Determination of Carbapenemase Producing of *Klebsiella pneumoniae* Isolated in Blood Cultures By Matrix Assisted Laser Desorption/Ionization-flight Time Mass Spectrometry

Kan Kültürlerinde Üreyen *Klebsiella pneumoniae* Suşlarında, Karbapenemaz Üretiminin Matriks Destekli Lazer Desorpsiyon/Iyonizasyon-Uçuş Zamanı Kütle Spektrometresi ile Belirlenmesi

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**ABSTRACT**

**Introduction:** *Klebsiella pneumoniae* can cause Healthcare Related Infections (HRIs), especially by carbapenemase producing microorganisms. Rapid methods are needed to determine carbapenem resistance. MALDI-TOF MS is a faster diagnostic method than conventional methods. It is expected that routine use and standardization of this diagnostic test in laboratories will contribute to the rapid determination of carbapenemase resistance. In this study, it was aimed to determine carbapenemase enzyme production by MALDI-TOF MS technique in *K. pneumoniae* strains.

**Materials and Methods:** Carbapenem susceptible (n= 40) and non-susceptible (n= 96) *K. pneumoniae* strains isolated in the Medical Microbiology Laboratory between January 2016 to December 2017 were included into the study. Detecting of production of carbapenemase enzyme was investigated by MALDI-TOF MS technique by comparing with the polymerase chain reaction (PCR).

**Results:** PCR analysis showed that 93 of the carbapenem resistant isolates had OXA-48 gene and three of them had NDM gene. In the MALDI-TOF MS analyzes of the 96 isolates with genotypic resistance, 88 were resistant and three were susceptible. Five isolates of carbapenem resistant with PCR could not be identified as resistant or sensitive by this method. Forty isolates susceptible to carbapenem were determined to be susceptible by MALDI-TOF MS. Sensitivity, specificity, positive predictive, negative predictive values were 96.7%, 100%, 100%, 93% respectively.

**Conclusion:** It appears that MALDI-TOF MS determines carbapenemase production with high specificity and sensitivity. We think it will be beneficial to complete standardization studies and use them in routine practice.

**Key Words:** Klebsiella pneumoniae; Carbapenemase; OXA-48; NDM; MALDI-TOF MS.
INTRODUCTION

Klebsiella pneumoniae, included in the Enterobacteriaceae family, is the microflora bacteria of the gastrointestinal tract and can cause Healthcare Related Infections (HRIs). These species, which are among the opportunistic pathogens, can cause various infections, especially in immunocompromised patients[1]. K. pneumoniae, the most important species in this genus, often causes hospital-acquired urinary tract infections, pneumonia, sepsis and soft tissue infections. Healthcare workers’ hands, mechanical ventilators apparatus, catheters and surgical wounds are important predisposing factors for Klebsiella infections and fecal-oral transmission is plausible[2,3].

Due to beta-lactamase enzymes synthesized by pathogens, difficulties are experienced in the treatment of infections caused by Enterobacteriaceae, including K. pneumoniae, which are generally treated with beta-lactam antibiotics. Beta-lactamases and carbapenemases are bacterial enzymes well-known for causing antibiotic resistance. Especially in the Enterobacteriaceae family, carbapenemase producing isolates have increased significantly in recent years[4].

Carbapenemases are one of the most broad-spectrum beta-lactam antibiotics, and treatment options are restricted since microorganisms have developed resistance to carbapenemases. The beta-lactamases hydrolyzing carbapenemases are classified in class A, B and D in the Ambler classification. K. pneumoniae carbapenemase (KPC), which is one of the first identified carbapenemases, is in the class A and is transferred via plasmid[5]. In class B, whereas Verona integron-encoded metallo-beta-lactamase (VIM) and imipenem-hydrolysing beta-lactamase (IMP) are found, and oxacillin-hydrolysing carbapenemase (OXA) enzymes are in class D. OXA-48 is the best known enzyme in the OXA group and is widely available in Turkey.
and worldwide[6,7]. New carbapenemases have been reported in Enterobacteriaceae and non-fermentative bacilli[7].

OXA-48 carbapenemase-producing strains are endemic in Turkey in terms of the spread of carbapenemase types among European countries. Furthermore, New Delhi metallo-beta-lactamase (NDM-1) type carbapenemases have rapid spread, and VIM type carbapenemases often cause outbreaks in hospitals. Several researches have reported that it is the focal point in India, United States, Israel, Greece and Italy for NDM, and Turkey and North African countries to OXA-48, and India for KPC. OXA-48 was first reported in our country in 2003 and continues to be reported intensively[7,8].

