Research Article / Araştırma Makalesi
doi: 10.18678/dtfd.1383748

Duzce Med J, 2024;26(1):15-20
Düzce Tıp Fak Derg, 2024;26(1):15-20

Detection of Carbapenem Resistance Using the Genotypic and Phenotypic Methods in *Klebsiella pneumoniae*

Klebsiella pneumoniae Suşlarında Karbapenem Direncinin Genotipik ve Fenotipik Yöntemler ile Saptanması

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Received / Geliş Tarihi : 31.10.2023
Accepted / Kabul Tarihi : 31.01.2024
Available Online / Çevrimiçi Yayın Tarihi : 06.03.2024

ABSTRACT

Aim: This study aimed to detect the carbapenem resistance of the *Klebsiella pneumoniae* strains, isolated from clinical specimens with genotypic and phenotypic methods.
Material and Methods: A total of 87 *Klebsiella pneumoniae* strains whose carbapenem resistance was determined by disc diffusion method were included in the study. Carbapenemase was investigated using the combined disk method and polymerase chain reaction (PCR).
Results: The evaluation of the PCR results demonstrated that OXA was detected in 60 (68.9%) samples, NDM was detected in 20 (22.9%), OXA + NDM in 5 (5.7%), and KPC was detected in 1 (1.1%) out of 87 clinical samples. Carbapenemase was not detected in one specimen with the PCR method. The results were found compatible with the combined disc test results for all isolates which were detected as only OXA, NDM, and KPC type carbapenemase positive. In 5 (5.7%) strains in which the co-existence of NDM and OXA type carbapenemases was detected by PCR, the combined disc method detected only OXA type carbapenemase.
Conclusion: The combined disk method is inadequate in the presence of strains that have multiple carbapenemases, and also have OXA which is the most frequently detected carbapenemase in our hospital. EUCAST recommends verification by other methods in the presence of OXA-48. Genotypic methods can be used for confirmation testing. The detections of strains with NDM, multiple carbapenemases, and the first detection of KPC were striking in the study. Monitoring the spread of these strains in the hospital will be necessary for infection control.
Keywords: *Klebsiella pneumoniae*; carbapenem; carbapenemase.

ÖZ

Amaç: Bu çalışmada, klinik örneklerinden izole edilen *Klebsiella pneumoniae* suşlarında karbapenem direncinin genotipik ve fenotipik yöntemler ile saptanması amaçlanmıştır.
Gereç ve Yöntemler: Disk difüzyon yöntemi ile karbapeneme dirençli bulunan toplam 87 *Klebsiella pneumoniae* suşu çalışmaya dahil edilmiştir. Karbapenemaz varlığı kombine disk yöntemi ve polimeraz zincir reaksiyonu (polymerase chain reaction, PCR) ile araştırılmıştır.
Bulgular: PCR sonuçları değerlendirildiğinde, 87 klinik örnekten 60 (%68,9) örnekte OXA tipi, 20 (%22,9) örnekte NDM tipi, 5 (%5,7) örnekte OXA + NDM tipi ve 1 (%1,1) örnekte ise KPC tipi karbapenemaz saptandığı görülmektedir. Bir örnekte ise PCR yöntemi ile karbapenemaz bulunamamıştır. Tek başına OXA, NDM ve KPC tipi karbapenemaz pozitifliği saptanan izolatların tamamı için sonuçların kombine disk testi ile uyumlu olduğu bulunmuştur. PCR yöntemi ile NDM ve OXA tipi karbapenemazın birlikte olduğu 5 (%5,7) örnekte ise kombine disk yönteminde sonuç OXA tipi karbapenemaz olarak bulunmuştur.
Sonuç: Kombine disk yöntemi, aynı anda birden fazla karbapenemaz bulunduran suşların bulunması ve hastanemizde en sık saptanan karbapenemaz tipinin OXA olması nedeni ile yetersiz kalmaktadır. EUCAST, OXA-48 varlığında diğer yöntemlerle doğrulanmasını önermektedir. Doğrulama testi olarak genotipik yöntemler kullanılabilir. NDM tipi karbapenemazın artmakta olduğu, birden fazla karbapenemazı taşıyan suşların görülmeye başlaması ve ilk defa KPC tipi karbapenemazın bulunması dikkat çekicidir. Bu suşların hastanede yayılımının takip edilmesi enfeksiyon kontrolü açısından önemli olacaktır.
Anahtar kelimeler: *Klebsiella pneumoniae*; karbapenem; karbapenemaz.

