areas of the isotope-labeled peaks and the "normal" peaks	No	RUO	Provides information on susceptibility and resistance to different antibiotics	Requirement for isotope-labeled medium; labor-intensive methodology; time-consuming methodology (~3 h to deliver results)
	Detection of resistance by semiquantification of bacterial growth at the breakpoint concn	MBT-ASTRA[c]	Ratio between the sum of areas of the peak profile of a microorganism incubated with and without antibiotic	No	RUO	Provides information on susceptibility and resistance to different antibiotics; easy handling using the microdroplet assay	Time-consuming methodology (~3 h to deliver results)
Measurement of resistance by analysis of the peak pattern of bacteria	Detection of KPC-producing *K. pneumoniae*	MBT Subtyping module for Compass software[a]	Identification of the 11.109-Da mass peak; identification of the 2.415-Da mass peak; identification of a series of mass peaks in the range of 4,000–5,500 Da	Not necessary[d]	RUO	No more steps are needed other than the minimum needed for identification; information provided after high-confidence identification; does not require further interpretation of spectra	Limited to a few resistance mechanisms; specificity is high, but sensitivity is related to prevalences in different geographic areas
	Detection of MRSA						
	Detection of *cfiA*-positive *B. fragilis*						

[a]Compass software (Bruker Daltonik GmbH, Germany) is an intuitive and integrated software for mass spectrometry analysis that allows modular application in packages. The MALDI Biotyper (MBT) Selective Testing of β-Lactamase Activity (STAR-BL) and the MBT Subtyping modules are two modules of the software.

[b]RUO, research use only; CE-IVD, European Conformity for *in vitro* diagnostics.

[c]MBT PeakShift prototype (Bruker Daltonik), MBT-Resist (Bruker Daltonik), and the MBT-ASTRA (Bruker Daltonik) are RUO software not incorporated into Compass software.

[d]No commercially available kit is necessary for analysis of mass peak profiles, as the procedure is the same as the one used for identification, and no extra reagents are employed.

Compass software (Bruker Daltonik) is an intuitive and integrated software for mass spectrometry analysis that allows a modular application in packages. It is included in the Microflex MALDI-TOF MS Biotyper IVD test. The MBT STAR-BL and the MBT Subtyping modules are two modules of the software. Vitek MS (bioMérieux) does not include any platform for detection of antimicrobial resistance in an automatable manner. Comparison of a profile spectrum acquired from an antibiotic with profile signatures stored in a reference library by visual inspection enables rapid determination of antibiotic susceptibility but requires certain expertise in interpreting mass spectra. Automated methods will allow and facilitate the implementation of the technique in clinical laboratories and support its application by users who are not trained in mass spectrometry techniques. The only commercially available kit for use with software for automated interpretation of spectra is the MBT STAR-Carba IVD kit (Bruker Daltonik) in the MBT STAR-BL software module (Bruker Daltonik). Samples are analyzed using imipenem to detect carbapenemase enzymes. According to the manufacturer, the kit contains enough reagents to test 20 to 58 samples depending on the assay batch size. The kit contains all the reagents required to perform the assay, except for a positive and a negative control, which must be provided by the user and used in all assays. Previous calibration of the equipment must be carried out in the mass range of spectrum acquisition, i.e., in a low-mass range (100 to 1,000 Da). The MBT STAR antibiotic calibration standard (MBT STAR-ACS), which contains a mixture of four small peptides that provide characteristic and well-defined MALDI-TOF mass spectrum signals, can be used for this purpose. The Carba assay can be used with microorganisms from plate cultures or from positive blood cultures. If using blood cultures, previous extraction with the Sepsityper kit (Bruker Daltonik) is necessary, and the procedure used is a slightly modified version of that used for identification. For a standard workflow, incubation of the blood samples with the antibiotic for 30 min is sufficient. However, for testing *Acinetobacter* spp., an incubation time of 60 min is recommended. The software automatically interprets the spectra by normalizing the intensities of the sample mass peaks relative to signal intensities obtained from positive- and negative-control strains. A report is generated with a color-coded plot that enables evaluation along with the logRQ value. Quantification reduces the risk of misclassifying isolates due to a lack of experience in the visual interpretation of mass spectra. Use of the software also reduces intra- and interassay (observer-related) variability, thus yielding standardized results. Moreover, the software enables the identification of the same target and assay of the same colonies being tested to detect carbapenemase activity. Dortet et al. evaluated the commercialized MBT STAR-Carba IVD kit with two in-house MALDI-TOF MS methods and Rapidec Carba NP for detecting carbapenemase activity in 175 isolates, including 120 carbapenemase producers (132). Overall, all systems have very similar performances, with sensitivities ranging from 95% to 100% and specificities ranging from 98.2% to 100%. The advantages of the STAR-Carba IVD kit are the turnaround time, the best for all systems evaluated ($<$1 h); the automated interpretation of results, which can be confusing for Rapidec Carba NP, especially for OXA-48-like enzyme producers with poor expression (133); and the possibility of coupling the software to the laboratory system so that results are directly connected and technical errors are minimized.

