OPINION

Rapid identification of Gram-negative pathogens from positive blood cultures using MALDI-TOF MS following short-term incubation on solid media

IOANNIS K. NEONAKIS[1] and DEMETRIOS A. SPANDIDOS[2]

[1]Department of Clinical Microbiology and Microbial Pathogenesis, University Hospital of Heraklion, 71201 Heraklion;
[2]Laboratory of Clinical Virology, School of Medicine, University of Crete, 71003 Heraklion, Greece

Abstract. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) enables the timely and reliable identification of microbes. The rapid identification of Gram-negative bacteria (GNB) in bloodstream infections is of critical importance. Several protocols have been proposed for the application of MALDI-TOF MS on samples from positive blood cultures (BCs) within the same day of BC positivity detection. The majority of these protocols include sample preparation steps with the use of chemicals or repeated centrifugations in order to avoid biases from human cells and proteins from the BC broth. These additional steps increase the hands-on processing time and the cost of identification. A different approach is to perform a MALDI-TOF MS analysis using biomass from briefly incubated subcultures on solid media. The present study discusses the findings of previous studies regarding the rapid identification of GNB from positive BC broth using MALDI-TOF MS following a short-term incubation period on solid media without any other additional steps or procedures.

Rapid identification of Gram-negative bacteria from positive blood cultures using MALDI-TOF MS

The rapid identification of Gram-negative bacteria (GNB) in bloodstream infections, particularly in septic patients, is of critical importance. Routinely, when a blood culture (BC) provides a positive signal, the broth is sub-cultured on solid

Correspondence to: Dr Ioannis K. Neonakis, Department of Clinical Microbiology and Microbial Pathogenesis, University Hospital of Heraklion, Voutes, 71201 Heraklion, Greece
E-mail: ineonakis@gmail.com

media and incubated 'overnight' for 18-24 h. The material from mature colonies is then processed for identification and antibiotic susceptibility testing (AST), usually requiring an additional 24-h period. Over the past few years, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been introduced in a number of microbiology laboratories, enabling the identification of pathogens within minutes. However, conventionally, it also requires material from mature colonies; thus, MALDI-TOF MS analysis can only be performed 1 day after the day of BC positivity. Several protocols have been proposed for the application of MALDI-TOF MS on samples from positive BCs within the same day of BC positivity detection. The majority of these protocols include sample preparation steps with the use of chemicals/consumables or repeated centrifugations in order to avoid bias from human cells and proteins from the BC broth (1-4). These additional steps increase the hands-on processing time and the cost of identification. A different approach is to perform a MALDI-TOF MS analysis using biomass from briefly incubated subcultures on solid media. This very simple and inexpensive strategy appears to have the potential to prevail (5). The present study discusses the findings of previous studies regarding the rapid identification of GNB using MALDI-TOF MS following a short-incubation period on solid media without any other additional steps or procedures even simple ones, such as 'flash spins' (6).

Idelevich *et al* (7), in 2014, plated two drops (50 μl; optimized inoculum) on and spread over the surface of a Columbia blood agar plate and performed MALDI-TOF MS analysis using the Microflex LT system (Bruker Daltonics GmbH & Co. KG) acquiring microbial biomass at the time-points of 1.5, 2, 3, 4, 5, 6, 7, 8 and 12 h of incubation. The experiment was continued until the time point of successful identification. Incubation was performed in air with 5% CO_2 at 36˚C. The MALDI-TOF MS analysis was performed in triplicate and the identification was considered valid at the genus level and at the species level when at least one of the three spots provided an identification score of ≥1.7 and ≥2, respectively. The mean identification time for aerobic GNB at the genus and species level was 1.7 and 2 h, respectively with the shortest mean time

at the species level being that for *Escherichia coli* (1.8 h) and the longest that for *Pseudomonas aeruginosa* (3 h). Following 4 h of incubation, 95.2% of all aerobic GNB (40/42) had been successfully identified (Table I). In particular, correct identification rates at the species level for these bacteria following an incubation period of ≤2, ≤4, ≤6, ≤8 and ≤12 h were 76.2, 95.2, 97.6, 97.6 and 97.6%, respectively.

