



MALDI-TOF bacterial subtyping to detect antibiotic resistance

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ABSTRACT

The spread of bacterial resistance has been continuously increasing in the recent decade. Multi-drug resistant (MDR) bacteria now represent one of the most worrisome public health issues, as they seriously complicate the treatment of infections, often leaving few therapeutic options.

Enterobacteria and *Staphylococcus aureus* are among the most common bacterial pathogens, while *Bacteroides fragilis* is the most frequent anaerobic pathogen. All of these species can cause severe and life-threatening infections, and represent the most frequent causes of antibiotic-resistant healthcare-associated infections worldwide, as they frequently exhibit resistance to various classes of antibiotics. Resistance to carbapenems, the last resort beta-lactam agent, is a particularly threatening problem. Achieved by different mechanisms, leads to total inefficacy of any beta-lactam agent.

During the recent years, MALDI-TOF mass spectrometry has become established as the reference method for bacterial identification in routine practice. It has proven to be a reliable and robust method to detect specific peaks in bacterial mass spectra, corresponding to specific resistance markers, enabling the instant detection of resistant isolates in real time during the standard routine identification process. Here, we investigated the performance of the subtyping module of the MALDI Biotyper system (Bruker Daltonik, GmbH) for the instant identification of KPC-producing *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus*, and carbapenemase-producing *Bacteroides fragilis* during the identification workflow. We evaluated accuracy and potential impact on turnaround time. Furthermore, we investigated the possibility to extend the subtyping for detection of the KPC-specific marker to bacterial species other than *K. pneumoniae*.

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1. Introduction

Bacterial resistance to antibiotics has been continuously increasing in the recent decade. Multi-drug resistant (MDR) bacteria now represent a concerning public health and economic threat [1,2], recently indicated by the WHO as one of the most serious public health issues of our time [3]. Moreover, few new antibiotics are under development [4], hence, images of a return to the pre-antibiotic era can be easily conjured.

Previously confined to hospitals, MDR bacteria are now also found in the community, environment and animals [5,6]. MDR bacteria can significantly complicate the treatment of infections, especially in critically ill patients, leaving clinicians with few and problematic treatment options [7–9].

Enterobacterales and *Staphylococcus aureus*, normally found as associated flora, are among the most clinically relevant bacterial pathogens, involved both in hospital-acquired and community-acquired infections. They play a major role among MDR organisms, exhibiting broad spectrum resistance to the various classes of antibiotics, and representing the most frequent causes of antibiotic-resistant healthcare-associated infections worldwide [10,11]. Resistance to beta-lactam antibiotics, especially carbapenems, is a particularly threatening problem. It is caused by different mechanisms in the two groups of bacteria, which are leading to total inefficacy of any beta-lactam agent.

Resistance to carbapenems in *Enterobacterales* has been dramatically increasing worldwide over the last decade [8,12–14]. This resistance involves various genera and species, and can be attributed to different molecular mechanisms [15]. Among them, bacteria producing carbapenem-hydrolyzing enzymes (carbapenemases) are the most worrisome, as they can spread easily and contaminate healthcare settings. The *Klebsiella pneumoniae* Carbapenemase

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(KPC) is the most clinically significant [12]. It is found predominantly in *K. pneumoniae*, and its global dissemination is a matter of great concern [16], given the high morbidity and mortality rates associated with invasive infection caused by these strains [12,13,17,18].

S. aureus resistance to carbapenems is exhibited by methicillin-resistant strains (MRSA), which have developed resistance to methicillin, and to all other beta-lactam agents, except for new anti-MRSA cephalosporins (ceftaroline and ceftobiprole), by horizontal transfer and natural selection of genes that code for a mutant penicillin-binding protein (PBP2a). PBP2a has a low affinity for beta-lactam molecules, and thus prevents the activity of beta-lactam drugs. Despite a declining frequency of MRSA in the last few years (ECDC 2016), it remains a major cause of healthcare-associated infection. Moreover, the transfer and spread of healthcare-associated MRSA clones into the community have been occurring [19].