Regarding the detection of antimicrobial resistance by analysis of the mass peak profiles of bacteria, the commercially available MALDI Biotyper Subtyping software module (Bruker Daltonik) enables the detection of proxies for specific resistance mechanisms in an automated workflow (134). The prerequisite for automation of the process for identifying resistance markers is high confidence (score of $>$2.0) in identification of the bacterium by the MALDI Biotyper. The process is quite simple, as no additional sample preparation is required after the samples have been directly transferred to MALDI target plates. Once the bacterium has been identified, the software automatically performs typing, comparing the spectrum obtained against a reference library and reporting the results. This module enables the identification of KPC-producing *K. pneumoniae* by searching for the p019 protein-related peak (11.109 Da) in the sample spectrum, which, if present, indicates that the sample is a presumptive

bla$_{KPC}$-positive sample. This module can also be used to detect methicillin-resistant *S. aureus* by detecting the PSM-*mec* peak (2.415 Da). Finally, the module can also be used to detect *cfiA*-positive *B. fragilis* by the detection of specific peaks associated with the protein expressed.

Automation has been achieved in the field of resistance detection by measuring the effects of the antibiotic on microorganism growth; however, these software packages are categorized as RUO and have not yet been included as modules for Compass software (Bruker Daltonik). The same happens for the detection of the AAC(Ib)-cr enzyme, which is available as prototype software and has not yet been released for the public in a commercialized software package.

The MALDI-TOF MS process of deposition of microorganisms on the MALDI target has already been developed by some companies. Chudejova et al. evaluated four different sample preparation methods: manual deposition, in-target extraction with formic acid, wet deposition (in-target extraction with the order altered, where formic acid is applied first and the colony is applied last), and automatic deposition using MALDI Colonyst (Biovendor Instruments, Brno, Czech Republic) (135). For clinical validation, the lowest scores were obtained by manual deposition of the target, and higher identification scores were obtained using automated processing by MALDI Colonyst for all tested microbial groups (Gram-positive bacteria, Gram-negative bacteria, anaerobes, and yeasts). Bruker Daltonik developed MALDI Biotyper Galaxy for automated deposition of colonies on the MALDI-TOF target. The system is coupled with on-board barcode reading so that the targets are matched to their corresponding projects, and traceability is guaranteed for every sample. The system also reduces contamination and frees personnel from routine work. There has not yet been a publication evaluating this system. Broyer et al. evaluated a benchtop instrument (bioMérieux, France) for automated processing of positive blood cultures (136). The yield was 83% correct identifications compared to manual methods. This prototype instrument is based on an "all-in-one" extraction strip. It allows less than 5 min of personnel time and provides identification results in less than 30 min. Moreover, it can be performed on demand and in small batches of positive blood cultures, allowing 24/7 service.

