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Short Communication

Rapid identification and detection of β-lactamase-producing *Enterobacteriaceae* from positive blood cultures by MALDI-TOF/MS

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ABSTRACT

Objectives: Current evidence suggests that early diagnosis of sepsis and timely detection of antimicrobial resistance are crucial to improve mortality rates among patients. The aim of this study was to evaluate a rapid method for the identification of Gram-negative bacteria from positive blood cultures (BCs), combined with the detection of extended spectrum β-lactamases (ESβL) and carbapenemases production, by means of MALDI-TOF/MS analysis.

Methods: During the study, all BCs positive for Gram-negative rods were selected. Starting from bacterial pellets obtained directly from BC broths, species identification and hydrolysis assays were achieved through MALDI-TOF/MS (Bruker). In particular, we performed a hydrolysis assays of cefotaxime (CTX) and ertapenem (ERT) for the rapid detection of resistance via ESβL and carbapenemases, respectively. These results were compared with the routine workflow, including BC subcultures and confirmation phenotypic methods. Finally, a comparison of the turnaround-time (TAT) between the two protocols was conducted.

Results: Overall, 185 BCs positive for *Enterobacteriaceae* were collected. In terms of species identification, we observed a concordance of 95.9% comparing MALDI-TOF/MS results to the subculture-based method. The sensitivity and specificity for CTX hydrolysis assay were 91.1% and 92%, respectively; ERT hydrolysis assay showed a sensitivity of 96.2% and a specificity of 99.2%. The TAT of the proposed MALDI TOF/MS-based protocol was significantly lower compared with the routine workflow ($P < 0.0001$).

Conclusions: The proposed protocol can provide reliable bacterial identification and data concerning β-lactam resistance in only 3 hours, positively improving management of patients in terms of antimicrobial stewardship.

1. Introduction

Sepsis is an important global health problem. The annual incidence of sepsis is 400 cases per 100 000 person-years, with a high mortality rate [1]. Current evidence suggests that early diagnosis and timely administration of appropriate antibiotics decrease mortality rates among patients [2]. In addition, the emergence of multidrug-resistant microorganisms, especially in *Enterobacteriaceae*, is an important problem for management of patients.

According to the European Antimicrobial Resistance Surveillance Network data, in Italy, the rate of resistance in Escherichia coli and Klebsiella pneumoniae invasive isolates is high [3]. The worldwide spread of extended-spectrum β-lactamase (ESβL)-producing Enterobacteriaceae organisms has led to an increased use of carbapenems, resulting in the emergence of plasmid-mediated resistance to these drugs [4]. Indeed, the rate of Gram-negative strains resistant to carbapenems has significantly increased in Europe over the years. Italy recorded an increase in carbapenem-resistant *K. pneumoniae* from 1.9% in 2008 to 26.8% in 2018 [3].

The resistance genes localized into mobile genetic elements can easily spread among patients and microorganisms, leading to problems with choice of antimicrobial therapy choices [4,5]. For these reasons, rapid detection of antibiotic-resistant *Enterobacteriaceae* in positive blood cultures (BCs) is therefore crucial for correct management of patients in terms of antimicrobial therapy.

In recent years, several methods for rapid species identification and detection of antimicrobial resistance starting from

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positive blood cultures have been described such as MALDI-TOF, Finger Print, LAMP, and chromogenic-based culture methods [6,7].

In this context, the aim of this study was to evaluate a rapid method for identification of Gram-negative bacteria from positive BCs, combined with the detection of extended spectrum β-lactamases and carbapenemases production, by means of MALDI-TOF mass spectrometry (MALDI-TOF/MS) analysis.

2. Materials and methods

2.1. Sample collection and workflow

A total of 185 blood cultures positive for Gram-negative rods were collected to evaluate the performance of the in-house MALDI-TOF/MS assays for identification and detection of resistance.

Briefly, the bacterial pellet obtained from BCs was directly used for MALDI-TOF/MS (Bruker Daltonics, Bremen, Germany) species identification. The same pellet was used in parallel for a hydrolysis assay with cefotaxime (CTX) (Cefotaxime, Mylan generics) and ertapenem (ERT) (Invanz®, MSD). CTX and ERT were used as markers for the detection of ESβLs and carbapenemases production, respectively.

Each bacterial pellet was suspended in 2.5 mL of NaCl 0.9% to a final turbidity of 4 McFarland, corresponding to ca. 1.2×10^9 CFU/mL. The suspension was then divided into two separate Eppendorf tubes with 1.5 mL and 1 mL of volume, respectively.

