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MALDI-TOF MS based procedure to detect KPC-2 directly from positive blood culture bottles and colonies.

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Abstract

In this study, we identified specific carbapenemase-producing isolates applying an easy and rapid protocol for the detection of mature KPC-2 β-lactamase by MALDI-TOF MS from colony and positive blood culture bottles. In addition, we evaluated the correlation of the ~11,109 Da signal as a biomarker associated with KPC-2 production. A collection of 126 well-characterized clinical isolates were evaluated (including 60 KPC-2-producing strains). Presence of KPC-2 was assessed by MALDI-TOF MS on protein extracts. Samples were prepared using the double layer sinapinic acid technique. In order to identify mature KPC-2, raw spectra were analyzed focusing on the range between *m/z* 25,000–30,000 Da. A single distinctive peak, at approximately *m/z* 28,544 Da was found in all clinical and control KPC-2-producing strains, and consistently absent in the control groups (ESBL producers and susceptible strains). This peak was detected in all species independently of where the gene $bla_{\text{KPC-2}}$ was embedded. Statistical results showed 100% sensitivity, CI95%: [94.0%; 100%] and 100% specificity, CI95%: [94.6%; 100%], indicating a promising test with a high discriminative power. KPC-2 β-lactamase could be directly detected from both colonies and blood culture bottles. On the other hand, the *m/z* 11,109 Da signal determinant was only associated with 32% of *Klebsiella pneumoniae* and *Escherichia coli* KPC positive isolates. This MALDI-TOF MS methodology has the potential to detect directly the widespread and clinically relevant carbapenemase, KPC-2, in *Enterobacterales* with a straightforward, low cost process, assuming MALDI-TOF MS is already adopted as the main identification tool, with clear clinical implications on antibiotic stewardship for early infection treatment.

1. Introduction

Resistance to carbapenems is one of the most relevant problems in current antimicrobial therapy for Gram-negative infections, limiting effective antimicrobial options and often leading to treatment failures (Ventola, 2015).

Class A carbapenemases are among the relevant resistance mechanisms in clinical isolates of *Enterobacterales*; KPC-2 is distributed in all continents, including community settings (van Duin and Doi, 2017). Coding genes for the major allelic variants of these β-lactamases are commonly carried on plasmids, with diversity being an important contributory factor for their spread (Cai et al., 2008, Giakkoupi et al., 2011, Gutkind et al., 2013, Navon-Venezia et al., 2006).

One of the key factors involving critical patients´ survival is a timely decision for an effective treatment, as delays may reduce the survival chances as much as 10 % daily (Luna et al., 2006). Therefore, among WHO recommendations, improvement (and acceleration) of diagnostic procedures is clearly needed (as an example, a dedicated call for better diagnosis is requested by the EU (https://www.jpiamr.eu/coming-soon-call-for-diagnostics-and-surveillance-africa-and-asia/). So, rapid and specific methods for carbapenemase detection in clinical microbiology are desirable in order to avoid treatment failure and prevent dissemination events (Rhodes et al., 2017).

Nowadays, preliminary screening for carbapenemase producers in clinical specimens is based on phenotypic tests. Traditional microbiological methods such as double disk synergy tests using β-lactam and β-lactamase-inhibitor disks, growth-based assays like modified Hodge test, and even the epsilometer tests (E-tests), remain convenient, easy to perform, but not fast methods for detecting these β-lactamase in Gram-negative bacilli (around 24-48 h after microorganism isolation). Manual and automated determination of minimal inhibitory concentration (broth microdilution assay and Phoenix or VITEK, respectively) are also reliable methods. Besides being time-consuming, some traditional microbiological methods have disadvantages such as interpretation difficulty and variability in the sensitivity/specificity parameters among different species (AlTamimi et al., 2017)

Other phenotype-based assays are available, including, immunochromatographic assays (ICAs) and rapid colorimetric-based tests (commercial versions of the Carba NP, CNPt-

Direct, and Blue-Carba) (Lima-Morales et al., 2018, Nordmann et al., 2012, Wareham et al., 2018). Also, polymerase chain reaction (PCR) used to confirm the presence of these resistance markers, is quite expensive (Mirande et al., 2015) and requires at least 3 h to obtain the results.

One approach that may shorten the time-to-result for antibiotic susceptibility testing is matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Incorporation of MALDI-TOF MS has been one of the most significant breakthrough technologies included in modern clinical microbiology, and is today routinely used in medium and high complexity laboratories for rapid species identification (Sandalakis et al., 2017).

Current studies demonstrate the possibility to apply a MALDI-TOF MS approach to predict β-lactamase production based on the detection of hydrolyzed β-lactam substrates. Although reliable, this detection is laborious. β-lactam hydrolysis is evaluated after at least one hour incubation of the bacteria in presence of a suitable substrate (Hooff et al., 2012, Oviano et al., 2016, Sparbier et al., 2012). Following this procedure, enzyme detection is feasible and reflects the presence of one or more enzymes with hydrolytic activity on the provided substrate.

Direct detection of a specific enzyme by analysis of the MALDI-TOF MS generated protein profile has been the subject of research in different studies. Among them, Papagiannitsis et al. detected CMY-2 enzyme in different enterobacteria after extraction of periplasmic proteins using a multiple steps procedure (Hart et al., 2015, Papagiannitsis et al., 2014). More recently, Espinosa et al. could detect a peak corresponding to CMY-2, analyzing the spectrum of proteins directly obtained from cellular lysates of *Escherichia coli* and other enterobacteria which displayed resistance to third generation cephalosporins (Espinosa et al., 2018).