In the future, as different procedures achieve clinical validation, more applications could be integrated into the software and will be available for the user. MALDI-TOF MS should occupy an outstanding place in the microbiology laboratory, coupled with automated systems for seeding, incubation, and digitized reading of plate cultures, thus yielding an integrated operating model with different innovative combinations and capacities.

FUTURE CHALLENGES

As demonstrated above, MALDI-TOF MS has gained value as a multifaceted instrument for clinical microbiology laboratories. Although tremendous progress in MALDI-TOF MS has been made in the past decade, particularly regarding the identification of microorganisms, antimicrobial resistance detection by MALDI-TOF MS has been proposed as a possible application. However, several major challenges must be overcome to enable further progress in clinical proteomics diagnostics in microbiology laboratories.

The following bottlenecks have been recognized: (i) limited MS sensitivity ($>10^5$ CFU/ml) for direct detection in clinical samples (43–45), (ii) the limited number of resistance mechanisms that can be identified by MALDI-TOF MS if only assays involving enzymatic activity of bacteria are chosen and a delayed response time if the effects of antibiotics on bacterial growth are measured, (iii) the need for public libraries of MALDI-TOF MS spectra for analysis of bacterial mass peak profiles, (iv) the lack of established and standardized routines for MALDI-TOF MS-based technology in detecting antimicrobial resistance, (v) the limited range of automated applications and commercially available kits, and (vi) the lack of studies on the cost-effectiveness of incorporating resistance detection by MALDI-TOF MS.

The balance between costs and savings is one of the main advantages of the new

technologies relative to traditional methods (137, 138). The most important clinical benefits of MALDI-TOF MS-based identification observed in the literature are the earlier administration of correct antimicrobial therapy, reduced morbidity and mortality, reduction in lengths of hospital stays, and reduced overall costs per hospitalized patient. However, no clinical benefits have yet been reported regarding the implementation of MALDI-TOF MS for resistance testing. This should be one of the main cornerstones of further research. In our opinion, the application of MALDI-TOF MS for detecting antimicrobial resistance will supplement the results obtained for the identification of microorganisms, providing more-accurate results to clinicians.

One of the first studies to evaluate the clinical impact of MALDI-TOF MS on patient outcomes was conducted by Huang et al. (139). These researchers proposed integrating MALDI-TOF MS into the workflow of an antimicrobial stewardship program. This study included 501 patients with bacteremia or candidemia for evaluation and 245 patients in the intervention group. For patients included in the MALDI-TOF MS group, the time to organism identification and the time to effective antibiotic therapy were reduced, optimal antibiotic therapy was provided, the mortality rate was lower, and the length of intensive care unit stay and the risk of recurrent bacteremia were reduced.

Clerc et al. conducted a prospective observational study regarding the clinical impact of MALDI-TOF MS for identifying positive blood cultures grown in samples from patients with bacteremia caused by Gram-negative bacilli compared with the information obtained by use of Gram staining (140). In 35% of the episodes ($n = 202$), the information generated by MALDI-TOF MS had a clinical impact by modifying empirical therapy. The most remarkable consequence was the correct broadening of the antibiotic spectrum in 43.7% of cases. In those cases in which MALDI-TOF MS did not have a documented impact, modification of therapy would have been possible for 24% of cases. Factors involved in not taking MALDI-TOF MS results into account included younger age of the patient, admission of the patient in an intensive care unit, male sex, and a lack of confidence shown by medical staff in the use of an unfamiliar technology. Verroken et al. evaluated the impact on antimicrobial prescription of a workflow in which the identification was provided by MALDI-TOF MS (141). Partial susceptibility results were performed regarding the previously obtained identification results and were provided on the same day as the identification of blood cultures positive for *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *S. aureus*. The mean time to identification was reduced by around 65%, the mean time to complete susceptibility results was reduced by 27%, and the mean time to optimal antimicrobial treatment was decreased to 16 h. Beganovic et al. evaluated the impact of MALDI-TOF MS in combination with antimicrobial stewardship intervention on patient outcomes (142). The primary outcome was the time to optimal therapy, which was significantly reduced by the use of MALDI-TOF MS. The mean time to microbiological clearance, the mean length of hospital stay, the mean length of stay in a critical care unit, the number of patients with recurrence of the same bacteremia, and the mean length of time of antimicrobial therapy were also reduced. Osthoff et al. evaluated the impact of MALDI-TOF MS on the management of bacteremic patients, by applying a single-center, open-label, controlled clinical trial with 425 patients during 1 year (143). Surprisingly, rapid identification by MALDI-TOF MS directly from positive blood cultures in this study did not affect the duration of intravenous antimicrobial therapy or the length of hospital stay; however, identification of contaminated blood cultures led to a shorter duration of therapy (mean time, 4.8 versus 7.5 days).