Bacteroides fragilis is the most frequent anaerobic pathogen, and can cause severe infections [20]. The species is split into two DNA homology groups, named Division I and Division II [21,22]. Resistance to carbapenems is associated with the *cfiA* gene-encoded metallo-beta-lactamases, constitutively present in Division II of the species [23], and represents an emerging problem [24,25].

The ability of clinical microbiology laboratories to detect resistant strains is crucial for diagnosis and treatment, as well as for epidemiological purposes. Rapid, reliable and cost-effective tests are required to improve patient outcome, and for the implementation of the adequate infection control practices [8,26].

Various phenotypic and genotypic methods are currently available to detect carbapenemase-producing *Enterobacteriales* and MRSA. Phenotypic methods are frequently inexpensive and easy-to-use, but are either slow, lack sensitivity and/or specificity [18], or are not suitable for epidemiological investigations, as many of them detect the presence of the enzymatic activity responsible for the antibiotic resistance, but don't enable discrimination among the different enzymes types that can be involved. On the other hand, PCR-based methods are accurate and reliable, but expensive, and sometimes laborious.

Matrix-Assisted Laser Desorption-Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) has become the universal reference method for microbial species identification in clinical microbiology. Its introduction into laboratories has significantly reduced the time required for identification of bacteria and fungi (now minutes versus hours to days), both with lower cost and increased accuracy [27,28].

Beyond identification, MALDI-TOF MS has further become a platform of investigation for antibiotic resistance. Recently, novel applications of this technology have facilitated the development of new methods for easy and rapid detection of bacterial antibiotic resistance. Specific peaks in the bacterial mass spectra were have been identified as antibiotic-resistance markers [29,30]. The development of dedicated algorithms for their detection, implemented into the commercially available MALDI-TOF MS system, have enabled the automated instant identification of resistant strains, in real-time during the standard species identification process. KPC-producing *K. pneumoniae* strains can be identified by the detection of a peak at 11109 *m/z*, specifically associated with one of the most common KPC-carrying plasmids [29]. A subgroup of methicillin resistant *S. aureus* strains can be identified by the detection of a peak at 2412 *m/z* [30]. The carbapenemase-producing (Division II) *B. fragilis* subgroup can be differentiated from Division I by a predictable shift in the MALDI mass spectral pattern [31].

Here, we investigated the performance and the diagnostic value of the automated detection of KPC-producing *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus*, and carbapenemase-

producing *Bacteroides fragilis* using the subtyping functionality of the MALDI Biotyper system (Bruker Daltonik, GmbH) implemented as part of the routine workflow. The reliability and the robustness of the method have been evaluated in previous studies, investigating datasets of molecularly well-characterized isolates [31,32], thus in this study we considered unnecessary any molecular confirmation of the results delivered by the MALDI approach.

2. Materials and methods

All routine clinical isolates of *S. aureus* and *B. fragilis*, and all surveillance and positive blood cultures isolates of *K. pneumoniae* collected in the laboratory of bacteriology of the University Hospital of Bologna Policlinico Sant'Orsola-Malpighi between 1st February – 15th May 2018 were included in this study. The strains were identified at species level by MALDI-TOF MS (MALDI Biotyper, Bruker Daltonik GmbH, Bremen, Germany), following the manufacturer's instruction. Subtyping for the detection of resistance markers was performed simultaneously with species identification by the subtyping functionality implemented into the Biotyper MBT Compass software.

Identification results with low confidence level (log score < 2.0) were excluded.

S. aureus, *B. fragilis* and *K. pneumoniae* isolates derived from blood cultures were cultivated on Tryptone Soy Agar with 5% sheep blood (Meus S.r.l, Piove di Sacco, Italy) for 24–48 h. Surveillance *K. pneumoniae* isolates were cultivated on CHROMagar KPC agar or CHROMagar ORIENTATION Meus S.r.l., Piove di Sacco, Italy).

2.1. KPC-producing *K. pneumoniae*

N = 684 clinical and surveillance isolates of *K. pneumoniae* were subtyped by the detection of a specific peak at *m/z* 11,109 related to the pKpQIL *bla*_{KPC} harbouring plasmid [29].

As the reliability and the robustness of the method has been previously assessed [32], when the KPC-related peak was detected, no further routine investigations were performed, besides the standard susceptibility testing (NM-EN51 panel, Microscan WalkAway, Beckman Coulter Inc., Brea, CA, USA).