Antibiotic resistance has also been predicted by MALDI-TOF MS by detection of biomarkers associated with a specific resistance determinant. The p019 protein, linked to the plasmid located Tn*4401a*, present in KPC-2 producing *Klebsiella pneumoniae* ST 258, can be detected as a 11,109-Da peak in MALDI-TOF MS and was used to predict KPC carbapenemase producers (Centonze et al., 2018, Gaibani et al., 2016, Youn et al., 2016).

Thus, prediction of this marker depends upon the surrounding genetic context, and as epidemiology of endemic or hyperendemic clones may vary across time and regions, detection of mature β-lactamase by MALDI-TOF MS, directly associated with the resistance pattern of bacteria, could be considered a more reliable marker.

Enterobacterales may account for up to 30% bloodstream infections (Tariq and Rasool, 2016). As reported by Kumar et al., if an ineffective treatment is applied in patients with septic shock, the mean survival rate decreased 7.6% per hour delayed (Kumar et al., 2006). The development of more rapid techniques for the prompt and accurate detection of resistance markers in the clinical laboratory should guide appropriate treatments.

The aim of this study was to apply an easy and quick methodology for the detection of mature KPC-2 β-lactamase by MALDI-TOF MS directly on Gram-negative bacilli, focusing on *Enterobacterales*. In addition, the correlation of the ~11,109 Da signal as a biomarker associated with the KPC-2 production was evaluated and the possibilities for direct KPC-2 detection from positive blood culture bottles by MALDI-TOF MS were analyzed.

2. Materials and methods

2.1 Control Strains

Recombinant and transformant strains expressing KPC-2 were used as controls.

To construct the recombinant plasmid, full-length $bla_{\text{KPC-2}}$ (882 bp) was amplified by PCR using custom designed primers KPC-2F (5´-TACGCGTCGACATGTCACTGTATCGCCGTC-3´) and KPC-2R (5´-CCGGAATTCTTACTGCCCGTTGACGCC-3´) containing *Sal*I and *Eco*RI restriction sites, respectively. The *Sal*I–*Eco*RI $bla_{\text{KPC-2}}$ fragment was cloned into the pK19 vector (recombinant plasmids pKPC-2-4), transformed in *E. coli* TOP10 (*E. coli* TOP10/pKPC-2-4) and selected on Tryptic Soy Agar (TSA) containing 1 µg/ml imipenem (Sigma-Aldrich, USA). Recombinant plasmid was confirmed by sequencing.

A wild-type plasmid carrying the $bla_{\text{KPC-2}}$ and $bla_{\text{CTX-M-14}}$ genes obtained from a clinical isolate (TFI-KPC) was electroporated into electrocompetent *E. coli* XL1-blue cells.

E. coli bla_{KPC}-positive transformant strains were selected on Mueller Hinton (MH) (Britania, Argentina) agar plates containing 1 µg/ml meropenem (Sigma-Aldrich).

The MALDI-TOF MS spectra range to detect the KPC-2 enzyme was specially focused in the range between m/z 25,000–30,000 Da based on the theoretical mass of mature KPC-2 protein.

2.2 Strain collection

A panel of 126 previously characterized isolates deposited in our laboratory collection in Buenos Aires, Argentina, was used. There was no epidemiological link among them. All bacteria were identified by MALDI-TOF MS (Bruker Daltonics, Germany) following the standard procedure (Matsuda et al., 2012). Sixty KPC-2-producing clinical isolates (*Klebsiella pneumoniae* (39), *Enterobacter cloacae* (7), *Escherichia coli* (4), *Serratia marcescens* (4), *Citrobacter braakii* (1) and *Pseudomonas aeruginosa* (5)) and 66 KPC-2-negative isolates were included. In addition to being the most commonly isolated species, the KPC-2-producing *K. pneumoniae* isolates also corresponded to multiple different ST that were circulating in our region (Table 1 and 2).

2.3 Antimicrobial susceptibility and genetic characterization

All isolates were previously characterized phenotypically by disk diffusion and synergy tests according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). Production of carbapenemases was investigated using boronic acid and EDTA by synergy tests (Lee et al., 2001, Tsakris et al., 2009). Further genotypic characterization was carried out by PCR focused on different β-lactamase gene families. PCR amplifications were performed on total DNA using primers and conditions described previously (Dominguez et al., 2018). Amplicons were sequenced on both strands using an ABI3730XL DNA sequencer (Table 1 and 2).

2.4 Bacterial typing

K. pneumoniae ST-258 clone was characterized by the *pilv-l* PCR amplification (Adler et al., 2014) while other STs were determined by the multilocus sequence typing (MLST) scheme (Diancourt et al., 2005). Characterization of the O25b-ST131 clone of *E. coli* was also done by allele-specific PCR assay based on detection of the *pabB* gene (Clermont et

al., 2009). The genetic environment of the *bla*$_{KPC-2}$ gene (isoforms of Tn*4401*) was explored by mapping as described previously (Cuzon et al., 2010, Kitchel et al., 2009, Naas et al., 2008) (Table 1). In order to evaluate the presence of the *p019* gene (which encodes for a hypothetical protein named p019) in the *bla*$_{KPC}$-producing strains, a simple PCR was performed using custom designed primers (p019F: 5´- AATATTGATACTGACCGC-3´ and p019R: 5´- AACCCATTTTTGCATCTG-3´) which rendered an amplicon of 328 bp.