The samples were centrifuged at 13 400 rpm for 2 min. The supernatant was discarded and the pellet obtained from the tube with 1.5 mL was suspended in 30 μL of ertapenem solution (0.5 mg/mL in deionized water), while the second tube was suspended in 30 μL of cefotaxime solution (0.5 mg/mL in deionized water).

As negative and positive controls, two different strains were used: a reference strain of *Escherichia* coli ATCC 25922 and a KPC-producing *E. coli* (previously analysed with molecular testing). For quality control of the CTX and ERT solutions and for detecting spontaneous hydrolysis, a control without bacteria (only with antimicrobials) was also included. All the controls were processed as described for BC samples.

All samples were vortexed and incubated at 35 °C under agitation (750 rpm) for 2 h. Afterwards, the suspensions were centrifuged at 13 400 rpm for 2 min and 2 μL of supernatant was spotted in duplicate on a polished steel MALDI target plate (Bruker Daltonics), left to dry and then overlaid with 1 μL HCCA matrix (10 mg/mL of α-ciano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid; Bruker Daltonics).

A MALDI TOF/MS measurement was performed using flexControl 3.0 software (Bruker Daltonics) of Microflex LT mass spectrometer (Bruker Daltonics) in the low mass-to-charge range (120–1000) with 60 Hz laser frequency and 200 laser shots. The matrix peaks (HCCA [M + H]+ at 190.04, and bradykinin peak $[M + H]^+$ at 757.40 Da and angiotensin II peak $[M + H]^+$ at 1046.54 Da) were used for calibration. Further analysis of mass spectra was performed by the flexAnalysis 3.3 software (Bruker Daltonics): first, the spectra were smoothed, and the baselines were subtracted. Then, the specific hydrolysed and non-hydrolysed peaks for cefotaxime (non-hydrolysed pattern: 395.9, 455.9, 477.9 Da; hydrolysed pattern: 370.1, 413.9 Da) and ertapenem (non-hydrolysed form: 476.5, 497.8, 514.5, 519.7, 536.5, 541.7 Da; hydrolysed form: 449.9, 471.9, 488.5, 493.8, 516.5, 537.8, 554.5 Da) were manually checked in the mass-to-charge range of 370–500 Da and 440–560 Da, respectively.

The results of these assays were compared with those obtained with the routine workflow. As reported in Fig. 1, conventional subcultures of the same sets of BC samples were performed to confirm the species identification. The results of the hydrolysis assay were compared with the reference antimicrobial susceptibility testing (AST) (Vitek 2 AST cards N201; bioMérieux, Marcy l'Étoile, France) and with two phenotypic methods, that is double-disk synergy test for the detection of ESβL and combination disk test for carbapenemase-producing *Enterobacteriaceae* (CPE) in accordance with EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines [8].

Finally, the turnaround-time (TAT) needed for the new proposed workflow was compared with that of the routine protocol.

2.2. Data analysis

Spectra obtained by the species identification were automatically analysed with Bruker's Biotyper software (version 3.0) according to the manufacturer's instructions. Identification scores >1.7 were considered valid to the genus level, whereas scores >2.0 were considered valid to the species level. As reported by Khot et al. [9], the results between 1.7 and 1.9 were correctly evaluated to species and genus levels only if the identification was the same for the first top 10 results or in case of at least a 10% score difference between the first and second results.

To estimate the results of the hydrolysis assays, logRQ values were calculated using the peak intensities as follows: logRQ = log (sum[hydrolysed peak intensities])/(sum[non-hydrolysed peak intensities]). Strains were classified as resistant if the intensities of the hydrolysed forms represented more than 50% of the non-hydrolysed forms [10].

2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego, CA, USA). A *t* test was used to determine significant differences in the time needed to reach species identification and antimicrobial resistance detection between the two workflows described. A P value < 0.05 was considered to be statistically significant.

3. Results and discussion

The high incidence of sepsis and the spread of multidrug-resistant Gram-negative microorganisms have a dramatic impact on patient outcome and represent an urgent healthcare issue from both a care management and an economic point of view. Rapid and accurate detection of antimicrobial resistances is essential to enable early treatment and prevent further transmission of these pathogens. Therefore, in this study we evaluated a MALDI-TOF/MS system for rapid identification and detection of β-lactamase-producing bacteria directly from positive BCs to provide results with a lower TAT.

