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REVIEW

Detection of carbapenemase producers by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS)

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Abstract
Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been recently applied in detection of carbapenemase-producing Gram-negative isolates. In the present study, we review the latest developments in this field.

Keywords Carbapenemase · MALDI-TOF · 11,109 *m*/*z* peak · 28,544 *m*/*z* peak · Carbapenemase-producing *Enterobacteriaceae* · Carbapenem resistance · DOT-MGA

The production of carbapenemases by Gram-negative bacteria is a major health concern worldwide. The timely detection of carbapenemase producers in clinical samples is of critical importance. Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has been shown to be an effective method with which this may be achieved [1, 2]. There are three different approaches: (a) the detection of carbapenem hydrolysis approach, (b) the detection of specific MS peaks approach, and (c) the evaluation of carbapenem MIC approach. The first is the most often implemented approach; the second includes the detection of the 11,109 and the 28,544 *m*/*z* peaks highly correlated to KPC production; and the third approach refers to the DOT-MGA assay.

In the present study, we review the latest developments in this field.

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The initial reports

The central hypothesis of the hydrolysis approach is as follows: carbapenem provides certain MS peaks and a certain MS profile. In order to determine whether a bacterial strain is a carbapenemase producer, this strain should be initially incubated in the presence of an indicator carbapenem and following the centrifugation of the mixture; the bacterial free supernatant should be processed with MALDI-TOF MS. If the known indicator carbapenem MS profile is present, then no hydrolysis has occurred and the conclusion is that the tested strain is not a carbapenemase producer. On the other hand, if the known indicator carbapenem MS profile disappears and the known MS peaks of its hydrolysis products are present, then there is sufficient evidence of carbapenemase production.

In the publication volume of September 2011 of the same medical journal, the first two reports of the detection of carbapenemase production by MALDI-TOF MS were published [1, 2]. Hrabák et al. efficiently detected carbapenemase production in *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates (sensitivity 96.67%, specificity 97.87%) by comparing the mass spectra obtained for native meropenem with that obtained following a 3-h incubation period of the bacteria in the presence of meropenem [1]. In the cases of carbapenemase production, a mass profile with lack of the known specific mass peaks was indicative of meropenem hydrolysis and the result was considered positive. Burckhardt et al. determined the characteristic mass spectrum of pure ertapenem [2]. They subsequently processed

carbapenemase- and non-carbapenemase-producing *Enterobacteriaceae* and *P. aeruginosa* strains, using an up to 2.5-h incubation protocol in the presence of ertapenem. Following this incubation step, the disappearance of the specific mass peaks for ertapenem was considered as a positive result for carbapenemase production. For the detection of certain carbapenemases, the incubation period needed was much lower than 2.5 h, namely 1.5 h for IMP-2, KPC-2, and VIM-1 and only 1 h for NDM-1 and IMP-1.

Validation and improvement of the method

In the years following the publication of the initial two reports mentioned above, this methodology was validated and improved by several research teams as regards the incubation time, the type of bacteria tested, the indicator carbapenem, the presence of inhibitors, or the matrix used. Among others, Kempf et al. applied the methodology in *Acinetobacter baumannii* clinical isolates for the first time in 2012 using imipenem as the indicator carbapenem [3]. In the same year, Hrabák et al. proposed modifications of their previously described meropenem hydrolysis assay (MHA), validating it in both *Enterobacteriaceae* and *A. baumannii* [4]. Sparbier et al. noted that the minimal change in the mass spectrum following the hydrolysis of a beta-lactam antibiotic was the disappearance of the molecular peak and its salt adducts [5]. However, it was underlined that in order to have more reliable results, it is preferable not only to monitor the disappearance of a mass peak but also to observe the corresponding products which are generated through hydrolysis or by subsequent reactions [5].

Comparing their results with those obtained by liquid chromatography-mass spectrometry, Carvalhaes et al. used MALDI-TOF for the detection of carbapenemase activity among SPM-1-, GIM-1-, and GES-5-producing *Pseudomonas aeruginosa* isolates and among OXA-143-, IMP-10-, and OXA-58-producing *A. baumannii* isolates for the first time [6]. They also noted that for the detection of carbapenem-hydrolyzing class D-lactamase (CHDL) activity in *Acinetobacter* spp., an extended incubation time was necessary. Álvarez-Buylla et al. aimed to optimize the bacterial inoculum and incubation time for carbapenemase detection in *Acinetobacter* spp. [7]. Moreover, the addition of dipicolinic acid (DPA) and Zn(2+) in the incubation solution made the differentiation between metallo-beta-lactamases (MBLs) and oxacillinases possible. Wang et al. developed specific software for the detection of carbapenemase producers [8]. Furthermore, as it had been previously reported, they noted that the time to the degradation of the indicator carbapenem is correlated with the enzyme carried, but not with the MICs of carbapenems [8].