Further cost-effective multicenter studies are required to evaluate the impact of resistance testing by MALDI-TOF MS using the above-reported methodologies and others yet to be developed. It will be very interesting to observe the clinical impact of these determinations and the situations in which the use of MALDI-TOF MS will be preferred for detecting antimicrobial resistance or when other similar tests, such as immunochromatographic techniques, biochemical tests, and/or molecular tests, should be used. Although further knowledge is needed to optimize the routine application of antimicrobial resistance detection by MALDI-TOF MS, the simplicity, rapidity, and

accuracy of this technology will enable MALDI-TOF MS to be included in the workflow of all antimicrobial stewardship programs.

Another potential approach that should be followed up is the detection of high-risk clones associated with multidrug-resistant bacteria. In this approach, the relatedness between clusters of bacteria can be measured faster and more cost-effectively than by molecular methods, and more information regarding antimicrobial resistance can be obtained directly or indirectly, i.e., by detecting biomarkers associated with resistance to antimicrobial agents (144–146). We should also take into account that whole-genome sequencing is evolving very rapidly, especially with nanopore technology (147, 148). The main advantage that MALDI-TOF MS offers is that information will be obtained at no additional cost besides those already incurred for identification purposes. Specific guidelines need to be standardized for the different species in order to interpret spectra for epidemiological purposes (149–152). In the future, we will see if MALDI-TOF MS has the ability to displace molecular techniques in infection control measures.

Automation in microbiology laboratories will enable the application of telebacteriology with expert systems and instrument interfaces and, thus, the creation of new routines (153, 154), for example, the use of robotic incubation and assignment via screen technology for specific follow-up for growing colonies in plate cultures. This technique could even be applied to positive blood cultures, with automated reading at any time of day and with subsequent downstream applications such as MALDI-TOF MS identification and antibiotic resistance detection. Samples could be examined in real time, and those requiring a rapid response in relation to antimicrobial therapy could be identified.

The effectiveness of MALDI-TOF MS makes this technology absolutely necessary in current microbiology laboratories, not only for the identification of microorganisms, as established above, but also for the detection of antimicrobial resistance and other applications. While advances are being made in automated technology, we must gain the maximum clinical benefit in its application. Even more importantly, the optimal workflow must be determined, particularly in relation to those clinical samples for which the greatest benefit will be obtained, the most suitable isolates, and the most suitable time of the day for performing the assays. Other questions that must be addressed are whether it is necessary for procedures to be carried out 24/7 in microbiology laboratories, whether it will be possible to stop routine identification to perform resistance assays, and what type of information is most urgent. One of the main issues that must be resolved is how to prioritize the different techniques that can be performed with MALDI-TOF MS by developing comparative studies of cost-benefit relative to other phenotypic, molecular, or biochemical assays. Although automation will speed up procedures and improve quality standards, the future of most microbiology laboratories will depend on the acquisition of at least two pieces of equipment to facilitate the workflow and to enable handling the growth in demand for diagnostic techniques. However, justifying the acquisition of a second instrument is not easy for nontertiary hospitals with limited budgets. In our experience, the use of MALDI-TOF MS for resistance detection has important value in having a positive impact on health care assistance, so it should justify to a certain extent the slowdown of microorganism identification results. In this regard, we are aware that MALDI-TOF MS cannot replace conventional antimicrobial susceptibility testing, and it must be done in addition to routine testing. This is mainly because the sample processing time is longer for applying MALDI-TOF MS techniques, because the whole process is not automated so far, and because the antibiotic MIC is not known for most of the above-described methods. Besides, it has the inconvenience of requiring technologists' time. Because of that, we think that the most appropriate position for antimicrobial resistance detection by MALDI-TOF MS is as a rapid technique to solve or confirm individual cases of antimicrobial resistance, such as carbapenemase detection, in a similar way as biochemical methods, like the Carba NP method, and molecular techniques are applied. Thus, the number of tests per day would not delay the results of identification in a

