Rapid Identification of Carbapenemase-producing *Klebsiella pneumoniae* strains by Matrix-Assisted Laser Desorption/Ionization-Time of Flight using Vitek® Mass Spectrometry System

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ABSTRACT

Objective: The analysis of the protein pattern of *Klebsiella pneumoniae* carbapenemase (KPC)-producing strains by Bruker Matrix-Assisted Laser Desorption Ionization (MALDI) Biotyper system has revealed the presence, in the majority of cases, of an 11.109 *m/z* peak. The peak corresponds to the gene product named p019 of the bla_{KPC}-bearing plasmids and has been suggested as a candidate for a biomarker that is able to distinguish KPC-producers from non-KPC-producers. The aim of this study was to evaluate the rapid detection of the 11.109 *m/z* peak of KPC-producer strains in the clinical laboratory routine by Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) technique, using the Vitek® Research-User-Only (RUO) Mass Spectrometry (MS) system without changing the instrument parameters.

Materials and Methods: Globally, 373 *K. pneumoniae* isolates were investigated and identified by MALDI-TOF MS analysis. KPC-producers were distinguished from non-KPC-producers by Antimicrobial Susceptibility Testing (AST) and phenotypic carbapenemase resistance assays.

Results: The MALDI-TOF Vitek MS RUO detected the 11.109 *m/z* peak in 95.7% of KPC-producers with 100% specificity before traditional test results became available.

Conclusion: Our approach is appropriate as a first screening step for the rapid identification of KPC isolates, which will help to improve infection control in clinical practice and prevent the outbreak and dissemination of resistant bacteria.

Keywords: Carbapenemase-producing strains, *Enterobacteriaceae*, *Klebsiella pneumonia*, MALDI-TOF MS, rapid identification

Introduction

Gram-negative bacteria, particularly *Enterobacteriaceae*, are widely known to be frequent causes of community- and hospital-acquired infections of the urinary and lower respiratory tracts and the bloodstream [1-4]. These pathogens can present genes encoding numerous antimicrobial resistance mechanisms, including extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases, and carbapenemases [5]. The preferred therapeutic intervention for serious infections is β-lactam antibiotics, while carbapenems are considered the last option. Nonetheless, in the last decade, the emergence and subsequent spread of carbapenem-resistant *Enterobacteriaceae* (CRE), especially *Klebsiella pneumoniae* strains, has led to the lack of therapeutic options [6, 7].

Carbapenemases are classified into three groups as follows; Class A: serine carbapenemases, Class B: Metallo-β-lactamases (MBL), and Class D: oxacillin (OXA) enzymes and OXA-like enzymes [8]. CRE are often resistant to antimicrobial agents belonging to other different classes and are related to higher mortality rates [5, 7]. In numerous countries, including Italy, Greece, and the U.S.A, the presence of carbapenemase-producing *K. pneumoniae* (KPC) strains is endemic [9-11]. Appropriate antibiotic therapy is mandatory in order to decrease the mortality of patients with infections due to KPC bacteria [12-14] and the rapid detection of KPC is essential to prevent the outbreak and dissemination of these strains. Several methods have been used for the detection of carbapenemases, such as phenotypic assays and molecular methods. Phenotypic assays consist of the modified Hodge test, the Carba NP tests, and the carbapenem inactivation method. Molecular methods, including multiple polymerase chain reaction, microarrays, and

whole-genome sequencing, identify mutated and acquired genes [7, 15-20].

Recently, Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (MS) has been introduced in clinical microbiology laboratories to detect gram-negative carbapenemase-producing bacteria [7, 21, 22]. In particular, several works reported the use of MALDI-TOF to determine the carbapenemase activity of *K. pneumoniae* strains by detecting the products of hydrolysis of carbapenems, such as imipenem and ertapenem [7, 21]. Gaibani et al. used MALDI Biotyper RTC software to detect a single-peak protein specific for KPC-positive strains [22]. Among 10 peaks potentially useful in differentiating between KPC-positive and KPC-negative isolates, an 11.109 *m/z* peak was identified in 88.2% of the KPC-producers, suggesting its high discriminatory power. The 11.109 *m/z* peak represents a cleavage protein product named pKpQIL_p019 (p019), which is encoded by the bla_{KPC}-containing pKpQIL plasmids and pKpQIL-like plasmids that have the propensity to carry KPC [23]. This protein is involved in carbapenem resistance and has already reported being associated with several numbers of CRE outbreaks [23-25].