Strains that did not show the KPC-related peak, but exhibited a reduced susceptibility to carbapenems (defined as MIC for at least one of the carbapenems higher than the epidemiological cut off) with routine susceptibility testing underwent verification of carbapenemase production by the reference method (disc-diffusion synergy test – KPC + MBL Confirm ID Pack, ROSCO Diagnostika, Taastrup, Denmark), to evaluate whether a carbapenemase other than the pKpQIL plasmid-related KPC was present.

The impact of this new method in terms of time-to-response for KPC-producing isolates was evaluated by comparing the reporting time of these samples with the reporting time of an equal number of routine samples randomly selected from the period of time prior to implementation of the Biotyper subtyping module. The former workflow included, as the first step, the Carba-NP test (Neo-Rapid CARB-Kit, Rosco Diagnostics, Taastrup, Denmark), in case of negativity, followed by a disc-diffusion synergy test).

2.2. Methicillin resistant *S. aureus* (MRSA)

S. aureus clinical isolates (n = 593) were subtyped by the Biotyper software through detection of a specific peak at *m/z* 2412. This peak corresponds to a PSM-*mec* peptide, related to one of the mechanisms that cause methicillin-resistance [30] – Fig. 1.

Classification into methicillin-resistant/susceptible was performed on the basis of susceptibility to oxacillin and to ceftoxitin, as specific screening beta-lactam antibiotics, included into the

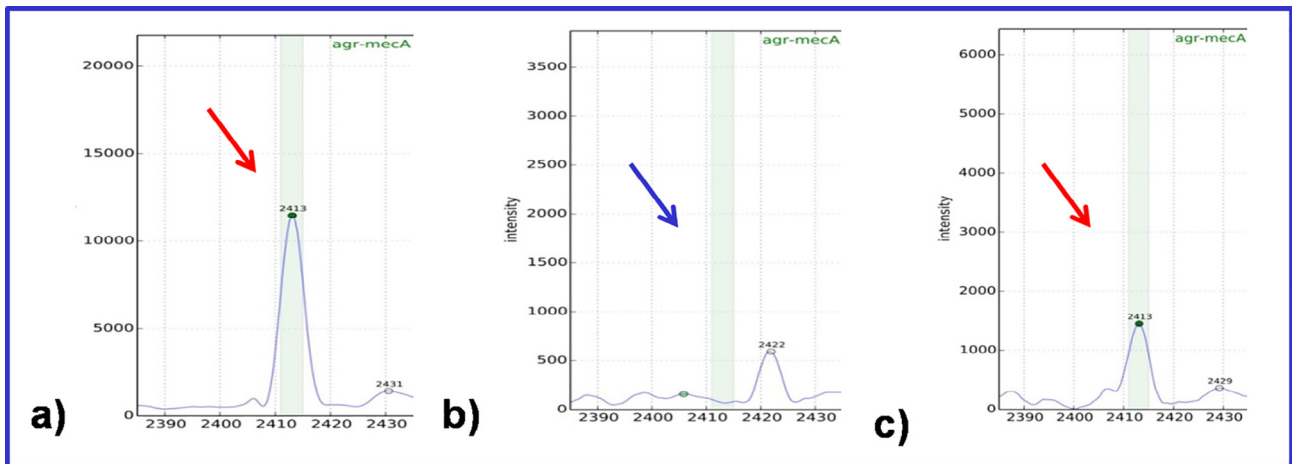


Fig. 1. Automated detection of the specific PSM peak at m/z 2411–2419. a) *S. aureus* ATCC 33591 (PSM-*mec* positive); b) clinical isolate of MRSA (PSM-*mec* negative); c) clinical isolate of MRSA (PSM-*mec* positive).

routine susceptibility test (PM STA-36 panel, Microscan Walk-Away, Beckman Coulter).