Presented orally at the 33rd ANKEM Rational Use of Antibiotics Congress (May 2-6, 2018; Muğla, Türkiye).

INTRODUCTION

Antibiotic resistance has recently become a serious public health problem worldwide, and gram-negative bacteria have an important role in this with their various antibiotic resistance mechanisms. Antibiotic resistance has become widespread, mainly owing to the extensive and inappropriate use of antibiotics (1,2).

Carbapenems are a group of beta-lactam antibiotics that have rapid bactericidal activity and the broadest spectrum. In recent years carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains have frequently been isolated in different regions of the world. Carbapenem-resistant *Klebsiella pneumoniae*-associated infections lead to the prolongation of hospital stay, together with higher mortality, and morbidity (3,4).

The minimum inhibitory concentration (MIC) values of carbapenems may vary depending on the type, and level of the carbapenemase enzyme and on the bacteria type. Therefore, the rapid and accurate detection of carbapenemase-producing bacteria has significant importance for the selection of the appropriate antimicrobial treatment and the application of infection control procedures (5).

Beta lactamases are divided into four molecular classes from A to D according to their amino acid structure (6). NMC- IMI, SME, GES and *Klebsiella pneumoniae* carbapenemase (KPC) are the four families of the Class A carbapenemases. KPC has emerged as a critical carbapenemase from gram-negative bacteria mostly from *K. pneumoniae* in the world (7). The differentiation of ESBL and AmpC from KPC is difficult when they are together with porin loss/change (8,9).

Besides, automated systems give inconsistent results in the detection of KPC-producing bacteria (3). Class B carbapenemases, also known as metallo-β-lactamases, include VIM, NDM, IMP, and NDM-1 (New Delhi metallo-β-lactamase) (10). NDM-1 was first identified in Sweden in 2008 from the *K. pneumoniae* strain isolated from a patient with a history of hospitalization in India then was reported in the USA, the United Kingdom, and many other countries especially related to traveling to India, and Pakistan (11,12). Class D consists of OXA-type carbapenemases. The OXA-48 was first reported from Turkey in 2003 (13). The enzyme is endemic in Turkey, Morocco, Libya, Egypt, and Tunisia (14).

Phenotypic and genotypic methods can be used in the detection of carbapenemases. Chromogenic media, modified Hodge test, inhibitor-based methods (double disk synergy, combined disc tests), biochemical methods, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), or immunochromatographic methods can be used as the phenotypic method. The polymerase chain reaction (PCR), sequencing, and oligonucleotide hybridization can be used as genotypic methods. Molecular methods are recommended for fast and accurate detection of carbapenemases. Phenotypic methods can be used in laboratories where molecular tests cannot be performed (5,15).

We aimed to investigate and perform the typing of the carbapenem resistance in *Klebsiella pneumoniae* strains isolated from the clinical specimens of the inpatients or the patients who applied to the outpatient clinic after being discharged using the phenotypic and genotypic methods in the present study. The obtained results will provide valuable information for appropriate antimicrobial therapy and infection control.

MATERIAL AND METHODS

Throat swab, rectal swab, tracheal aspiration, bronchoalveolar lavage, hemoculture, urine, sputum, drainage fluid, pleural fluid, and abscess samples of inpatients who were admitted to the outpatient clinic of Istanbul University Istanbul Medical Faculty Hospital between 2015 and 2017 were evaluated. A total of 87 isolated *Klebsiella pneumoniae* strains that had been found resistant to carbapenem by the disk diffusion method were included in the study. No clinical discrimination was performed, and examples from all services were included in the study. A single clinical sample of each patient was included in the study.