The goal of our study was to assess the sensitivity and specificity of MALDI-TOF Vitek® MS Research-User-Only (RUO) system (bioMérieux, Marcy l'Etoile, France) for first rapid detection of the 11.109 *m/z* peak using several unrelated *K. pneumoniae* isolates collected at Desio Hospital, Desio (MB), Italy. Early detection could contribute toward preventing the dissemination of resistant bacteria and implementing appropriate infection control measures in hospitals.

Materials and Methods

Isolates and Resistance Detection

K. pneumoniae strains used in this work were isolated from blood and other specimens such as central catheters, pus, scabs, surgical wounds, sputum or tracheal aspirate, using Columbia agar with 5% sheep blood (*COS,* bioMérieux, Marcy l'Etoile, France) and MacConkey agar plates (MCK, bioMérieux, Marcy l'Etoile, France), and from urine samples and urinary catheter using chromID®CPS® Elite agar plates (bioMérieux, Marcy l'Etoile, France). COS, MCK, and CPS agar plates were incubated for 24-48 h at a temperature of 36°C±1°C under aerobic conditions. The species were identified using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Vitek® MS RUO system (bioMérieux, Marcy l'Etoile, France). Samples collected from patients known or suspected to be carriers of carbapenem-resistant *Klebsiella pneumoniae* included perirectal or nasopharyngeal swabs as well as stool, sputum or tracheal aspirate. These samples were inoculated on MacConkey agar with the addition of a meropenem disk (10 µg) (Oxoid Limited, Basingstoke, Hampshire, UK) to isolate carbapenemase-resistant *Enterobacteriaceae*. A diameter of inhibition smaller than 30 mm was the criterion to suspect carbapenem resistance [26].

Antimicrobial Susceptibility Testing (AST) and resistance detection of the clinical isolates were determined using Vitek AST-N202 cards (bioMérieux, Marcy l'Etoile, France). An isolate was considered resistant when intermediate or complete resistance to one of the carbapenems (ertapenem, imipenem, or meropenem) was registered. To confirm the identification of a carbapenemase-producing strain, the isolate was then inoculated on selective chromogenic medium CARBA-SMART (bioMérieux, Marcy l'Etoile, France), and incubated for 24-48 h at a temperature of 36°C±1°C under aerobic conditions. Moreover, meropenem β-lactam inhibitor Combination Disk Test (CDT) (ROSCO Diagnostica A/S, Taastrup, Denmark) was performed on Muller-Hinton agar plates (bioMérieux, Marcy l'Etoile, France) to determine the presence of carbapenemase resistance phenotype [27].

A total of 373 clinical *K. pneumoniae* isolates were obtained from subjects hospitalized at Hospital from April 2017 to September 2017. Fresh overnight cultures of *K. pneumoniae* ATCC BAA 1705 (bla_{KPC}-positive) and *K. pneumoniae* ATCC BAA 1706 (bla_{KPC}-negative) were used as controls for the tests.

Sample and Target Plate Preparation

Two different methods were carried out for MS sample preparation of *K. pneumoniae* isolates: direct deposition and identification after ethanol/formic acid/acetonitrile protein extraction. For direct-spotting experiments (method **1**), one bacterial colony was spotted directly on the Vitek® MS-DS target slide (bioMérieux, Marcy l'Etoile, France) and overlaid with 1 µL of formic acid and 1 µL of saturated α-Cyano-4-hydroxycinnamic acid (CHCA) (Vitek® MS-CHCA, bioMérieux, Marcy l'Etoile, France). For protein extraction (method **2**), fresh bacterial isolates were suspended in 1 mL of 70% ethanol, vortexed for 1 min, and centrifuged at 13,000 *rpm* for 2 min. The supernatant was removed completely, and the pellet was vortexed for 10s with 50 µL of 70% formic acid and 50 µL of 100% acetonitrile. After incubation for 15 min at room temperature and centrifugation at 13,000 *rpm* for 2 min, 1 µL of the supernatant solution was spotted onto a target plate, air dried and covered with 1 µL of CHCA matrix solution.