2.4 Protein extraction protocol for β-lactamase detection by MALDI-TOF MS

Pure cultures were obtained on MH agar plates without antibiotics and incubated overnight at 37 °C. Samples were prepared using a modified extraction protocol for β-lactamase detection (formic acid- isopropyl alcohol – water, 17:33:50 ratio by volume), as previously described (Espinosa et al., 2018).

2.5 Detection of mature KPC-2 protein from colonies

The protein extraction with an organic solvent mentioned above was performed on colonies. The samples were loaded once in three different spots on the stainless steel MALDI target plate using the double layer sinapinic acid technique. Every spot was measured in duplicate (6 spectra per sample). Mass spectra were obtained using a Microflex LT mass spectrometer by flexControl 3.4 software (Bruker Daltonics, Germany) within the mass range of 17,000 to 50,000 Da as previously described (Espinosa et al., 2018). Data were automatically acquired using the AutoXecute acquisition control software (Bruker Daltonics) and spectra were analyzed using flexAnalysis 3.4 software.

2.6 Detection of hypothetical p019 protein (~11,109 Da peak) by MALDI-TOF MS

Detection of the ~11,109 Da peak was carried out in the 2,000 – 20,000 Da range for all 126 isolates. Briefly, one µl of the protein extraction for β-lactamase detection was applied in triplicate onto a MALDI target, the dried spots were overlaid with 1 µl of HCCA matrix (10 mg/ml α-cyano-4-hydroxy-cinnamic acid) (Bruker Daltonics) and acquired twice for a total of six spectra (Centonze et al., 2018, Youn et al., 2016). An external Standard BTS (Bruker Daltonics) was used for mass calibration during parameter optimization. Spectra were generated on a Microflex LT (Bruker Daltonics) MALDI-TOF MS system operated in linear mode with a total of 240 laser shots. Data acquisition and further analysis were performed as detailed above after baseline subtraction.

2.7 Detection of KPC-2 from blood culture (BC) bottles using MALDI-TOF MS

Direct KPC-2 detection from positive blood culture bottles was performed on 39 samples: 16 KPC-2-producing clinical isolates of *K. pneumoniae* (9), *E. cloacae* (1), *E. coli* (2), *S. marcescens* (3), and *C. braakii* (1); 23 non-KPC-2 producing clinical isolates were also included (Table 1). Briefly, 1 ml of a bacterial suspension (approximately 10^4 CFU) was inoculated in a pediatric blood culture bottle (BD BACTEC™ Peds PlusTM/F Culture Vials, Argentina) previously inoculated with 5 ml of human blood. Blood cultures were incubated at 37 °C for 18 h. Direct KPC-2 detection was carried out using the protocol previously described (Espinosa et al., 2018). An extra-washing step with distilled water on the pellet was added prior to solvent extraction in order to remove cell debris. Samples were tested in triplicate using the double layer sinapinic acid technique as described above.

2.8 Statistical analysis

Spectra of each strain after automatic calibration and normalization were analyzed with the software ClinProTools 3.0 (Bruker Daltonics) (Ketterlinus et al., 2005). Statistical analysis was performed using the full raw spectra (17,000 to 50,000 Da) with the "Peak Statistic Calculation" tool. The area under the curve (AUC) of the ROC curve was evaluated for the specific peak (28,544 Da) between KPC-2 positive and negative strains, to establish the sensitivity, specificity and the discriminative power.

2.9 Detection index

The detection index (DI) was calculated for each isolate taking into account the peak intensities at 28,544 Da ($PI_{28544\ Da}$) as follows: DI = [$PI_{28544\ Da}$ mean for each clinical isolate/$PI_{28544\ Da}$ mean of 31 spectra of *E. coli* ATCC 25922]. *E. coli* ATCC 25922 spectra were used as the negative control. Data that did not follow a normal distribution, were presented as median and interquartile range. Also, 95% confidence intervals (CI95%) were calculated. Comparisons between groups were performed using the Mann-Whitney test (GraphPad Prism Version 5).

2. Results and discussion

The MALDI-TOF MS spectra range to detect the KPC-2 enzyme was modified, from the bacterial identification range, to 17,000 - 50,000 Da, specially focusing on the range

between *m/z* 25,000–30,000 Da. *E. coli* TOP10 carrying the recombinant plasmid pK19-KPC-2 (called pKPC-2-4) and their corresponding controls (*E. coli* TOP10 and *E. coli* TOP10 + pK19) were processed. A characteristic peak at around 28,544 Da (slightly differing from the expected value for the mature KPC-2 enzyme, i. e. without signal peptide, 28,477 Da) was only observed in the recombinant pKPC-2-4 strain (Figure 1a). In addition, the KPC-2 producing *E. coli* DH5α (electroporant) called TFI-KPC, displayed the same characteristic peak which was absent in the isogenic strain (*E. coli* DH5α) (Figure S1). These KPC-2-producing strains were used as positive controls each time when the different clinical isolates were analyzed.

The protein extraction protocol for β-lactamase detection was conducted for all 126 collection isolates included in the study. To confirm bacterial identification these extracts were assessed by MALDI-TOF MS using the standard procedure. Subsequently, all the protein extracts were analyzed by MALDI-TOF MS in order to detect the presence of KPC-2. Comparison among mass spectral profiles showed that two groups could be differentiated after peak analysis. A single distinctive peak with different intensities, at approximately *m/z* 28,544 Da was found in all previously characterized KPC-2-producing *Enterobacterales* and was consistently absent in the KPC-2 negative isolates (Figure 1b). The presence of this distinctive peak was also observed in KPC-2-producing isolates of *P. aeruginosa* (Table 1 and Figure 1c). Analysis of replicate cultures of all isolates gave consistent and straightforward mass spectral profiles within this range.