Apart from only detecting carbapenemase production in *Enterobacteriaceae* and *Pseudomonas* spp. with MALDI-TOF MS, Hoyos-Mallecot et al. managed to discriminate the metallo-beta-lactamases using ethylenediaminetetraacetic acid (EDTA) as an inhibitor [9]. Johansson et al. proposed an assay based on ertapenem using 2,6-pyridinedicarboxylic acid for VIM/NDM and 3-aminophenylboronic acid (APBA) as inhibitors for KPC [10]. They found that ertapenem hydrolysis by KPC was detectable after only 15 min, whereas for MBLs a 2-h incubation period was needed. They suggested that the addition of inhibitors in the assay may not be necessary, as the time to detection was highly specific for the separation of KPC from MBL enzymes [10]. In the same year, Sauget et al. used an optimized MALDI-TOF MS imipenem hydrolysis assay to detect OXA-48-like-producing *Enterobacteriaceae* [11].

It should be noted that the development of the detection of carbapenemase production with the MALDI-TOF MS methodology was in large based on the use of the Bruker MALDI-TOF MS system and the corresponding software (Bruker Daltonics). Knox et al., using the VITEK MS research use only (RUO) system (bioMérieux, France) with an imipenem hydrolysis assay, reported a sensitivity of 87% and a specificity of 100% in detecting carbapenemase-producing *Enterobacteriaceae*, stating that for clinical laboratories using Vitek MS that have access to the appropriate software, the method described in their study could be relatively easily implemented [12]. Furthermore, Carvalhaes et al. using the VITEK MS platform reported detection rates of 95% of class A and 87% of class B carbapenemase-producing isolates after 60 min of incubation with ertapenem as the indicator [13].

Studentova et al. and Papagiannitsis et al. provided evidence that the addition of NH_4HCO_3 to the reaction buffer significantly improved the ability of the assay to detect OXA-type producers [14, 15]. Moreover, Papagiannitsis et al. evaluated the performance of the MTB STAR/BL prototype software (Bruker Daltonics) for the automatic analysis of raw spectra with good results for *Enterobacteriaceae* [15]. In the same year, Mirante et al. used faropenem as an indicator compound for the first time [16].

Monteferrante et al. reported 100% sensitivity and specificity for the detection of KPC, NDM, and OXA-48-like positive isolates using a protocol involving cellular lysis and enzyme extraction from the bacterial cells [17]. Extracts were made in order to obtain a higher yield of beta-lactamase enzymatic activity and thus a better MS detection. For characterization experiments, the inhibitors phenylboronic acid and 2,6-pyridinedicarboxylic acid were used for KPC+ and NDM strains, respectively [17]. Ramos et al. observed that false-negative results were more frequently found when carbapenemase-producing *A. baumannii* isolates were grown on MacConkey agar medium, even when the incubation period with ertapenem was extended to 4 h [18].

For the rapid detection of carbapenemase activity in *Enterobacteriaceae* by MALDI-TOF MS, Oviano and Bou proposed the use of the novel combination of imipenem-

avibactam with good results [19]. Knox and Palombo reported an improved detection of MBL-producing strains without compromising the detection of other carbapenemase types with the addition of $ZnSO_4$ in the MALDI-TOF MS–based imipenem hydrolysis assay [20]. A modified MHA was proposed by Calderaro et al. and it was validated using a large collection of 981 carbapenemase-producing *Enterobacteriaceae* [21]. They also observed that different shipment conditions of the indicator antibiotic (meropenem) could affect the drug degradation and this was taken into account for the improvement of the interpretation of the results. For the better detection of carbapenemase-producing *P. aeruginosa* strains, a new MALDI-TOF MS imipenem hydrolysis assay was proposed by Miltgen et al. [22]. This research group managed to correctly discriminate carbapenemase producers ($n = 74$) from non-producers ($n = 95$) in 99.4% of cases. They obtained these results by allowing beta-lactamases to leak out bacterial cells by partial disruption of the outer membrane barrier using a combination of high concentration of colistin and a freezing-thawing step [22]. In 2018, Chang et al. developed a novel method using detonation nanodiamond as a platform for the concentration and extraction of *A. baumannii* carbapenemase–associated proteins prior to MALDI-TOF MS analysis [23]. As mentioned by the authors, this approach could directly detect the carbapenemase-associated proteins of *A. baumannii* within 90 min and did not require the addition of carbapenemase substrate, which was required in other mass spectrometric methods [23].