As regards the identification, 92.4% of BCs were monomicrobial (Table 1). As shown in Table 1, a MALDI-TOF/MS score $\geq$ 2.0 was obtained for 78.4% of the samples. Regardless of the MALDI-TOF/MS score, 164/171 (concordance: 95.9%) monomicrobial blood cultures showed a concordant result with the final identification achieved by the routine workflow. The proposed in-house workflow showed even better performances, when compared with the results of a commercially available kit used for MALDI-TOF/MS identification of positive BCs (Sepsityper, Bruker) [11]. The sensitivity of the proposed method was in line with other in-house systems for blood culture analysis, reporting concordance rates ranging from 67% to 100%, especially for Gram-negative organisms [12–16].

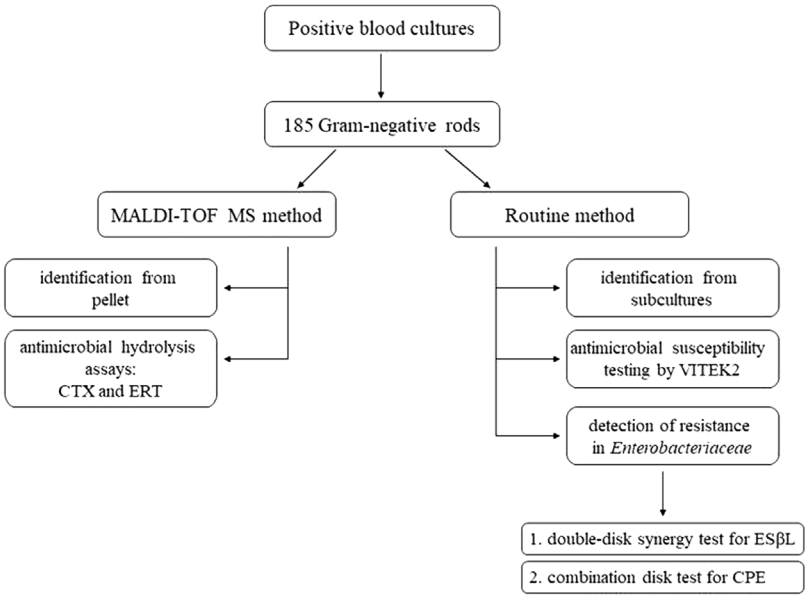

Fig. 1. Study flowchart. Laboratory flowchart for direct identification and detection of beta-lactams resistance from positive blood cultures. For species identification, isolates grown from subcultures were used to confirm MALDI-TOF MS direct identification from bacterial pellet. Moreover, antimicrobial susceptibility testing and traditional methods for resistance detection (double-disk synergy test and combination disk test) were compared to MALDI-TOF MS-based antimicrobial hydrolysis assays. CTX = cefotaxime; ERT = ertapenem; ESβL = Extended-spectrum β-lactamase; CPE = Carbapenemase-producing *Enterobacteriaceae.*

Table 1
Performance of MALDI-TOF/MS in bacterial identification.

Subculture identification		MALDI-TOF/MS identification				
Monomicrobial cultures	Total BCs	0–1.699	1.7–1.999	≥ 2.0	ND	% Agreement
Enterobacter aerogenes	4	0	1	3	0	100% (4/4)
Enterobacter cancerogenus	1	0	0	1	0	100% (1/1)
Enterobacter cloacae	9	1	5	2	1	88.9% (8/9)
Escherichia coli	79	3	13	61	2	97.5% (77/79)
Klebsiella oxytoca	4	0	2	2	0	100% (4/4)
Klebsiella pneumoniae	65	0	6	56	3	95.4% (62/65)
Proteus mirabilis	5	0	0	5	0	100% (5/5)
Providencia stuartii	1	0	1	0	0	100% (1/1)
Serratia marcescens	3	0	0	2	1	66.7% (2/3)
Polymicrobial cultures						
Escherichia coli + *Klebsiella oxytoca*	1	0	0	1[a]		
Escherichia coli + *Klebsiella pneumoniae*	3	0	0	3[b]		
Escherichia coli + *Staphylococcus haemolyticus*	1	0	0	1[a]		
Klebsiella oxytoca + *Streptococcus pneumoniae*	1	1[e]	0	0		
Klebsiella oxytoca + *Enterobacter cloacae*	1	0	0	1[c]		
Klebsiella pneumoniae + *Enterococcus faecalis*	1	0	0	1[d]		
Klebsiella pneumoniae + *Proteus mirabilis*	2	0	0	2[c]		
Klebsiella pneumoniae + *Proteus vulgaris*	1	0	0	1[c]		
Proteus mirabilis + *Klebsiella pneumoniae* + *Enterococcus faecium*	1	0	0	1[c]		
Proteus mirabilis + *Klebsiella pneumoniae*	1	0	0	1[e]		
Providencia stuartii + *Morganella morganii*	1	0	0	1[f]		
TOTAL	185	5	28	145		