Thus, the visualization and identification of the 28,544 Da peak corresponding to the KPC-2 mature protein was successful using the supernatant obtained after applying the extraction protocol for β-lactamase detection (formic acid, isopropyl alcohol, water).

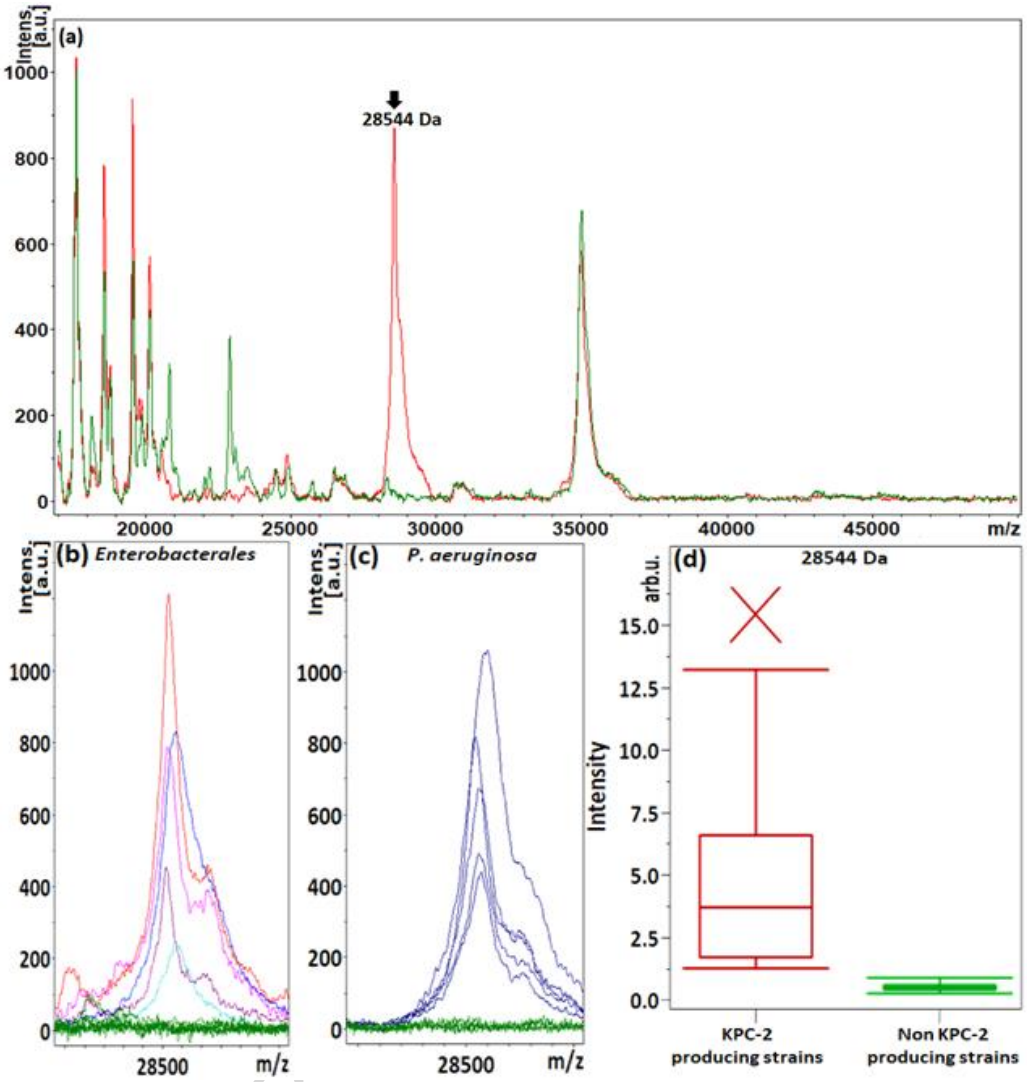

Figure 1. Peaks of MALDI-TOF mass spectra of the KPC-2 and non-KPC-2 producing strain. **a)** Peak detection of ~28,544 Da in *E. coli* with recombinant plasmid (*E. coli* TOP10 + pKPC-2-4) is indicated by red line; Absence of the ~28,544-*m/z* peak in the recipient strain (*E. coli* TOP10+pK19) is indicated by green line. Peak corresponding to KPC-2 is indicated with arrows. **b)** Spectra show the presence or absence of the KPC-2 peak in different *Enterobaterales*. Specific color (red, blue, light blue, magenta, and violet) was assigned to particular species (*K. pneumoniae*, *E. cloacae*, *E. coli*, *S. marcescens*, and *C. braakii* respectively). Green spectra correspond to non-KPC-2-producing strains (susceptible to β-lactams or TEM-1-, CMY-2- or CTX-M- producers). **c)** Dark blue spectra correspond to KPC-2 producing *P. aeruginosa* and green correspond to non-KPC-2-producing *P. aeruginosa*. **d)** Box plot showing median and interquartile range is calculated

from the intensities of the 28,544 Da peak in the KPC-2 and non-KPC-2 producing strains (red and green box, respectively). *x* and *y* axes show *m/z* values and intensity (in arbitrary units – a.u.), respectively.