In the same year, Bhatti *et al* (8) presented the first study employing pre-warmed plates for rapid identification using the Vitek MS system (bioMérieux). Samples appearing in the Gram-staining to be polymicrobial or having yeasts were excluded from the study. They used trypticase soy agar with 5% sheep blood agar plates (BAPs) that were prewarmed at 37°C for 1 h. Among other methods requiring additional centrifugation steps, they compared the efficacy of two simple methods namely the direct streak method and the direct spot method. In the first method, a single drop was dispensed onto a BAP and streaked into four quadrants, whereas in the second method, a drop was just dispensed onto a BAP without streaking. The incubation was performed in a 5% CO_2 atmosphere at 37°C and MALDI-TOF MS analysis was performed at 2, 4, 6 and 8 h. If identification was achieved, no further time points were analyzed. Among 47 GNB, there were 34 Enterobacteriaceae. The identification rates among GNB using the direct streak method at 2, 4, 6 and 8 h were 53, 79, 87 and 89%, respectively, whereas when using the direct spot method, the rates were 45, 87, 91 and 91%, respectively. The identification rates among Enterobacteriaceae using the direct streak method at 2, 4, 6 and 8 h were 47, 79, 91 and 94%, respectively whereas when using the direct spot method, the rates were 47, 94, 94 and 94%, respectively (Table I). The identifications provided until the 8-h time point were at the species level with the exception of two *Salmonella* sp. and one *Achromobacter* sp. (8).

Hong *et al* (9), also in 2014, used broth from positive aerobic BCs from two different types of BC systems, namely the BD BACTEC FX Blood Culture System (Becton, Dickinson and Company) and the BacT/ALERT 3D System (bioMérieux). The samples were dispensed on BAP and incubated in an environment with 5% CO_2 at 35°C for 4 and 6 h. MALDI-TOF MS analysis was performed using the VITEK MS Axima Assurance mass spectrometer (bioMérieux). A total of 86 monomicrobial specimens containing GNB were included in the study. Among these 86 cases, 82 (82/86, 95.3%) and 84 (84/86, 97.7%) were correctly identified at the species level after 4 and 6 h of incubation, respectively (Table I). Among the 35 *E. coli* isolates, 34 (34/35; 97.1%) and 35 (35/35; 100.0%) were correctly identified at the time points of 4 and 6 h, respectively; however, regarding *Klebsiella pneumoniae*, the rates were 18/20 (90.0%) and 19/20 (95.0%), respectively. The authors of the study reported that there were no statistically significant differences in the identification rate for this plating procedure depending on the blood culture systems (9).

A 5-h incubation period protocol using Columbia agar plates was used in the study by Verroken *et al* (10). A temperature of 37°C and a 5% supplemented CO_2 atmosphere was used for the incubation of aerobic pathogens. The MicroFlex platform (Bruker Daltonics) was used for the MALDI-TOF MS procedure and the analysis was performed according

to the manufacturer instructions using the cut-off of 1.7 for acceptable identification to the species level. Among the overall 323 aerobe GNB, correct identification at the species level was achieved in 292 cases (90.4%). Out of the 275 Enterobacteriaceae tested, 255 (92.7%) were correctly identified (Table I). Among these, 156/164 *E. coli* (94.0%), 39/45 *K. pneumoniae* (86.7%), 15/17 *Enterobacter cloacae* (88.2%) and 16/16 *Klebsiella oxytoca* (100.0%). Out of the 34 non-fermenters included in the study, 32 (94.1%) were correctly identified. Among these, 22/23 *P. aeruginosa* (95.7%) and four out of five *Acinetobacter baumannii* (94.1%). Apart from Enterobacteriaceae and non-fermenters, poor results were obtained for the remaining of the aerobic GNB (including *Capnocytophaga sputigera*, *Haemophilus influenzae*, various species of *Moraxella* and *Neisseria meningitidis*) with an overall identification rate of 35.7% (5/14).

Zabbe *et al* (11) evaluated the MALDI-TOF MS bacterial identification using bacterial growth from a chocolate agar following 3 h of incubation at 37°C in 5% CO_2. The inoculum included one drop from the blood bottle. Identification was validated at species level either if the score was >2 or if the same identification matched several times in all cases that the score was between 1.7 and 2. Biotyper 3.0 software (Bruker Daltonics) was used. Among the Enterobacteriaceae, 72/78 (92.3%; with a score >2) and 75/78 (96.15%; if adding three more cases that had a score between 1.7 and 2.0 with more than two matches) were correctly identified along with four out of five *Pseudomonas* sp. (two had a score between 1.7 and 2.0) and one out of one *Acinetobacter* sp. (Table I).