The species identification obtained from routine workflow (subculture on solid media) was compared with MALDI-TOF/MS identification from bacterial pellet. A total of 171 monomicrobial and 14 polymicrobial blood cultures were analysed. In the polymicrobial cultures only one microorganism was identified: a) *Escherichia coli*; b) two identified as *Escherichia coli* and one as *Klebsiella pneumoniae*; c) *Klebsiella pneumoniae*; d) *Klebsiella oxytoca*; e) *Proteus mirabilis*; f) *Providencia stuartii.* BC = blood cultures.

In all polymicrobial BCs (7.6% of positive BCs) only one of the bacterial species was identified by the MALDI-TOF/MS: in particular, in 92.9% of them the identified microorganism was characterized by an identification score > 2.0. As reported by other authors [11,13], one possible explanation for this result could that the MALDI-TOF/MS method is capable of identifying only the most abundant species. Furthermore, Gram-positive bacteria are not usually identified as frequently as Gram-negative organisms [15,16].

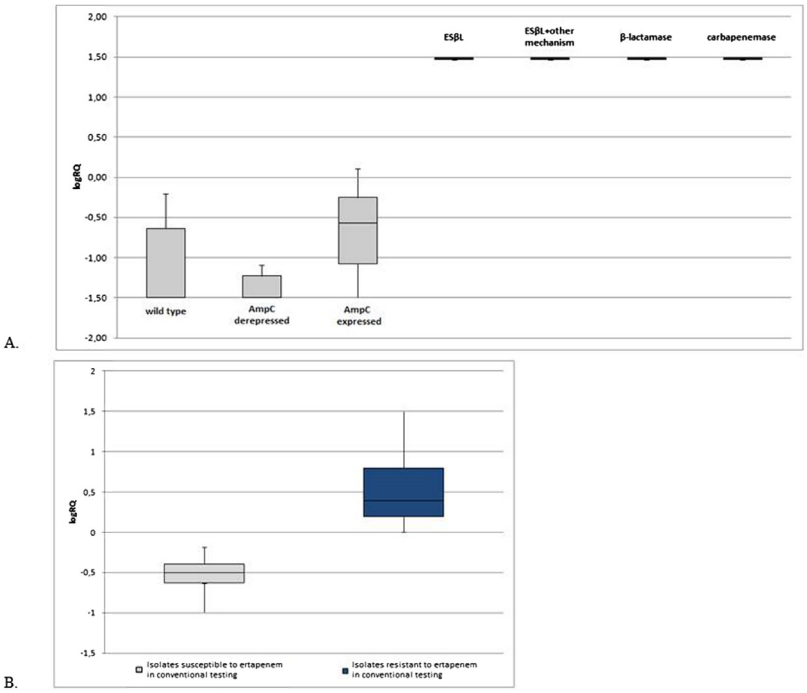

Fig. 2. Results of hydrolysis assay. (A) Cefotaxime hydrolysis assay. Boxplots showing the logRQ values of different isolates. Cefotaxime-resistant isolates producing class A β-lactamases (all showing logRQ values >1) were clearly separated from the other strains. (B) Ertapenem hydrolysis assay. The logRQ values for ertapenem are displayed in a boxplot diagram. The results of MALDI-TOF/MS hydrolysis assay were compared with those from conventional susceptibility testing (susceptibility/resistance to ertapenem). All isolates susceptible to carbapenems (grey box) showed logRQ values <0. Ertapenem-resistant strains are displayed with a blue box. The boxplots represent the median value and 75th and 25th percentiles.