Statistical results showed a *p*-value < 0.000001 by Anderson-Darling test and Wilcoxon/Kruskal-Wallis test which confirmed a significant difference for the ~28,544 Da peak when both groups were compared (KPC-2 positive vs KPC-2 negative isolates) (Figure 1d). Consistently, the area under the curve (AUC) of the ROC curve for this specific peak was 1 (100% sensitivity, CI95%: [94.0%; 100%] and 100% specificity, CI95%: [94.6%; 100%]). It should be noted that representative isolates of the main *K. pneumoniae* sequence types which are disseminated worldwide were included (such as ST258, ST307, ST25, ST11, ST392, ST340, ST101, ST13) suggesting that this peak detection may be effective on different strains. Furthermore, the ~28,544 Da peak detection was useful on different species where KPC-2 is less frequent.

The DI of KPC-2-producing isolates from colony assays varied between 171.3 - 12.0 (median: 33.0, CI95%: [26.1; 39.9]) while for negative strains, between 5.0 - 0.2 (median: 1.5, CI95%: [1.2; 1.7]) (Figure 2a). Values of this parameter could be used to define a cut-off if it were possible to implement an automated procedure for the detection of clinically relevant β-lactamases.

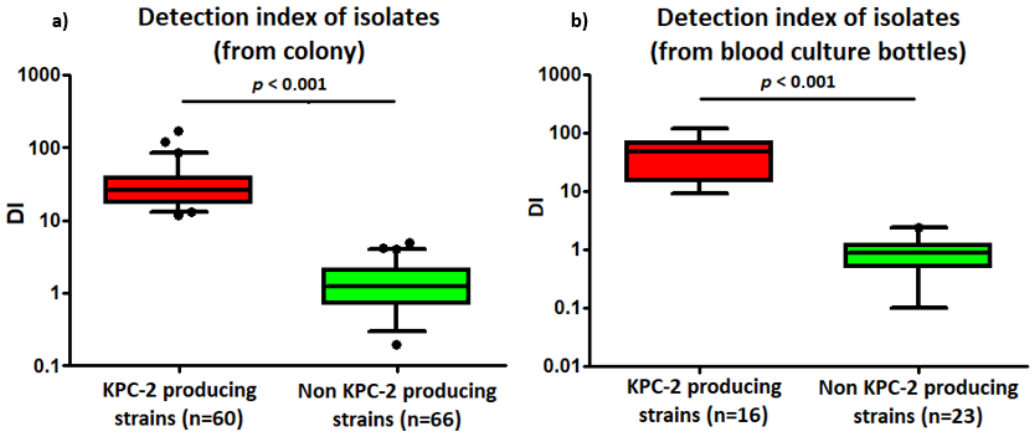

Figure 2. Box and whisker plot showing DI of KPC-2-producing strains (red) and non KPC-2-producing strains (green) (GraphPad Prism). The box always extended from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The

whiskers are drawn down to the 5th percentile and up to the 95th. Points below and above the whiskers – outliers - are drawn as individual points. *p*-value obtained with Mann-Whitney test shows a significant difference between the two groups. a) Box-plot of DI from colony analysis. b) Box-plot of DI from blood culture bottle analysis.

Only eleven out of 39 KPC-2-producing *K. pneumoniae* (28%) were positive for the ~11,109 Da peak and all of them were also positive for the *p019* gene by PCR. All of these 11 strains showed the presence of Tn*4401* element corresponding to isoform a, and 10/11 (91%) belonged to ST258 which was identified extensively worldwide (Table 1). The peak of ~11,109 Da was not observed in the rest of KPC-2 producing *K. pneumoniae* isolates (72%, 28/39). Four out of 28 isolates had Tn*4401* (not isoform a) and three out of those four corresponded to the ST258 (Table 1). In the remaining isolates, $bla_{\text{KPC-2}}$ was associated with a truncated structure of this transposon or to a non-Tn*4401* element (NTE).

Of the four KPC-2-producing *E. coli* clinical isolates (all characterized as ST131), two were positive for the ~11,109 Da peak coinciding with the presence of the *p019* gene in the latter. As expected, Tn*4401* (isoform a) was also detected in these two strains (Table 1). The remaining KPC-2 positive species (*E. cloacae*, *S. marcescens*, *C. braakii* and *P. aeruginoasa*) gave negative results for the ~11,109 Da peak, *p019* gene and Tn*4401*. In these cases, $bla_{\text{KPC-2}}$ would be associated with an NTE (Table 1).

Detection of the ~11,109 Da peak was considered a promising test for the rapid detection of KPC-2-producing strains by MALDI-TOF MS (Gaibani et al., 2016, Youn et al., 2016). In our study, the $bla_{\text{KPC-2}}$ environment in the circulating strains showed a low prevalence of the *p019* gene. This is not surprising considering that different genetic platforms can replace Tn*4401*, and the rearrangements that often occur in plasmids leads to the genetic evolution of these strains and the emergence of several successful clones. As previously reported, the *p019* gene is part of the insertion sequence IS*Kpn31* which is inserted or is a constituent of the Tn*4401a* (Centonze et al., 2018). In this study, $bla_{\text{KPC-2}}$ was not always associated with the Tn*4401a* isoform and was frequently associated with different environments. In good agreement with our results, previous research has also revealed that strains circulating in different regions possess various KPC environments (Partridge, 2014, Stoesser et al., 2017). Thus, the protein p019 or its gene should be

considered a biomarker with low sensitivity in our region and others where the KPC-2 gene is embedded in diverse surrounding environments.