Further validation and improvements of the methodology have been provided by several other research groups [12, 24–30], and the field has also been previously reviewed [31, 32].

A commercial assay

The MBT STAR-Carba kit (Bruker Daltonics) was the first commercial assay to detect carbapenemase production by Gram-negative bacteria using MALDI-TOF MS and it was evaluated by Rapp et al. [33]. A collection of *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp. clinical isolates was tested. Bacterial colonies were mixed with the provided antibiotic solution and incubated for 30 (*Enterobacteriaceae* and *P. aeruginosa*) and 60 min (*Acinetobacter* spp.). Following centrifugation, the supernatant was applied on a MALDI target plate and overlaid with the provided matrix. The final spectra were automatically analyzed by the software and results were obtained. The assay correctly identified all carbapenemase-positive *Enterobacteriaceae* (31/31) and *P. aeruginosa* isolates (4/4), while it failed to detect half of the carbapenemase-positive *A. baumannii* isolates (4/8) harboring *blaOXA*$_{-23}$-like and *bla*$_{OXA-24}$-like genes. The extended incubation time (2 h) did not improve the results regarding these false-negative isolates. All carbapenemase-negative isolates were correctly tested negative by the assay [33]. A further evaluation of the assay was reported in 2018 by Dortet et al. [34]. A collection of 175 *Enterobacteriaceae* that have previously been characterized for their beta-lactamase content at the molecular level were included in the study. A sensitivity of 100% and a specificity of 98.2% were reported. Furthermore, the repeatability of the assay was evaluated by repeating the test three times for each strain using a 7-day interval between repetitions. An almost perfect repeatability of the technique was found [34].

Direct detection of carbapenemase production from blood cultures using MALDI-TOF MS

Due to its critical importance, many efforts have been made to detect carpanemase production with MALDI-TOF MS directly from positive blood cultures. In 2012, Sparbier et al. reported that the characteristic peak pattern found for the discrimination of carbapenemase-positive- and carbapenemase-negative-plated bacteria could also be seen for bacteria from fresh positive blood cultures [5]. Initial efforts to detect beta-lactamase activity directly from positive blood cultures were made in 2014 by Jung et al. using aminopenicillin and third-generation cephalosporin as indicator antibiotics for beta-lactamase activity [35]. In the same year, Carvalhes et al. provided evidence that MALDI-TOF MS was able to detect carbapenemase production directly from positive blood culture vials for all KPC-2-producing and SPM-1-producing isolates [36]. Hoyos-Mallecot et al. reported carbapenemase detection and pathogen identification from blood cultures in 4.5 h with excellent sensitivity (100%) and specificity (100%) [37]. High rates of carbapenemase detection from positive blood cultures were reported by Fernández et al. following a 4-h subculture in Mueller-Hinton [38].

Further validation along with improvements of this methodology has also been provided by other research groups [39–41].

Oviano et al. proposed a universal method for detecting automatically the carbapenemase producers among *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. from positive blood cultures within 30 min [32, 42]. This was achieved by the addition of imipenem to a buffer containing NH_4HCO_3 (better detection of OXA-48 producers), $ZnCl_2$ (better detection of some MBLs in *Pseudomonas* species) and sodium dodecyl sulfate (SDS; better reactivity for *Acinetobacter* spp.). For the extraction from the blood culture, a Sepsityper kit (Bruker Daltonik) was used applying a modified protocol. An overall sensitivity of 98% and a specificity of 100% were reported.