MALDI-TOF Mass Spectrometry Analysis

All *K. pneumoniae* isolates were prepared using the two methods described above, and analyzed by MS in duplicate. Spectra were obtained on a MALDI-TOF Vitek® MS RUO system (bioMérieux, Marcy l'Etoile, France) in a positive linear mode in the mass range of 2000–20000 *m/z*, without changing the instrument parameters. The results were analyzed using the Saramis™ database (Spectral ARchive And Microbial Identification System) (Version 4.10, AnagnosTec, Potsdam, Germany) and Shimadzu Biotech Launchpad® software (Shimadzu Corporation, Kyoto, Japan). Data analysis was performed following the manufacturers' instructions; if the percentage was between 75% and 99.9%, the identification was considered valid at the species level and if the percentage was below 75%, no identification was obtained. These confidence references were based on the accuracy of fit-to-weighted consensus reference spectra for a given taxon. *Escherichia coli* ATCC® 8739 was used as a control strain. The operators were trained in Saramis software for the visual inspection of the spectra and the detection of 11.109 *m/z* peak in each spectrum was acquired by MALDI-TOF MS.

Statistical Analysis

Sensitivity, specificity, and other statistical analyses were carried out with MedCalc for Windows software (Version 15.0, MedCalc Software, Ostend, Belgium) [28].

Results

Among the 373 *K. pneumoniae* strains that were isolated from April 2017 to September 2017 and subjected to phenotypic assays, 163 were characterized as non-carbapenemase-producers, while 210 were characterized as carbapenemase-producers by antimicrobial susceptibility and CDT phenotypic assays. Using the Vitek® MS RUO system for the rapid identification of KPC-producers by detecting the 11.109 *m/z* peak and considering both the direct deposition and the complete protein extraction protocol, the 11.109 *m/z* peak was found in 201 (95.7%) spectra of KPC-positive and in none of the KPC-negative spectra (Figure 1). The peak was not detected in only 9 KPC-producers (4.3%) (Table 1). Overall, the peak detection achieved a sensitivity of 95.7% and a specificity of 100% (Table 1).

The mean *m/z* value (± standard deviation (SD)) of the p019 peak using *K. pneumoniae* ATCC