In good agreement with our previous results, the distinctive peak of ~28,544 Da, corresponding to KPC-2 was visually observed in all spectra of KPC-2-producing isolates in the blood culture bottles tested, but not in the carbapenem-susceptible strains (Figure S2a). In order to validate this methodology in the clinical setting and estimate if the biomass present at the time the blood culture becomes positive allows for sufficient KPC-2 detection, a trial with the participation of different laboratories will be necessary. Statistical results showed a 100% sensitivity and specificity (AUC: 1) (Figure S2b) with all included strains under the assayed conditions. Moreover, when the DI was evaluated in the blood culture bottles assays, KPC-2-producing isolates ranged between 118.1 – 9.3 (mean: 47.3, CI95%: [31.2; 63.4]) and the KPC-negative isolates between 2.4 - 0.1 (mean: 1.0, CI95%: [0.6; 1.2]) (Figure 2b). Presence of the biomarker (~11,109 Da peak) was also assessed directly from the blood culture bottles and it was detected only in the p019 positive isolates (3/16).

One of the advantages of the use of MALDI-TOF MS for KPC-2 detection from colony or blood culture bottle is the substantial decrease in the turnaround time (TAT) compared to automated and traditional microbiological phenotypic methods. Although β-lactamase detection by ICAs display a shorter TAT (Riccobono et al., 2018) than MALDI-TOF MS resistance detection, the latter is a low cost procedure once the technology is in place (Tran et al., 2015).

At the present time, any β-lactamase activity can be predicted using MALDI-TOF MS by detection of hydrolysis products of β-lactams. However, it is not possible to establish an accurate resistance profile, unless complementary hydrolysis tests with several substrates requiring 1 hour incubation time each are performed (Hooff et al., 2012, Sparbier et al., 2012). In this sense, the characterization of the resistance marker that is achieved with our protocol could be considered as another advantage. It is important to highlight that, beyond KPC-2, the procedure used in this work proved to be useful for the detection of different β-lactamases such as CMY-2 (Espinosa et al., 2018) and preliminarely on different CTX-Ms (Figueroa et al. 28th ECCMID, 2018).

It is well known that colorimetric tests are easy to perform and cost-effective methods but, in contrast with our method, the identification of β-lactamases involved is not possible.

Applying the proposed 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 less than 1 h (usually 30 minutes), employing the HCCA and SA matrices, respectively.

3. Conclusions

In this study, we demonstrated that MALDI-TOF MS is able to detect directly the most clinically relevant carbapenemase, the KPC-2-enzyme, on different Gram-negative bacilli, mainly *Enterobacterales*, both from colony material and directly from positive blood culture bottles, and at the same time that bacterial identification becomes available. The rapid and accurate detection of KPC-2, as well as any other relevant β-lactamase that are still being explored by MALDI-TOF and other techniques might have extensive clinical implications in resistance diagnosis, antibiotic stewardship for early infection treatment, and implementation of procedures aimed at carbapenem resistance containment.

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Transparency declarations

None to declare

Conflicts of interest:

The authors declare that they have no conflicts of interest

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Table 1. Characterization of KPC-2 producing strains.

Strain	Isolate Origen	Resistance Marker	Peak at m/z ~28,477 Da	Peak at m/z ~11,109 Da	*p019* gene	KPC element (Tn*4401*)	Sequence Type (ST)
K. pneumoniae 720	Human/ Urine	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 952	Human/ Urine	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 729	Human/ Tracheal fluid	$bla_{KPC-2}/bla_{CTX-M-2}$	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 139	Human/ Swab	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 439	Human/ Swab	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 176	Human/ Swab	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 791	Human/ Swab	$bla_{KPC-2}/bla_{CTX-M-15}/ bla_{TEM-1a}/ bla_{OXA-1}/ bla_{SHV-11}$	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 069	Human/ Abdominar fluid	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 070 **BC**	Human/ Liver abscess	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae 805	Human/ Urine	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	340
K. pneumoniae UTI-7	Human/ Blood	$bla_{KPC-2}/bla_{CTX-M-15}/bla_{TEM-1b}$ bla_{SHV-11}	(+)	(-)	(-)	NTE_{KPC}	392
K. pneumoniae 74I **BC**	Human/ BAL	$bla_{KPC-2}/bla_{CTX-M-2}$	(+)	(-)	(-)	NTE_{KPC}	ND
K. pneumoniae 197 **BC**	Human/ Swab	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	ND
K. pneumoniae CX27	Human/ Urine	$bla_{KPC-2}/bla_{CTX-M-G1}/ bla_{CTX-M-2}$	(+)	(-)	(-)	NTE_{KPC}	11
K. pneumoniae HJMC5 **BC**	Human/ Soft tissue	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
K. pneumoniae HSM 456	Human/ Urine	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	25
K. pneumoniae A12 **BC**	Human/ Tracheal fluid	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	SLV 13
K. pneumoniae A24 **BC**	Human/ Catheter	bla_{KPC-2}/bla_{CTX-M}	(+)	(-)	(-)	Yes[b]	258
K. pneumoniae A23	Human/ Catheter	$bla_{KPC-2}/bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	SLV 13
K. pneumoniae A25	Human/ Catheter	bla_{KPC-2}/bla_{CTX-M}	(+)	(-)	(-)	Yes[b]	258
K. pneumoniae A27	Human/ Catheter	bla_{KPC-2}/bla_{CTX-M}	(+)	(-)	(-)	Yes[b]	258
K. pneumoniae M1	Human/ Blood	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	392
K. pneumoniae M2	Human/ Blood	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
K. pneumoniae M3	Human/ Blood	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	13
K. pneumoniae M4	Human/ Blood	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	392
K. pneumoniae M5	Human/ Blood	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	392
K. pneumoniae M6	Human/ Blood	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	101
K. pneumoniae M7	Human/ Blood	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
K. pneumoniae M9 **BC**	Human/ Soft tissue	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	392
K. pneumoniae M10	Human/ Urine	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	392
K. pneumoniae M14 **BC**	Human/ Urine	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
K. pneumoniae M15 **BC**	Human/ Urine	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
K. pneumoniae M17	Human/ Urine	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	392
K. pneumoniae M22	Human/ Blood	bla_{KPC-2}	(+)	(-)	(-)	Yes[b]	307
K. pneumoniae M23	Human/ Abdominar fluid	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
K. pneumoniae 2-10	Human/ Urine	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
K. pneumoniae 16-10	Human/ BAL	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
K. pneumoniae 19-10	Human/ Urine	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258