Carbapenem-resistant microorganism infections are associated with high mortality and morbidity due to limited availability of treatment options. However, the increase of carbapenem resistance provides depletion of antibiotic alternatives necessary for the treatment of these infections and an environment for the rapid dissemination of resistance. In Turkey, the rate of increase in infections caused by carbapenemase-producing Klebsiella species is higher than the other regions of the world[7,9,10].

Identification of carbapenemase-producing clinical isolates is necessary for the treatment of infections caused by these isolates, to prevent spread of resistance genes, and for prevention of hospital infections[11]. For the identification of carbapenemase-producing bacteria, conventional methods such as Modified Hodge gradient test (MHT), double disk synergy test, and using the inhibitors and molecular methods have been utilized. In addition to these tests, Matrix assisted laser desorption/ionization-flight time mass spectrometry (MALDI-TOF MS) is one of the new methods used in this field[12-16].

MALDI-TOF MS is a method that analyzes different flight behavior of the released molecules by providing ionization of the sample with a laser that can determine the production. The peak diagram of meropenem can be shown in carbapenem-sensitive isolates. But these peak diagrams decrease in carbapenemase producing strains[12-16].

Difficulties have been experienced in identifications of OXA-48. The presence of these enzymes should be confirmed by other methods since no inhibitor is present for OXA-48-like enzyme-producing bacteria[17]. Therefore, it is needed to develop rapid identification systems such as MALDI-TOF MS to determine of OXA-48 like carbapenamase enzymes.

In this study, it was aimed to determine carbapenemase enzymes produced by K. pneumoniae strains by using MALDI-TOF MS technique used in the identification of microorganisms in microbiology laboratories.

**MATERIALS and METHODS**

K. pneumoniae strains were isolated from the blood samples in Medical Microbiology Laboratory of Necmettin Erbakan University Medical Faculty Hospital between January 2016 and December 2017. Ninety-six of the isolates resistant to imipenem and meropenem and 40 isolates sensitive to imipenem and meropenem according to EUCAST criteria were included into this study. All bacteria were stored at -20°C in 15% glycerin-containing stock. Ethical review and approval were sought from Meram Faculty of Medicine Research and Ethics Committee (Approval No: 2017/1092).

Identification of the bacteria was performed with VITEK® MS MALDI-TOF (BioMerieux, France), an automated identification system and the system’s software was used (SARAMIS v4.12 program). K. pneumoniae strains that were defined with above 98% safety by the evaluation (giving specific peaks in Figure 1) of the system in the database were included into the study. Bacteria identified with safety lower than this were not included into the study. Confirmations of 96 K. pneumoniae isolates that were imipenem and meropenem resistant were performed by gradient test methods.

The synergy test with meropenem discs combined with aminophenyl boronic acid, phenyl boronic acid, dipicolinic acid and cloxacillin was performed. If synergy was not observed and temocillin disc (30 μg) formed a lower zone at 10 mm, phenotyping determinant for OXA-48 was accepted.
Polymerase chain reaction (PCR) analysis of these isolates was performed to determine the resistance genes OXA-48 and NDM. In order to identify bacterial resistance genes and see growth curves in real-time PCR, the primer was determined and the following primer sequences were used. For NDM-1; forward primer: 5'-TTGGCCTTGCTGTCCTTG-3', reverse primer: 5'-ACACCAGTGACAATATCACCG-3', and for OXA-48; forward primer: 5'-TGTTTTTGCTGGCATCGAT-3', reverse primer: 5'-GTAAMRATGCTTGGTCCGC-3' primers were used. DNA amplification was performed using a real-time PCR system (Roche Light Cycler 480 Instrument II, Switzerland).

When samples in stock media were at room temperature, they were then cultivated on blood agar and incubated overnight at 35°C. Suspensions were prepared to study of VITEK® MALDI-TOF MS. Three mL of 150 mM NaCl, and 20 mM Tris-HCl solution was transferred to each test tube. Bacterial suspensions were prepared to four McFarland densities. One ml each of these prepared three ml suspension was transferred to microcentrifuge tubes. The samples were then centrifuged at 4000 rpm for three minutes. After centrifugation, the supernatants were discarded, 50 µL solution containing 150 mM NaCl, 20 mM TRIS HCl and 20 mM meropenem were added to the tubes. This solution was incubated at 35°C for 3 hours. Subsequently, the composition was centrifuged at 14000 rpm three min. In order to study MALDI-TOF, one µL of the supernatant was taken and the preparation was prepared.