2.3. Carbapenemase-producing *B. fragilis*

B. fragilis clinical isolates ($n = 35$) were subtyped into Division I/II (*cfiA*-negative/positive) by their specific pattern in the mass spectra, as previously described [31]. Susceptibility to carbapenems was investigated as part of a routine diagnostic workflow to determine the Minimum Inhibitory Concentration (MIC) of one of the carbapenems (meropenem) by the MIC gradient strip methodology (M.I.C. Evaluator, Oxoid, Basingstoke, UK) according to EUCAST guidelines, version 8.1 (www.eucast.org).

2.4. KPC subtyping of enterobacteria other than *K. pneumoniae*

The KPC-related peak was searched in other *Enterobacterales* species (all species for which at least one KPC-producing isolate was found in the Bacteriology laboratory of the University Hospital of Bologna since the appearance of the first KPC-producing isolate in 2010).

MALDI-TOF mass spectra of 8801 clinical and surveillance isolates of clinically relevant *Enterobacterales* species, other than *K. pneumoniae*, were investigated for the presence of the KPC-related peak at 11109 m/z described in KPC-producing *K. pneumoniae* (see Section 2.2). The spectra were collected in the Bacteriology laboratory of the MVZ Lab in Dortmund ($n = 7694$) and in Bologna ($n = 1107$), and exhibited different susceptibility patterns to carbapenems (Table 1).

All strains had undergone routine susceptibility testing by Vitek2 (bioMérieux, Marcy L'Etoile, France), and confirmation of carbapenemase-production by a disk-diffusion synergy test (KPC

+ MBL Confirm ID Pack, Rosco Diagnostics) in case of reduced susceptibility to carbapenems.

In detail, *E. coli* ($n = 3502$), *E. cloacae* ($n = 2249$) complex, *K. aerogenes* ($n = 414$), *K. oxytoca* ($n = 1460$), *Citrobacter* spp. ($n = 639$) and *S. marcescens* ($n = 537$) clinical and surveillance isolates were included (Table 1).

As this functionality was not part for the MALDI Biotyper software at the time of this study, for automated detection of the KPC-related peak in non-*K. pneumoniae* enterobacteria, a specific software algorithm was developed for each species (*E. coli*, *E. cloacae*, *E. kobei*, *E. asburiae*, *E. ludwigii*, *K. aerogenes*, *K. oxytoca*, *C. freundii*, *S. marcescens*). The peak detection was based on results of visual analysis of spectra performed with the flexAnalysis® software version 3.4 (Bruker Daltonik, Bremen, Germany) – Fig. 2. Spectra were normalized and smoothed with standard settings. For precise detection, the spectra were screened for species-specific potential peaks for an internal recalibration, as described earlier [33]. After internal recalibration, intensities 3-times higher than the surrounding noise were counted as peaks. If a peak was detected in a window $\pm 5 m/z$ around the previously described mass of 11109 m/z , the algorithm accepted the detection of the KPC-related peak. Examples of the automated detection results are shown in Fig. 3.

3. Results

3.1. KPC-producing *K. pneumoniae*

371 *K. pneumoniae* strains were subtyped as “presumptive KPC”.

Among the 313 strains subtyped as “non-KPC”, 24 strains resulted positive for the production of a class A carbapenemase to synergy test (KPC). Therefore, sensitivity of KPC detection was

Table 1

Dataset of enterobacteria other than *K. pneumoniae*, and sensitivity of KPC-production by MALDI Biotyper subtyping for the different species.

Species	Tot.	Carbapenem-S		carbapenem-R Italy	KPC-producing	KPC-peak detected
		Italy	Germany			
<i>E. coli</i>	3502	398	2735	369	146	126 (86.3%)
<i>K. aerogenes</i>	414	18	362	34	11	9 (81.8%)
<i>E. cloacae</i> complex	2249	90	2085	74	5	5 (100%)
<i>K. oxytoca</i>	1460	31	1413	16	5	3 (60%)
<i>C. freundii</i>	639	30	587	22	9	8 (88.9%)
<i>S. marcescens</i>	537	19	512	6	6	4 (66.7%)
	8801	586	7694	521		