Strain	Isolate Origen	Resistance Marker	Peak at m/z ~28,477 Da	Peak at m/z ~11,109 Da	*p019* gene	KPC element (Tn*4401*)	Sequence Type (ST)
K. pneumoniae 20-10	Human/ Urine	bla_{KPC-2}	(+)	(+)	(+)	Yes[a]	258
E. cloacae 680 [BC]	Human/ Urine	bla_{KPC-2}/$bla_{CTX-M-G1}$	(+)	(-)	(-)	NTE_{KPC}	ND
E. cloacae A26	Human/ Catheter	bla_{KPC-2} /$bla_{CTX-M-G9}$	(+)	(-)	(-)	NTE_{KPC}	ND
E. cloacae 016	Human/ Swab	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
E. cloacae 043	Human/ Swab	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
E. cloacae 21051	Human/ Swab	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
E. cloacae 029	Human/ Swab	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
E. cloacae 91051	Human/ Swab	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
E. coli 519	Human/ Soft tissue	bla_{KPC-2}/$bla_{CTX-M-G9}$	(+)	(+)	(+)	Yes[a]	131
E. coli 151 [BC]	Human/ Blood	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	131
E. coli 547	Human/ Soft tissues	bla_{KPC-2}/$bla_{CTX-M-G9}$	(+)	(+)	(+)	Yes[a]	131
E. coli 075 [BC]	Human/ Peritoneal fluid	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	131
S. marcescens AMP162-I [BC]	Human/ Catheter	bla_{KPC-2}/$bla_{CTX-M-2}$	(+)	(-)	(-)	NTE_{KPC}	ND
S. marcescens AMP162-II	Human/ Catheter	bla_{KPC-2}/$bla_{CTX-M-2}$	(+)	(-)	(-)	NTE_{KPC}	ND
S. marcescens Sm759 [BC]	Human/ Blood	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
S. marcescens Sm216 [BC]	Human/ Tracheal fluid	bla_{KPC-2}/$bla_{CTX-M-2}$	(+)	(-)	(-)	NTE_{KPC}	ND
C. braakii Cbra007 [BC]	Human/ Urine	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
P. aeruginosa 2047	Human/ Nosocomial	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	654
P. aeruginosa 4270	Respiratory secretions	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	654
P. aeruginosa 4753	Respiratory secretions	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	654
P. aeruginosa Mart1	Human/ Nosocomial	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
P. aeruginosa 537	Human/ Nosocomial	bla_{KPC-2}	(+)	(-)	(-)	NTE_{KPC}	ND
E. coli DH5α	Laboratory Strain	*gyrA96*	(-)	(-)	(-)	(-)	n/a
E. coli TFI-KPC (Tc)	Laboratory Strain	*gyrA96*/bla_{KPC-2}/$bla_{CTX-M-9}$	(+)	(+)	(+)	Yes[a]	n/a
E. coli TOP10	Laboratory Strain	*rpsL*	(-)	(-)	(-)	(-)	n/a
E. coli TOP10 + pk19	Laboratory Strain	*rpsL*/*aph*(3')-Ia	(-)	(-)	(-)	(-)	n/a
E. coli TOP10 + pKPC-2-4) (RC)	Laboratory Strain	bla_{KPC-2} /*rpsL*/*aph*(3')-Ia	(+)	(-)	(-)	(-)	n/a

NTE_{KPC}: bla_{KPC}-bearing partial or non-Tn*4401* element, [a] Tn*4401*a isoform, [b] Tn*4401* isoform different to Tn*4401*a, [BC] Blood Culture assay strains, $bla_{CTX-M-G1}$, $bla_{CTX-M-G2}$ and $bla_{CTX-M-G9}$: correspond to bla_{CTX-M} gene group 1, 2 or 9, respectively, SLV: single locus variant, ND: Not determined, n/a: not applicable.

Table 2. Characterization of non KPC-2 producing strains.