These results suggest that MALDI-TOF/MS protocol from positive BCs is optimal in settings where the incidence of Gram-negative sepsis is particularly high and in case of monomicrobial BCs. Afterwards, a test for the detection of third-generation cephalosporins and carbapenemases resistance was performed by a hydrolysis assay using CTX and ERT as indicator drugs. This assay evaluates the drug degradation from the presence of a hydrolytic enzyme, suggesting a susceptible/resistant phenotype to CTX and ERT. This information is crucial for correct management in terms of antimicrobial stewardship (e.g. switch to a carbapenem or continuation with the first-line administered antimicrobial agent).

This assay applies better in epidemiological contexts where the prevalence of infections caused by ESβL and carbapenemase-producing bacteria is known to be high. Italian epidemiological data showed that CTX-M and KPC are the most common enzymes determining ESβL and carbapenemase-producing phenotypes [17]. It is worth underlining that these β-lactamases are highly active on cefotaxime and ertapenem. Therefore, using CTX and ERT as drug markers, it is possible to identify most of the resistant strains in line with the local epidemiology. Indeed, in our study, based on the routine method, more than half of the isolates were cephalosporin-resistant (54.6%) and ca. 30% were resistant to ertapenem.

Analysing the results of CTX hydrolysis, we found high logRQ values (1.5) in ESβL and carbapenemase-producing bacteria: these strains were characterized only by spectra of CTX hydrolysed forms corresponding to a 100%-hydrolysis activity. As reported in Fig. 2, a clear separation between resistant isolates and susceptible strains was noticed. Only one of the eight strains expressing AmpC showed a complete CTX hydrolysis. Globally, the 49 carbapenemase-producing bacteria showed a mean activity of CTX degradation of 96.9% (Table S1). Only in two cases (one *Klebsiella oxytoca* MβL and one *K. pneumoniae* KPC) was the hydrolysis activity < 50%.

Overall, this assay showed a sensitivity of 91.1% and a specificity of 92% with positive predictive value of 92% and negative predictive value of 89.4%. However, considering that Italian and global epidemiology of ESβL shows a high prevalence of CTX-M family, the use of CTX is probably the optimal choice for screening purposes [18,19].

When comparing ERT results between the phenotypic method and the hydrolysis assay, we found a clear separation of the isolates into two different groups (Fig. 2).

The ERT-resistant strains were characterized by mean logRQ values of 0.4, with only 1 K. pneumoniae OXA-48-type carbapenemase showing a borderline logRQ value (-0.07) (Table S1). A possible explanation for this result lies in the slower hydrolytic activity shown by OXA-48 enzymes on carbapenems [20]. Globally, the sensitivity and specificity for ERT hydrolysis assay were 96.2% and 99.2%.

Results consistent with phenotypic methods were also obtained in all polymicrobial cultures.

Currently, the TAT needed for phenotypic methods does not satisfy the demand for timely information about the antimicrobial susceptibility of a patient's isolate. In contrast, molecular methods are rapid but expensive and able to provide information only about the absence or presence of a selection of specific genes, without a full correlation to the phenotype.

Our data show that this proposed workflow starting from positive BCs could speed up the TAT providing clinically relevant and reliable information. The new workflow was able to obtain a bacterial species identification in 35 min, whereas 140 min (±10.3 min, standard deviation) were needed to perform both hydrolysis assays. In contrast, with the routine workflow, the mean time required from incubation of BC to AST reporting was 33 h (±0.27 h, standard deviation). Overall, the difference between the two workflows in terms of time required for identification and susceptibility testing was statistically significant ($P < 0.0001$) (Fig. S1).

Immunochromatographic tests could be even faster in detection of resistance mechanisms [6]. However, these tests can detect only limited and selected mechanisms, compared with the

hydrolysis assays, able to highlight the effect of an enzyme, irrespective of the specific resistance determinant.

These results could be crucial in terms of timely diagnosis of bacteraemia and prompt evaluation of ongoing empirical antimicrobial therapy. Although this assay holds promise for the future, further studies are needed to confirm its integration into a laboratory workflow and to evaluate the potential clinical impact on management of septic patients.

We are fully aware that the absence of typing of ESβL and some carbapenemase determinants is a major limitation of the study: further experiments will be needed for an in-depth evaluation of the performance of the hydrolysis assays based on resistance mechanisms at a more specific level (i.e. subtyping of ESβLs and MβLs).

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Competing interests

None declared.

Ethical approval

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. All the samples were kept anonymous throughout the duration of the study.

Author contributions

GR, SA and MCR conceived and designed the study. GR and CF analysed the data and wrote the paper. All authors read, reviewed and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2020.12.015.

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