The sample prepared from the supernatant was analyzed by VITEK® MS MALDI TOF (SARAMIS v4.12 program) (BioMerieux, France). The peaks of meropenem and bacteria were assessed in the MALDI-TOF MS. The results were compared with genotypic evaluation.

RESULTS

OXA-48 gene in 93 isolates and NDM gene in three isolates were revealed in genotypic analysis of the 96 carbapenem resistant isolates.

In the MALDI-TOF MS analysis, the peaks in the MALDI-TOF MS formed in meropenem only are shown in Figure 2. The peaks formed by carbapenem-sensitive isolate are shown in Figure 3. The peak diagram of the carbapenem-resistant isolate is shown in Figure 4. As shown in Figure 4, if the meropenem peak was observed to fall (a lack of the m/z 380-383 peaks), the tested
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Figure 2. Representative MALDI-TOF MS spectra of meropenem without bacteria.

Figure 3. Representative MALDI-TOF MS spectra of meropenem and of carbapenem susceptible isolate.

Figure 4. Representative MALDI-TOF MS spectra of meropenem and of carbapenem resistant isolate.
strain was considered to have carbapenemase production. If both the bacterium and meropenem peaks were as seen in Figure 3 (a decrease in bacteria peak over time), it was accepted to produce no carbapenemase.

In the MALDI-TOF MS analysis of 96 isolates which have genotypic resistance genes, 88 of them were found to be resistant to carbapenem (producing carbapenemase) and three of them were found to be no resistant although they are resistant genetically. MALDI-TOF MS analysis could not observe whether five isolates were resistant or sensitive. Sensitivity of five isolates was not detected by MALDI-TOF MS analysis. No false positive results were seen.

This method had a sensitivity of 96.7%, specificity of 100%, positive predictive value (PPV) of 100% and negative predictive value (NPV) of 93% for the detection of producing carbapenemase compared with genetically analyzing (PCR). Failure to make any comments for five isolates reduces the efficiency of the test.

**DISCUSSION**

It is frequently observed that the members of carbapenemase-producing *Enterobacteriaceae* have low levels of carbapenem resistance, which can lead to misleading results related to MIC values. The detection of low-level carbapenemase-expressing infectious agents is not always possible according to the limit values determined by international guidelines. Especially, OXA-48-expressing strains are generally low levels of carbapenemase producers and there is no phenotypic method with a high diagnostic value that can be used for the detection of OXA-48 in carbapenem-resistant strains[18]. Therefore, it is rationally predictable that a single carbapenem screening criterion cannot be used to specifically identify carbapenemase-expressing strains. Thus, confirmatory tests is essential for the detection of especially carbapenemase-producing *Enterobacteriaceae*[18,19]. This situation causes problems such as increase in workload, economic loss and most importantly, longer detection period.

Currently, there are studies reporting that the sensitivity of conventional methods in the detection of beta-lactamases and carbapenemases is generally high. For example, Mirigou et al., using MHT method to detect carbapenemase in *Enterobacteriaceae* species, have observed that the test had a sensitivity of 95-100%[20]. Ribeiro et al. aimed at assessing the performance of a quantitative interpretation of MHT in a study of concerns about difficult interpretation and widespread false positive results in the presence of beta-lactamases[21]. At the end of the study, the sensitivity of the test was obtained as 99.4%. Similarly, Legese et al. have reported that they had 90.9% sensitivity of the double-disc synergy test used in the detection of beta-lactamase and carbapenemase production in their study in the members of *Enterobacteriaceae* causing infection in pediatric patients[22]. Pasteran et al. have reported that they obtained 86-100% sensitivity in the detection of carbapenemase-producing bacteria in their study using the combined disc synergy test, which is a phenotypic method[23].

The most important disadvantage of conventional methods with high sensitivity percentages in the detection of beta-lactamase/carbapenemase is the longer incubation time. At the same time, there is a need for long time for analyzing by PCR in practice. However, the cost of consumables is very low, but the cost of installation and maintenance of MALDI-TOF MS is high. On the other hand, with automated systems such as MALDI-TOF MS, results with high sensitivity percentages can be obtained in a short time. In addition to allowing identification directly from the patient sample, antibiotic susceptibility detection and enzyme level distinctions such as carbapenemase may be detected in the future[24].