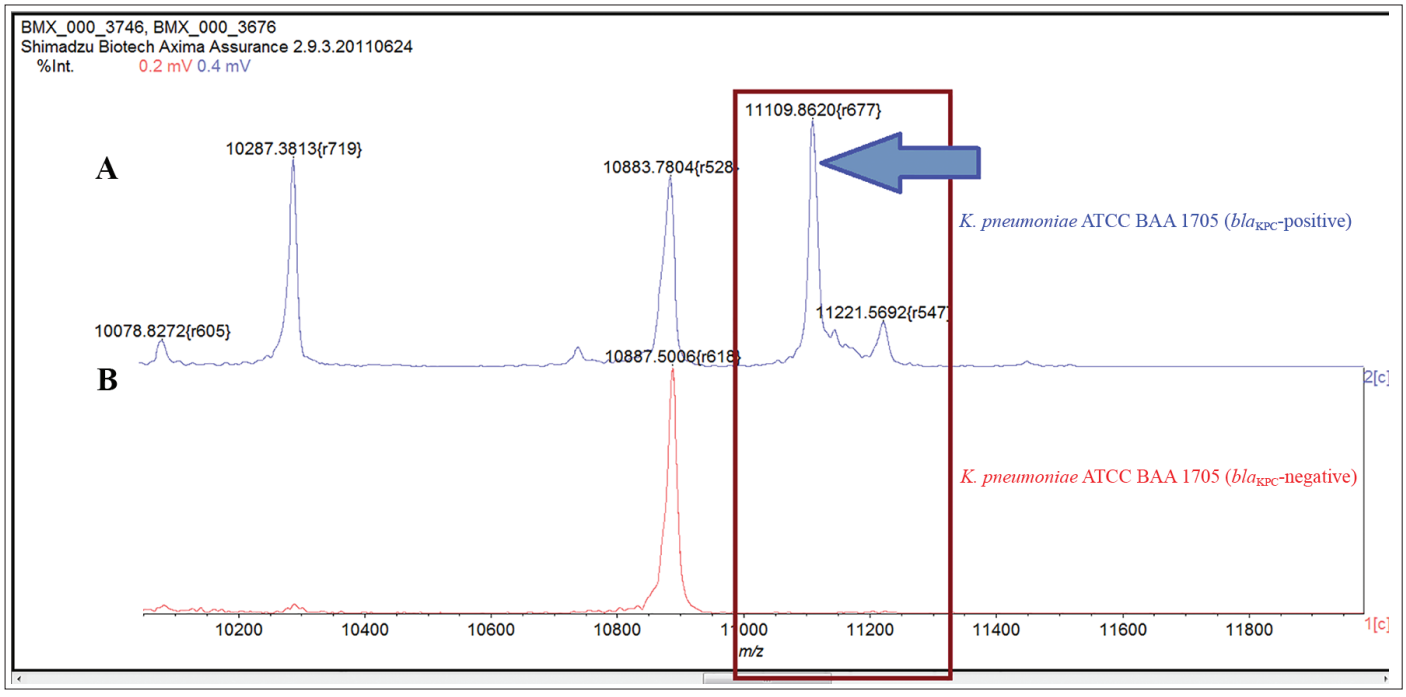

Figure 1. a, b. MALDI-TOF Vitek® MS RUO detection of 11.109 *m/z* peak in (a) *K. pneumoniae* ATCC BAA 1705 (bla_{KPC}-positive) strain and (b) *K. pneumoniae* ATCC BAA 1706 (bla_{KPC}-negative). The arrow indicates the 11.109 *m/z* peak detected in *K. pneumoniae* KPC-positive isolates.

Table 1. Performances of the two protocols of MALDI-TOF identification for KPC isolates

Methods	KPC isolates (*n*)	Presence of 11.109-Da peak (*n*)	Absence of 11.109-Da peak (n)	Mean relative peak intensity[b] [Arb. unit (±SD)]	Sensitivity	Specificity
Direct deposition[a]	210	201	9	0.20±0.10	95.7	100
Complete protein extraction[a]	210	201	9	0.62±0.20		

[a]For the direct deposition, one bacterial colony was gently scraped from each agar plate using a 1µL disposable plastic loop picked on the same Vitek® MS-DS target slide (bioMérieux), covered with 1 µL of formic acid, air dried, and covered with 1 µL of saturated α-Cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 2.5% trifluoroacetic acid matrix solution (Vitek® MS-CHCA, bioMérieux). For the second procedure, one bacterial colony was gently scraped from the agar plate with a 1µL disposable plastic loop and suspended in 1 mL of 70% ethanol. The sample was centrifuged at 13,000 *rpm* for 2 min in a bench top centrifuge; the pellet was suspended in 50 µL of 70% formic acid, vortexed, and then 50 µL of 100% of acetonitrile was added. The suspension was vortexed and after incubation for 15 min at room temperature, centrifuged at 13,000 *rpm* for 2 min. 1 µL of the supernatant solution was finally spotted onto a target plate, air dried and covered with 1 µL of CHCA matrix solution.

[b]The difference between the mean relative peak intensities of the two methods is significant ($p<0.0001$).

KPC: *Klebsiella pneumoniae* carbapenemase (KPC)-producing; Arb. unit: Arbitrary Unit; SD: Standard Deviation.

BAA 1705 (bla_{KPC}-positive) was 11.110±6 *m/z*, while the one obtained from the clinical specimens investigated in this study was 11.111±18 *m/z* ($p<0.0001$). The direct deposition on the target plate and the complete protein extraction protocol yielded the same results in detecting the 11.019 *m/z* peak, although a few differences were observed. The complete protein extraction method generated a reduced background highlighting the 11.019 *m/z* peak, and thus higher mean relative peak intensity values [0.62±0.20 Arb. unit (±SD), Arbitrary Unit] compared to the direct deposition with formic acid [0.20±0.10 Arb. unit (±SD)] ($p<0.0001$).