Isolates were identified using conventional methods. After morphologic examination, Gram staining was performed on suspicious colonies, and the identification of the Gram-negative rods was performed using the catalase, oxidase, motion examination, VP reaction, citrate, indole, urea, H2S formation, lysine, and ornithine decarboxylase tests. Antibiotic susceptibility was investigated using the disk diffusion method (Oxoid-United Kingdom) according to the recommendations of the Clinical and Laboratory Standards Institute (16).

The combined disc method (D70C, Mast Group, United Kingdom) was used as the phenotypic method in the detection of carbapenemase. Meropenem (10 μg), meropenem (10 μg) + MBL inhibitor, meropenem (10 μg) + KPC inhibitor, meropenem (10 μg) + AmpC inhibitor, and temocillin (30 μg) discs (TEM30C, Mast Group, United Kingdom) were used in the test.

Two different real-time multiplex PCR kits were used as the genotypic method: MDR KPC / OXA Real-TM (Sacace, Italy) kit KPC and OXA-48/162 (with no distinction), MDR MBL (VIM, IMP, NDM Real-TM (Sacace, Italy) kit identify the VIM, IMP, and NDM.

All procedures were approved by the ethical standards of the Ethics Committee of the Istanbul University Istanbul Faculty of Medicine (25.11.2016, 1355).

Statistical Analysis

The IBM SPSS v.21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp.) program was used for statistical analysis of the data. In descriptive statistics, number and percentage values were calculated. The chi-square test was used for comparison between groups. The results were evaluated at the 95% confidence interval. A p value of <0.05 was considered as statistical significance.

RESULTS

Thirty-seven carbapenem-resistant strains from 2015, 26 from 2016, and 24 from 2017 were evaluated. The samples were taken during the successive period. No significant difference was detected between the years in the evaluation of the enzyme results ($p=0.153$).

The evaluation of the PCR results demonstrated that OXA-48/162 was detected in 60 (68.9%) samples, NDM was detected in 20 (22.9%) samples, OXA-48/162 + NDM in 5 (5.7%) samples, and KPC was detected in 1 (1.1%)

sample out of 87 clinical samples. No enzyme was detected in one specimen in the PCR and combined disc method.

This strain was found resistant only to ertapenem. All isolates that were detected as single OXA, NDM, and KPC type carbapenemase with PCR were found compatible with the combined disc test results. In five (5.7%) samples where the coexistence of NDM and OXA type carbapenemase were determined with PCR, the result was found as OXA type carbapenemase in the combined disk method.

The investigation of the distribution of the OXA and NDM positive 80 samples showed that OXA positivity was found in 3 (42.9%) strains in the neonatal intensive care unit, 7 (100%) strains in anesthesiology and reanimation, and 7 (100%) strains in cardiovascular surgery clinics. The statistical investigation showed that the lower detection of OXA positivity in the neonatal intensive care unit, and higher in the anesthesiology and reanimation, and cardiovascular surgery units were significant (p=0.031). The distribution of carbapenemases by years is shown in Figure 1, and the distribution of the clinics is shown in Figure 2.

The antibiotic susceptibility test results of our study showed that 3 (5%) strains were susceptible to cefotaxime, 6 (10.7%) were susceptible to ceftazidime, 7 (10.6%) strains were susceptible to cefepime, and 1 (1.5%) strain was intermediately susceptible to cefepime. No carbapenemase was detected in the tests performed on the strain which was intermediately susceptible to cefepime. OXA-type carbapenemase was detected in all strains that were found susceptible to one or more of cefepime, cefotaxime, and ceftazidime.

Tobramycin resistance was detected as 100% in the strains with NDM and OXA+NDM and 74% in the strains with OXA. Gentamicin resistance was detected as 87.8% in the strains with NDM and OXA+NDM, and 66% in the strains with OXA. Amikacin resistance was detected as 75% in the strains with NDM and OXA+NDM, and 26.6% in the strains with OXA. Three (6.1%) strains were intermediately susceptible to amikacin.