Direct detection of carbapenemase production in urine clinical samples using MALDI-TOF MS

In order to detect carbapenemase-producing *Enterobacteriaceae* directly in clinical urine samples, Oviaño et al. developed an interpretative automated MALDI-TOF MS–based method [43]. The samples for the study were selected using a flow cytometry cutoff value of $\geq 1.5 \times 10^5$ bacteria/ml and they were processed with a Sepsityper kit for bacterial extraction. Imipenem was used as an indicator and the results were automatically interpreted with the MALDI-TOF Biotyper Compass software (Bruker Daltonik). Excellent sensitivity (100%) and specificity (100%) were reported. Despite the fact that the method required a large volume (10 ml) and furthermore, it could be applied to only monomicrobial infections, the final results were obtained within 90 min of sample reception, which was a great advantage of the method [43].

The 11,109 *m/z* MS peak approach

Although Lau et al. did not directly detect carbapenemase production from *K. pneumoniae* outbreak isolates, they managed to reveal the presence of a bla_{KPC}-containing plasmid (pKpQIL) by identifying a ~11,109 *m/z* MALDI-TOF MS peak corresponding to the cleavage product of pKpQIL_p019, a protein encoded by the pKpQIL plasmid [44]. This specific MS peak was present in the spectra of all 18 clinical *K. pneumoniae* outbreak isolates resistant to carbapenems and absent from 44 control bla_{KPC}-negative clinical *Enterobacteriaceae* isolates. They additionally demonstrated that the detection of this specific MS peak directly from simulated (spiked) positive blood cultures was possible. In pKpQIL-like plasmids, the bla_{KPC} gene is located within a Tn*4401* transposon sequence, whereas the pKpQIL_p019 gene is located adjacent to the Tn*4401* transposon sequence between transposase and resolvase genes and therefore appears to be closely linked to the bla_{KPC} gene [44]. It is significant that the pKpQIL_p019 protein sequence is present not only within the pKpQIL-like plasmids but also to other bla_{KPC} Tn*4401*-containing plasmids not closely related to the pKpQIL family [44]. Although the presence of the bla_{KPC} gene is not always a synonym to carbapenemase activity, the great advantage of this methodology is that MALDI-TOF MS data routinely collected for organism identification could be directly analyzed for the presence of the bla_{KPC}-containing plasmid.

The same research group provided a second study, evaluating the clinical performance of MALDI-TOF MS to detect pKpQIL_p019 (p019) protein and proposed an in-house software script for the rapid detection of the relevant 11,109 *m/z* MS peak [45]. As reported, blind analysis of 140 characterized *Enterobacteriaceae* isolates using two protein extraction methods (plate extraction and tube extraction) and two peak detection methods (manual and automated) showed sensitivities and specificities ranging from 96 to 100% and from 95 to 100%, respectively. The authors underlined that this methodology can provide the real-time detection of certain carbapenem-resistant bacteria, which contain a bla_{KPC}-harboring plasmid using mass spectra acquired as part of routine organism identification without placing additional constraints on laboratory resources [45]. The correlation between the 11,109 *m/z* peak and the bla_{KPC}-harboring pKpQIL-like plasmids was also confirmed by Gaibani et al. using a collection of 34 well-characterized unrelated *K. pneumoniae* strains collected in Italy [46]. The peak was detected in 88.2% of the KPC producers. Moreover, this research group analyzed the whole genomes of 159 KPC-producing *K. pneumoniae* strains isolated from different countries and demonstrated that *p019* gene was present in 135 (97.8%) of 138 genomes harboring Tn*4401*a and absent in 20 KPC-producing *K. pneumoniae* genomes harboring the isoform Tn*4401*b [46]. They suggested that the 11,109 *m/z* MS peak approach is suitable as a first step for the direct screening of KPC-producing *K. pneumoniae*.

Recently, Centonze et al. provided the first evaluation of this approach using the MALDI-TOF VITEK MS system (BioMerieux) [47]. They confirmed the correlation between KPC production and the presence of the specific MS peak, reporting its presence in the spectra of 99.4% (175/176) of the KPC-producing strains compared with the controls, which all lacked the peak [47].

Investigating a large collection of 6209 *K. pneumoniae* isolates from Italy and Germany by applying the 11,109 *m/z* peak methodology, Cordovana et al. managed to instantly detect the KPC-producing strains simultaneously during the standard routine species identification process [48]. This was achieved by integrating a specific algorithm into the MALDI Biotyper system (Bruker Daltonik). The automated peak detection provided an excellent specificity (100%) and a good sensitivity (85.1%) [48]. The methodology, which was further applied directly to positive blood cultures, provided a very fast detection of KPC-producing strains [48].