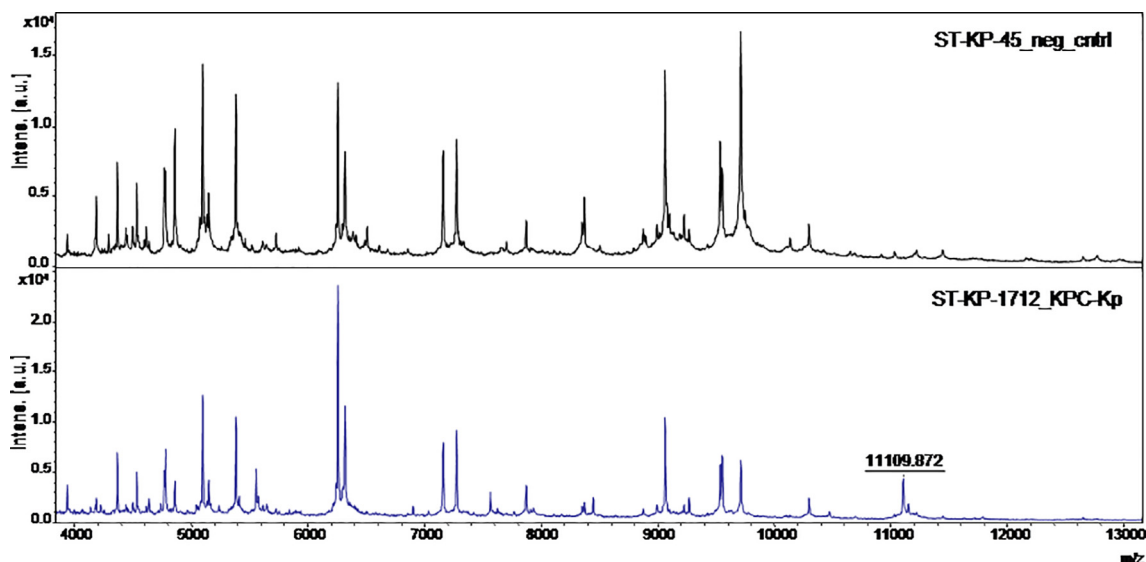


Fig. 2. The KPC-related peak at 11.109 m/z in the bacterial MALDI-TOF MS mass spectra of a KPC-producing isolate (lower spectrum), in comparison with a non-KPC producing isolate (upper spectrum).

calculated as 94% (371/395) for the tested sample collective (Table 2).

The method in this study enabled us to report the presence of KPC-producing isolates earlier compared to the former routine approach. The time saved ranged from 1.5 to 24 h, depending on which of the methods included in the multistep routine workflow detected the positive result (Carba NP or disc-diffusion synergy test – KPC, respectively).

3.2. Methicillin resistant *S. aureus* (MRSA)

28 *S. aureus* strains were subtyped as “presumptive PSM-positive MRSA”, corresponding to 15.7% (28/178) of the total number of MRSA detected by standard susceptibility testing. None of the 415 methicillin-susceptible strains were subtyped as “presumptive PSM-positive MRSA”.

The “presumptive PSM-positive MRSA” warning provided by the instrument during routine practice allowed a reduction in the time to reporting by one day.

3.3. Carbapenemase-producing *B. fragilis*

32 (91.4%) *B. fragilis* strains were subtyped as Division I, and 3 (8.6%) as Division II. The result of routine susceptibility testing for meropenem was coherent with the Biotyper classification. The MALDI subtyping into Division I/II (carbapenem-susceptible/resistant, respectively) enabled a reduction in the time-to-response, regarding susceptibility to carbapenems, by 24–48 h in comparison with the MIC determination.

3.4. KPC subtyping of other enterobacteria

The same KPC-related peak at 11109 m/z described in *K. pneumoniae* was detected in 126/146 (86.3%) *E. coli*, 5/5 (100%) *E. cloacae* complex, 9/11 (81.8%) *K. aerogenes*, 3/5 (60%) *K. oxytoca*, 8/9 (88.9%) *C. freundii*, and 4/6 (66.7%) *S. marcescens* KPC-producers (Table 1).