Strain	Isolate Origen	Resistance Marker	Peak at m/z ~28,477 Da	Peak at m/z ~11,109 Da	Sequence Type (ST)
K. pneumoniae I26 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-G9}}$	(-)	(-)	ND
K. pneumoniae HAP13	Human/ Urine	$bla_{\text{CTX-M-G2}}$	(-)	(-)	ND
K. pneumoniae HAP33	Human/ Urine	$bla_{\text{CTX-M-G1}}$	(-)	(-)	ND
K. pneumoniae HAP121	Human/ Urine	$bla_{\text{CTX-M-G1}}$	(-)	(-)	ND
K. pneumoniae B4	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	11
K. pneumoniae CM4	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	11
K. pneumoniae I3 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	11
K. pneumoniae I4	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	11
K. pneumoniae I11	Human/ Nosocomial	$bla_{\text{CTX-M-G1}}$	(-)	(-)	ND
K. pneumoniae I17 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-G1}}$	(-)	(-)	ND
K. pneumoniae I21 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-G1}}$	(-)	(-)	ND
K. pneumoniae T8	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	11
K. pneumoniae C14 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
K. pneumoniae I18 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
K. pneumoniae N1	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
K. pneumoniae C10 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
K. pneumoniae CV1 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}/bla_{\text{CTX-M-15}}$	(-)	(-)	11
K. pneumoniae CM1	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	11
K. pneumoniae I1	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
K. pneumoniae N2 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
K. pneumoniae Ck [BC]	Human/ Nosocomial	$bla_{\text{CMY-2}}$	(-)	(-)	ND
E. coli CM3	Human/ Nosocomial	$bla_{\text{CTX-M-14}}$	(-)	(-)	ND
E. coli N8	Human/ Nosocomial	$bla_{\text{CTX-M-14}}$	(-)	(-)	ND
E. coli C17 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-14}}$	(-)	(-)	ND
E. coli SM8	Human/ Nosocomial	$bla_{\text{CTX-M-14}}$	(-)	(-)	68
E. coli L4 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	131
E. coli M1	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	410
E. coli N2	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	ND
E. coli N4	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	410
E. coli SM5 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	131
E. coli T3	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	131
E. coli T1	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	131
E. coli V91	Pet/Cat	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
E. coli V109	Pet/Cat	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
E. coli 24-28 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
E. coli N1 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
E. coli Itu2	Human/ Urine	$bla_{\text{CTX-M-2}}/mcr\text{-}1$	(-)	(-)	ND
E. coli N14	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND

Strain	Isolate Origen	Resistance Marker	Peak at m/z ~28,477 Da	Peak at m/z ~11,109 Da	Sequence Type (ST)
E. coli SM3	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
E. coli VTEM-38	Pet/Dog	$bla_{\text{TEM-1}}$	(-)	(-)	ND
E. coli VTEM-86	Pet/Dog	$bla_{\text{TEM-1}}$	(-)	(-)	ND
E. coli IACA17	Human/ Urine	NDβ	(-)	(-)	ND
E. coli CEM4	Human/ Urine	NDβ	(-)	(-)	ND
E. coli 1510	Human/ Urine	$bla_{\text{CMY-2}}$	(-)	(-)	ND
E. coli 856	Human/ Abdominar fluid	$bla_{\text{CMY-2}}$	(-)	(-)	ND
Cbra CX16 [BC]	Human/ Soft tissue	NDβ	(-)	(-)	ND
Cbra HdeC2 [BC]	Human/ Urine	NDβ	(-)	(-)	ND
Sm I2 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
Sm T9 [BC]	Human/ Nosocomial	$bla_{\text{CTX-M-2}}$	(-)	(-)	ND
Sm 947 [BC]	Human/ ND	$bla_{\text{SME-1}}$	(-)	(-)	ND
Sm CAR653	Human/ Soft tissue	NDβ	(-)	(-)	ND
Sm CX46	Human/ Soft tissue	NDβ	(-)	(-)	ND
Sm CX17 [BC]	Human/ Soft tissue	NDβ	(-)	(-)	ND
Sm HdeC30	Human/ Tracheal fluid	NDβ	(-)	(-)	ND
Sm HAP54	Human/ Bile	NDβ	(-)	(-)	ND
Sm SOA15	Human/ Blood	NDβ	(-)	(-)	ND
Sm VSm102	Pet/Dog	NDβ	(-)	(-)	ND
Sm VSm117	Pet/Dog	NDβ	(-)	(-)	ND
Ecl 1042 [BC]	Human/ Blood	*act*7 /$bla_{\text{TEM-1}}$	(-)	(-)	ND
Ecl I28	Human/ Nosocomial	$bla_{\text{CTX-M-15}}$	(-)	(-)	ND
Ecl V206	Pet/Cat	Ind AmpC	(-)	(-)	ND
Ecl V211	Pet/Cat	NDβ	(-)	(-)	ND
Ecl V207 [BC]	Pet/Dog	$bla_{\text{CTX-M-15}}$	(-)	(-)	ND
P. aeruginosa 24	Human/ Nosocomial	NDβ	(-)	(-)	ND
P. aeruginosa 2054	Human/ Nosocomial	NDβ	(-)	(-)	ND
P. aeruginosa ATCC 9027	Laboratory Strain	NDβ	(-)	(-)	ND

[BC] Blood Culture assay strains, $bla_{\text{CTX-M-G1}}$, $bla_{\text{CTX-M-G2}}$ and $bla_{\text{CTX-M-G9}}$: correspond to $bla_{\text{CTX-M}}$ gene group 1, 2 or 9, respectively, NDβ: Non-detectable β-lactamases, Ind: Inducible β-lactamase, ND: Not determined

Highlights

- KPC-2-enzyme was detected by MALDI-TOF MS in different Gram-negative bacilli
- A distinctive peak around 28,544 Da was found in all KPC-2 producers
- Direct KPC-2 detection on positive blood culture bottles was also achieved
- The proposed accompanying 11,109 Da peak was a bad predictor for KPC-2 presence