significant way. However, as we have cautioned above, each laboratory should evaluate its own situation and evaluate the diagnostic demand for improving health care assistance.

Manufacturers must be able to rapidly adapt to evolution in the laboratory, by improving systems and adapting novel protocols. The time-consuming hands-on portion of the process should need further automation so that we can reduce the preanalytical steps. We think that this is possible by simplifying centrifugation steps and the automated deposition of the analyte on the MALDI-TOF MS target. It is very important that users keep their equipment supervised by expert engineers so that maintenance is performed according to the technical specificities and results are optimized to the maximum level. If manufacturer recommendations are not followed strictly, users could observe poor spectral quality, with variations in signal intensities, signal-to-noise ratios, resolution, and so on, that can finally result in false-positive or -negative results.

In our opinion, the second most interesting application of MALDI-TOF MS in microbiology laboratories is for the detection of high-risk clones harboring specific antimicrobial resistance mechanisms, as it actually simplifies currently used typing methods, and what is more important, no extra hands-on time is needed beyond identification. However, this technique should still be applied with caution. Antimicrobial resistance is a complex process that is the result of, but not limited to, chromosomic mutations, acquisition of resistance determinants, different levels of expression, efflux pumps, and loss of porins, etc. MALDI-TOF MS will not replace any of the preexisting techniques for detection of antimicrobial resistance but will add accurate and valuable information at a very low cost in a limited time.

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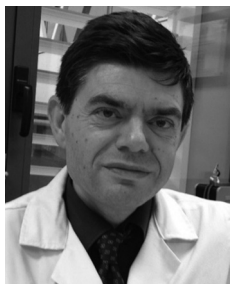
Marina Oviaño, Ph.D., graduated in Chemistry in the Faculty of Chemistry in Oviedo, Spain. She completed her residence in clinical microbiology at the University Hospital A Coruña and her doctoral studies in the Health Sciences University of A Coruña, Spain. Dr. Oviaño served as a researcher at the National Health System in Spain (2016 to 2017), and at present, she works as a clinical microbiologist at the Microbiology Department of the University Hospital A Coruña. She has been working on research in the field of bacterial resistance to antibiotics, focused on the use of MALDI-TOF MS as a rapid technology for diagnostic use.



Germán Bou received his Ph.D. from the Molecular Biology Center (CSIC)-Autonoma University, Madrid, Spain. He also completed a residence in clinical microbiology at Ramon y Cajal Hospital. Afterwards, he was granted a postdoctoral position with the Fulbright Scholarship Program to work in the Department of Laboratory Medicine and Pathology at Mayo Clinic, Rochester, MN. Dr. Bou then served as an investigator at the National Health System in Spain (2001 to 2005) and as a consultant in clinical microbiology (2005 to 2010), and at present, he is the Head of the Microbiology Department of the University Hospital A Coruña. Since 2009, he has been associate professor of medical microbiology at Santiago of Compostela University. Dr. Bou's research focuses on the understanding of the molecular basis for antimicrobial resistance in human pathogens, developing rapid tests for detecting resistant organisms, and designing and developing bacterial vaccines. So far, he has published more than 200 international peer-reviewed manuscripts on these topics as well as obtained 7 related patents. Recently, he was appointed with the honorary title of ESCMID fellow for professional excellence and service to society.