In contrast with the abovementioned good results by the application of the 11,109 *m/z* peak approach, in a very recent study, Figueroa-Espinosa et al. found only a 32% association of the 11,109 *m/z* signal with *K. pneumoniae* and *Escherichia coli* KPC-positive isolates [49].

Although this novel approach using the specific signal as a biomarker for KPC production provides indirect evidence of carbapenemase activity and is related only to KPC production, it has the advantage of the instant detection of KPC-producing bacteria during the routine identification process in a clinical microbiology laboratory, thus allowing for an instant clinical and epidemiological response. However, even in the case of KPC, its sensitivity largely depends on the KPC environment that predominates in a certain region, leading to instability. If

the isoform Tn*4401a* predominates, then good results will be obtained. If other KPC genetic contexts prevail, then the results will be poor. Additional studies validating this approach within different clinical settings are anticipated in the near future.

Direct detection of KPC-2: the 28,544 *m/z* MS peak

Recently, Figueroa-Espinosa et al. reported 100% sensitivity and 100% specificity regarding KPC-2 detection by identifying the specific 28,544 *m/z* MS peak provided by the KPC-2 enzyme [49]. The signal was present in all 60 *Enterobacteriaceae* and *P. aeruginosa* KPC-2-producing clinical isolates tested independently of where the gene bla_{KPC-2} was embedded. Furthermore, this methodology could be applied to both colonies and blood culture bottles. Following a protein extraction step, the samples were loaded on the MALDI-TOF target plates and mass spectra within the mass range of 17,000 to 50,000 *m/z* were obtained and analyzed. The spectra revealed that the presence or absence of the 28,544 *m/z* MS peak was correlated with the presence or absence of KPC-2. As the authors stated, by applying this protocol using a single sample preparation method, it was possible to carry out the bacterial identification (even the p019 resistance biomarker) and detection of KPC production in < 1 h (usually 30 min), employing different matrices [49]. It is a very promising approach and additional studies of larger scale are anticipated.

MALDI-TOF MS–based direct-on-target microdroplet growth assay (DOT-MGA)

In 2018, a novel approach in the detection of antibiotic resistance by MALDI-TOF mass spectrometry was proposed by Idelevich et al. and furthermore, with two subsequent studies, this research group reported the implementation of this assay in detecting ESBL and AmpC in clinical isolates along with the detection of carbapenem resistance directly from positive blood cultures [50–52]. The assay has recently been reviewed [53].

The principle of this methodology is as follows [53]: the microorganisms are incubated with and without (growth controls) the index antibiotic in nutrient broth as microdroplets directly on MALDI-TOF MS target spots. An antibiotic concentration gradient can be achieved in a series of consecutive spots. In order to avoid evaporation of the microdroplets, the target is incubated in a simple plastic transport box (Bruker Daltonik), using it as a humidity chamber with the addition of 4 ml water onto the bottom of the plastic box. After the incubation period, the broth is separated from microbial cells by just contacting the microdroplets with an absorptive material. MALDI-TOF can detect the presence or absence of the strain growth in each spot and identify the microorganism. Since the exact concentration of the antibiotic in each droplet spot is known, the MIC can be evaluated and the microorganism can be characterized as susceptible or not. With this assay and using a panel of different antibiotics, not only can the susceptibility status of an isolate be determined but also the underlining resistance mechanisms be revealed. Apart from its easiness, rapidity, and practicality, this methodology has the great advantage of generalizability. Its validation by larger scale studies and future developments is highly anticipated.

Due to its simplicity, its low cost, and its rapid turnaround time, MALDI-TOF MS has been established as the major methodology for bacterial identification. Every day, an increasing number of microbiology laboratories worldwide acquire a MALDI-TOF MS system and successfully incorporate it into the routine diagnostic workflow. Over the past few years, its use has been greatly expanded. One of its most important developments is its use in the detection of resistant pathogens and particularly those producing a carbapenemase. Rapid detection of carbamenamase producers in clinical samples and especially in positive blood cultures is of critical clinical and epidemiological importance, as it allows for a correct and timely antimicrobial treatment, prevents outbreaks and the spreading of dangerous clones, and significantly improves the overall patient management. Since the first report of this methodology in 2011, the detection of carbapenemase producers with MALDI-TOF MS has been substantially improved and results can be obtained within minutes. This methodology is highly established and its application is strongly advocated.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethic approval No ethical approval was required.

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