MALDI-TOF MS is an automated method increasingly used in the identification of various infectious agents at routine works and in research laboratories. There are many advantages of this method, including high sensitivity and specificity, short analysis time, and etc. This method that analyzes different flight behavior of the released molecules by providing ionization of the sample with the laser can determine the production of carbapenemase in a short time by the detection of the hydrolytic products of carbapenem in the case of carbapenemase expression of the infectious agent, too[25,26].
Optimizing the MALDI-TOF MS method by diversifying the solutions used in the extraction, the antibiotic to be used in the analysis, and incubation times will be important milestones in laboratory diagnosis of carbapenemase detection. Thus, the method will be used more widely for carbapenemase detection\textsuperscript{[13,27]}.

In a study using the MALDI-TOF MS method, MALDI-TOF MS method has been confirmed in \textit{Enterobacteriaceae} members and \textit{Pseudomonas aeruginosa} with carbapenemase-mediated carbapenem resistance with other methods\textsuperscript{[28]}. Of the 124 strains included in the study, 30 were observed carbapenemase-producing by conventional methods. When investigated producing carbapenemase with MALDI-TOF MS, the sensitivity and specificity of the method were determined as 96.67\% and 97.87\%, respectively. The results have showed that this method can be routinely used to detect carbapenemases in \textit{Enterobacteriaceae} members and \textit{Pseudomonas} species\textsuperscript{[28]}.

In another study on the rapid detection of carbapenemase-producing \textit{Enterobacteriaceae} members using MALDI-TOF MS, a total of 105 strains of various carbapenemase-producing and non-producing strains have been identified and tested using this method. Imipenem hydrolysis analyses followed by mass spectrometry to determine the breakdown of the antibiotic were used. As a result, when the data were checked by PCR, the tests showed 87\% sensitivity and 100\% specificity\textsuperscript{[29]}.

Similarly, direct carbapenemase activities from blood culture samples have been determined using MALDI-TOF MS. A total of 100 blood culture samples have been analyzed for carbapenemase by MALDI-TOF MS and compared with molecular methods. Of the 110 isolated bacterial isolates, when 29 were found to produce carbapenemase with molecular methods, 21 were found with MALDI-TOF MS (72.4\%)\textsuperscript{[30]}.

In another study, in performed MALDI-TOF MS analysis using ertapenem in \textit{P. aeruginosa} strains, it could be able to detect strains having KPC only after 15 minutes of incubation, but only 8 (57\%) of 14 isolates having VIM were identified correctly\textsuperscript{[31]}.

As seen in the above studies, studies on this subject are in progress.

In the present study, OXA-48 was shown in 93 out of 96 isolates, and NDM-1 was found in three of them. This study showed that resistance development in the carbapenemase-producing \textit{K. pneumoniae} strains was associated with OXA-48 in our region generally. Previously, in the Konya region, OXA-48 had been observed in four \textit{K. pneumoniae} strains and two of them had NDM-1 at the same time. No other study has been found in the literature related to these bacterial species in our region\textsuperscript{[32]}. Considering the difficulties in demonstrating carbapenem resistance in OXA-48 strains, it is important to demonstrate a new method in this study\textsuperscript{[17]}.

In this study, sensitivity was obtained as 96.7\%, and specificity and PPV were 100\% and NPV was 93\%. Sensitivity and other data obtained in our study are similar to other studies. Failure to make any comments for five isolates reduced the efficiency of the test. This method is more advantageous than other methods because of high sensitivity and specificity, and short analysis time. It seems that complete standardization can not be achieved due to the recent use of the method in this field and the lack of sufficient data in the literature yet.

MALDI-TOF MS, which has been used in identification and antibiotic susceptibility studies, is a relatively new application in this area, and it has been observed that the sensitivity of determination of carbapenem resistance is high and it is observed faster than phenotypic methods. On the other hand, new studies are needed to determine standardization, specificity, and sensitivity of antimicrobial susceptibility detection and determination of carbapenemase.

To conclude, it appears that MALDI-TOF MS determines carbapenemase production with high specificity and sensitivity. We think it will be beneficial to complete standardization studies and use them in routine practice.

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ETHICS COMMITTEE APPROVAL

Ethical review and approval were sought from Meram Faculty of Medicine Research and Ethics Committee (Approval No: 2017/1092).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORSHIP CONTRIBUTIONS

Concept/Design: MD
Analysis/Interpretation: AYB
Data Acquisition: AYB
Writing: MD
Final Approval: MD

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