4. Discussion

In this study, we investigated the performance and the diagnostic value of the automated detection of KPC-producing *Kleb-*

siella pneumoniae, methicillin-resistant *Staphylococcus aureus*, and carbapenemase-producing *Bacteroides fragilis* using the subtyping functionality of the MALDI Biotyper system (Bruker Daltonik, GmbH) and its suitability to be implemented into the routine workflow. *K. pneumoniae* isolates were subtyped for the presence of the *bla*_{KPC} pKpQIL carrying a plasmid-related peak present only in KPC-producing strains. *S. aureus* isolates were subtyped for the presence of the PSM peak, related to a subgroup of methicillin-resistant strains. *B. fragilis* isolates were subtyped into Division I and II by the detection of a specific mass spectral pattern.

MALDI-TOF MS subtyping proved to be a reliable and promising method to recognize resistant strains simultaneously with identification at species level, in a short time, and without requiring any further tasks besides classical routine identification procedures. The implementation of KPC subtyping further simplified and shortened the laboratory workflow, as it reduced the handling time and the number of samples that required further analytical steps.

Sensitivity of this approach was different for the three groups of bacteria investigated (~95% for KPC-*K. pneumoniae*, ~20% for methicillin-resistant *S. aureus*, and ~100% for carbapenemase-producing *B. fragilis*). This difference can be easily explained with some epidemiological considerations. In fact, subtyping for *S. aureus* and *K. pneumoniae* depends on the epidemiological situation, as it relies on the detection of one of the possible mechanisms for that given resistance, and thus, it was shown to be related only to the prevalence of the resistance type detectable by MALDI among all the circulating resistant strains (prevalence of the KPC-producing strains harbouring the pKpQIL plasmid among all the KPC-producing *K. pneumoniae* circulating clones, and prevalence of the subgroup of methicillin-resistant *S. aureus* harbouring the *mecA* cassette containing the gene which encodes the psm-peak related small protein).

In contrast, for *B. fragilis*, subtyping relies on the detection of strains that belong to one of the two DNA-homology groups in which the species is divided. Division II harbors the only mechanism for resistance to carbapenems currently known for *B. fragilis*. Hence, sensitivity overlaps with prevalence of the Division II subfamily among all the *B. fragilis* isolates and was found to be 100%.

For the three groups investigated, specificity was found to be excellent (100%), as the specific peaks/pattern were not found in any of the susceptible isolates.