Discussion

In this study, we successfully used the commercial MALDI-TOF Vitek® MS RUO system for the rapid detection of p019 protein in carbapenemase-producing *K. pneumoniae* isolates. Overall, we observed that 210 out of 373 isolates were phenotypically identified as KPC, and the sensitivity and specificity of our MALDI 11.109 *m/z* peak detection were 95.7% and 100%, respectively. Our results confirmed that this approach provides a more rapid response (24 h) to clinicians compared to other phenotypic tests, such as AST (48 h) and selective agar plate tests (48-72 h). Moreover, this method can be used to detect the presence of the protein product of *p019* gene in all *Enterobacteriaceae* species as previously described [23, 25], in order to control the spread of carbapenemase-producing bacteria among hospitalized patients.

The analytical performance of the two procedures applied to prepare MALDI target plates, i.e. direct deposition with formic acid and complete protein extraction, showed that the 11.109 *m/z* peak was clearly detected in 95.7% of KPC strains. However, a variable intensity of the 11.109 *m/z* peak and run-to-run *m/z* range was observed. Using the direct protein deposition, MALDI-TOF provided spectra with a suitable resolution, but the number of mass ions present in the profiles was elevated and the background noise was high, probably due to the typical mucous-like consistency of *K. pneumoniae* colonies or to the contaminations in the culture medium. The variability of peak detection could also have been caused by a partial protein extraction in the direct deposition procedure, and by the differences in protein expression of isolated strains. Conversely, the protein extraction protocol generated spectra of higher quality, thus improving the signal-to-noise ratio, accuracy, intensity, and identification of the 11.109 *m/z* peak in most of the KPC-positive isolates. Only in 9 KPC-producing *K. pneumoniae* isolates was the 11.109 *m/z* peak absent, which was confirmed by multiple runs performed on the same day as well as on different days. Our data agree with the results previously obtained by Gaibani

et al. [22], who verified the presence of the 11.109 *m/z* peak in 88.2% of KPC-producing *K. pneumoniae* strains analyzed in their study. The absence of 11.109 *m/z* peak was most probably due either to the very low protein expression of p019 in these isolates or to the lack of *p019* gene in their plasmids. In fact, detailed genetic analyses showed that the *p019* gene is situated on a broad range of *Inc* group plasmids that have a propensity to carry carbapenemase resistance. It is particularly related to the isoform 'a' of transposon Tn4401and is absent in plasmids carrying other Tn4401 isoforms, such as Tn4401b and Tn4401d [22, 23]. Further analyses of KPC-p019 negative plasmids could be useful for the identification of genes, and the corresponding proteins, involved in alternative carbapenemase resistance mechanisms.

A limitation of this study was that it was based on a 'one center, one collection' approach. The sample group needed to include characterized strains from other hospitals or from national reference centers/institutes. Another limitation was that only *K. pneumoniae* isolates were used for our analysis because of their greater isolation frequency, however, it could be useful to extend our method to all *Enterobacteriaceae* species, even if they are uncommon. Therefore, the accuracy of our results could be improved using a larger number of species, which would contribute to the implementation of infection control measures to prevent a wide range of outbreaks. Finally, the correct identification of the isolates was confirmed using different conventional phenotypic CRE detection approaches to minimize errors rather than the highly efficient molecular detection tests.

In conclusion, our study confirms Gaibani's results using a different MALDI-TOF MS system. Further, we have demonstrated that the complete protein extraction procedure that requires only a few additional minutes of preparation compared to the direct deposition method, improves the quality of detection of the p019 protein and thus of the identification of KPC-producers, thereby reducing the time it takes to report to clinicians. This approach could thus complement current traditional assays as a suitable first step in the routine workflow of clinical laboratories to rapidly detect KPC-producers and prevent the dissemination of resistant pathogens.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Desio Hospital, Desio (MB), Italy.

Informed Consent: N/A.

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