Altun *et al* (12) transferred five drops of broth from positive BCs on chocolate agar plates and three drops per side on dual blood/cystine lactose electrolyte-deficient agar plates. MALDI-TOF MS analysis using the system of Bruker Daltonics was performed following 2.5 h of incubation and if there was no identification (using identification score ≥1.7) the analysis was repeated after an overall 5.5 h of incubation. The study included a large number of clinical samples. Among the 229 GNB, 180 (78.6%) could be correctly identified at the first time point of 2.5 h. Among these, Enterobacteriaceae were identified at a percentage of 82.8% (173/209) along with 3/6 (50%) for aerobic non-Enterobacteriaceae GNB and 4/14 (28.6 %) for anaerobic GNB (Table I). After 5.5 h of incubation 34 additional GNB were correctly identified leading to an overall 93.4% identification rate for GNB (Table I). Identifications were provided at the species level with the exception of four *Salmonella* sp. The authors accepted these MALDI TOF MS identifications at the genus level as successful ones since all *Salmonella* sp. were also identified only at the genus level by conventional methods at ≥24 h (12).

By plating the positive BC broth on chocolate agar plates and incubating this for 4 h (37°C, 5% CO_2), Kohlmann *et al* (13) succeeded in correctly identifying at the species level 97.6% (82/84 cases) of GNB, including Enterobacteriaceae, non-fermenters and Gram-negative anaerobic bacteria. They used the modified cut-off value of ≥1.7 instead of the recommended ≥2 by the manufacturer (Bruker Daltonics). However, even with the later cut-off value the identification rate was 96.4% (81/84). In either cases, no Gram-negative rod was misidentified. Using the modified cut-off value, all of the

Table I. Species-level identification results obtained by MALDI-TOF MS systems using short-term bacterial incubation method compared with those of conventional methods.

Microbia	Incubation time	Medium	% ID	Additional information	(Refs.)
aGNB	4 h	Columbia	95.2	*E. coli* m.t.: 1.8 h/*P. aeruginosa* m.t.: 3 h	(7)
Enterobacteriaceae	4 h	BAP	94.0	With direct spot method	(8)
Enterobacteriaceae	4 h	BAP	79.0	With direct streak method	(8)
aGNB	4 h	BAP	95.3	Samples from 2 BC systems	(9)
Enterobacteriaceae	5 h	Columbia	92.7	Non-fermenters: 94.1%	(10)
Enterobacteriaceae	3 h	Chocolate	92.3	With ID c.s. >2; 96.15% with ID c.s. ≥1.7	(11)
Enterobacteriaceae	2.5 h	Various	82.8	GNB: 78.6%; GNB: 93.4% after 5.5 h i.t.	(12)
Enterobacteriaceae	4 h	Chocolate	98.7	With ID c.s. ≥1.7; GNB: 97.6%	(13)
Enterobacteriaceae	5 h	Columbia	93.1	aGNB: 87.5%	(14)
Enterobacteriaceae	6 h	BAP	90.4	System of Bruker Daltonics; ID c.s. ≥1.7	(4)
Enterobacteriaceae	6 h	BAP	84.0	System of bioMérieux; ID c.s. ≥90%	(4)
aGNB	3 h	Columbia	92.0	ID c.s. ≥2; 95% with ID c.s. ≥1.7	(15)
aGNB	3 h	MAC	97.0	ID c.s. ≥2; 98% with ID c.s. ≥1.7	(15)
aGNB	3 h	Chocolate	92.0	ID c.s. ≥2; 95% with ID c.s. ≥1.7	(15)

BAP, trypticase soy agar with 5% sheep blood agar plates; BC, blood culture; Chocolate, Chocolate Agar; Columbia, Columbia Agar with 5% sheep blood; ID, identification; ID c.s., ID confidence score; i.t., incubation time; aGNB, aerobic Gram-negative bacteria; MAC, MacConkey agar for Gram-negative rods.

non-fermenters (7/7, 100.0%) and almost all of the bacteria among Enterobacteriaceae (74/75, 98.7%) were correctly identified at the species level (Table I).