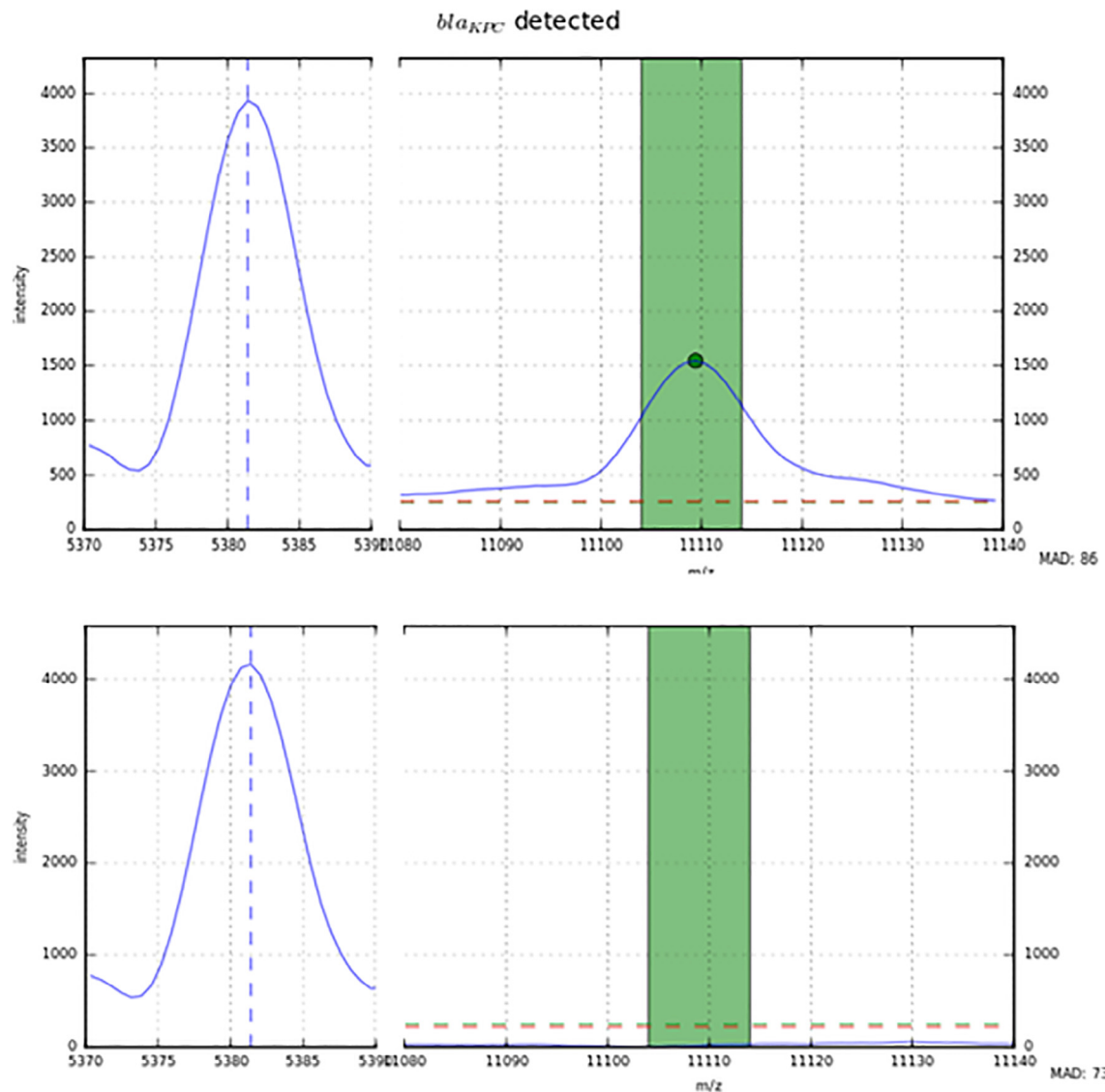


Fig. 3. Detection of the KPC-specific peak by the automated algorithm. For each species, the dedicated specific algorithm recognized an internal calibration peak, related to the species, and the KPC-related peak at 11,109 m/z , if present.

Table 2

Sensitivity and specificity of detection of KPC-production in *K. pneumoniae* by MALDI Biotyper subtyping.

<i>K. pneumoniae</i>	All isolates (n = 684)	
	KPC-peak detected	KPC-peak NOT detected
KPC+ (n = 395)	371 (93.9%)	24
KPC- (n = 289)	–	289 (100%)

5. Conclusions

The MALDI subtyping approach enabled a significantly faster time to report in comparison to the routine procedure, saving between 1 and 24 h for KPC-producing *K. pneumoniae*, 24 h for methicillin-resistant *S. aureus*, and 24–48 h for carbapenem-resistant *B. fragilis*. Thus, the method could enable an earlier adoption of proper surveillance measures. Moreover, the shortened time to report in KPC-producing strains from blood cultures might have the potential to significantly reduce the time to therapeutic escalation.

Furthermore, this study showed that KPC subtyping can be successfully extended to further species of enterobacteria. The same KPC-related peak described in *K. pneumoniae* was detected in the majority of the KPC-producing strains of all the other species of *Enterobacteriales* investigated (providing interesting hints for further investigations regarding the transfer of this genetic determinant among the different species). The prevalence of the KPC-specific peak in KPC-bearing strains of these other species was similar to the one in KPC *K. pneumoniae*.

These findings on one hand prove that MALDI subtyping for detection of antibiotic resistance markers is a valid and useful method, mature enough to be implemented into the routine practice. On the other hand, this approach could have a huge potential to be expanded to other combinations of bacteria/resistances, as soon as specific resistance markers are identified.

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Declaration of Competing Interest

MC, ABP and SA have any conflicts of interest to disclose.

Authors with financial interests or relationships to disclose are listed with their details below: MK is employee of Bruker Daltonik GmbH, manufacturer of the MALDI Biotyper system.

Corresponding author confirms here the conflict of interest statements provided during the initial submission of the manuscript.

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Glossary

MDR: Multi-Drug Resistant

KPC: *Klebsiella pneumoniae* Carbapenemase

MRSA: Methicillin-resistant *Staphylococcus aureus*