In addition, by performing MALDI-TOF MS assay with the VITEK MS system (bioMérieux) at 3, 5 and 24 h of incubation, Curtoni *et al* (14) analyzed, among others, 32 selected positive monomicrobial BCs with aerobic GNB isolates, including 29 Enterobacteriaceae and 3 non-fermenters. The protocol included the spreading of two drops of positive BC broth on Columbia Agar with 5% Sheep Blood followed by incubation at 36±1°C in 5% CO_2 for 3, 5 and 24 h. Identification was considered valid when the confidence level provided by the VITEK MS was ≥99.9%. A 78.1 and 75.0% correct identification rate for GNB at the genus and species level, respectively were achieved at 3 h; furthermore, the rates increased to 90.6 and 87.5%, respectively at 5 h (Table I). As regards Enterobacteriaceae, a 79.3% correct identification rate was achieved at both the genus and species level at 3 h, and a 93.1% at both the species and genus level at 5 h.

Ha *et al* (4) performed MALDI-TOF MS analysis with both the MicroFlex LT (Bruker Daltonics) and VITEK MS (bioMérieux) systems using bacterial colonies that grew on BAPs after plating one drop of positive BC broth and incubating for 6 h. The identification confidence scores used were ≥1.7 and ≥90% for MicroFlex LT and Vitek-MS, respectively (4). Among 111 mono-microbial aerobic samples with GNB, 99 (89.2%) and 106 (95.5%) were correctly identified at the species and genus level, respectively with MicroFlex LT, whereas correct identifications with Vitek-MS were 94 (84.7%) and 100 (90.9%), respectively. Among 94 Enterobacteriaceae 85 (90.4%) and 90 (95.7%) were correctly identified at species and genus level, respectively with MicroFlex LT, whereas with Vitek-MS the rates were 84.0% (79/94) and 90.4% (85/94), respectively (Table I).

Froböse *et al* (15) compared the performance of MALDI-TOF MS analysis (with MicroFlex, Bruker Daltonics) after streaking an inoculum of two drops in three different types of plates for GNB, namely Columbia blood agar (CBA), chocolate agar (Choc) and MacConkey agar for Gram-negative rods (MAC). The incubation conditions were: 5% CO_2 atmosphere and temperature at 36.6±1°C and all plates had been previously incubated overnight under the same conditions for prewarming and sterility control. A total of six plates of each type were used in order to obtain bacterial biomass independently at six time points, namely 2, 3, 4, 5, 6 and 24 h without interrupting the incubation of the remaining plates in the meantime of analysis. The results for both low-confidence (score of ≥1.7 to <2) and high-confidence identification criteria (score of ≥2) were included. When a score of ≥2 was obtained, the respective colonies were no further analyzed at a later time point. Obligate anaerobes were excluded from the study. A total of 63 Gram-negative rods were included in the analysis. The shortest median time to both low-confidence and high confidence identification was achieved on MAC (2.0 h). At the time point of 2 h, the proportions of low-confidence identification for CBA, MAC and Choc were 87, 89 and 79%, respectively, whereas the proportions of high-confidence identification were 65, 78 and 57%, respectively. At the time point of 3 h, the proportions of low-confidence identification were 95, 98 and 95%, respectively, whereas the proportions of high-confidence identification were 92, 97 and 92%, respectively (Table I). At the time point of 4 h, the proportions of low-confidence

identification were 98, 100 and 98%, respectively, whereas the proportions of high-confidence identification were 98, 100 and 98%, respectively. As the authors reported only the proportion of high-confidence identification of Gram-negative rods was significantly higher with MAC than with Choc at 2 h (78 vs. 57%; P=0.013, χ^2 test), whereas for the remaining time points, the proportions did not differ significantly.

As shown by the reviewed studies, performing MALDI-TOF MS analysis for the identification of GNB using biomass from briefly incubated subcultures on solid media is a very reliable method. It can be very easily incorporated into the workflow of a modern microbiology laboratory with an operating MALDI-TOF MS system, since the method does not require specific expertise or expensive extra consumables. The additional hands-on processing time is very low and the whole procedure is very simple. The method has the additional advantage of providing microbial biomass for susceptibility testing after the identification of the pathogen (12,16). Moreover, selective media with antibiotics can be used for rapid detection of resistant pathogens (7). The method also has the potential to be combined with advanced modern technical procedures such as automated camera reading or robotized picking-up of biomass (7). Based on all these advantages, the method is highly expected to prevail within the coming years.

Availability of data and materials

Not applicable.

Authors' contributions

IKN was involved in designing the study, analyzing the data, drafting, editing and reviewing the manuscript. DAS was involved in the conception and design of the study and in critically revising the work. Both authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. IKN declares that he has no competing interests.

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