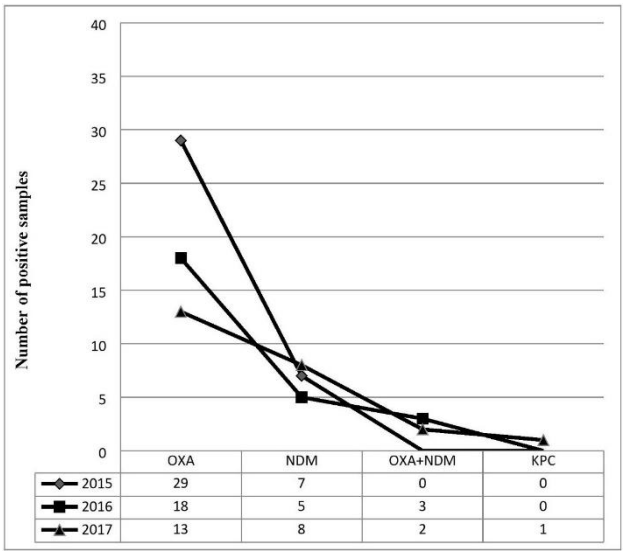

Figure 1. The distribution of carbapenemases by years

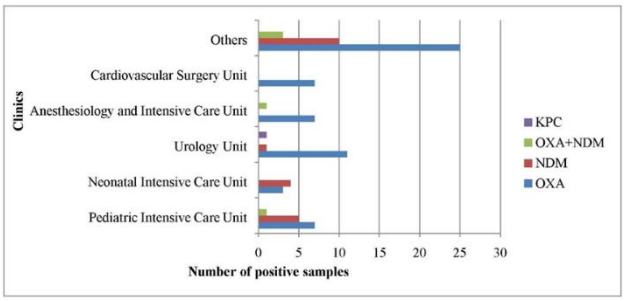

Figure 2. Distribution of carbapenemases by clinic

DISCUSSION

There has been a rapid spread of antibiotic resistance worldwide in recent years. Immediate and accurate detection of carbapenemase-producing bacteria is particularly crucial in the treatment of severe, life-threatening infections and infection control.

Molecular methods are recommended for rapid, and accurate detection of carbapenemases. Phenotypic methods can be used in laboratories where molecular tests cannot be performed (5,15). The sensitivity and specificity of the combined disc method were determined as 78-100%, and 93-100% depending on the carbapenemase type of the isolates (15,17).

PCR and combined disc tests were compatible in detecting the carbapenemase in isolates that had OXA, NDM, and KPC only. No enzyme was found in one sample through the use of PCR and combined disc method. This strain was found resistant to ertapenem only. The sensitivity of ertapenem was very high in carbapenemase screening; however, its specificity was lower.

In five (5.7%) samples where the coexistence of NDM and OXA-type carbapenemase was determined with PCR, the result was found as OXA-type carbapenemase in the combined disk method. The detection of the co-existence of carbapenemase enzyme types has been detected at increasing rates (18-20).

OXA-48 was found in the *K. pneumoniae* 11978 strain, isolated in the Hospital of Istanbul Faculty of Medicine in 2001. OXA-48 was suggested to originate and spread from Turkey to the world (13). OXA-48 is the most frequently detected carbapenemase enzyme in Turkey and is also endemic in Morocco, Libya, Egypt, and Tunisia.

We found 60 (68.9%) out of 87 carbapenem-resistant *K. pneumonia* strains as OXA-48 or OXA-162 positive in our study. The distribution of OXA within the years was 80% in 2015, 69% in 2016, and 54% in 2017. The results might be in OXA-48 predominance because OXA-48 was first detected in our hospital. The ratio of OXA-type carbapenemase is consistent with similar studies conducted in Turkey (18,21-23). The decrease in the last two years was found insignificant. However, in two different studies conducted in our hospital in the following years, OXA-type carbapenemase rates were found to be 16% and 25.8% (24,25). It is seen that OXA-type carbapenemase rates continued to decrease in the following years.

NDM-1 was first identified in the *K. pneumoniae* strain in Sweden in 2008 in a patient who was previously hospitalized in India (12). The first NDM-1-positive *K. pneumoniae* strain in Turkey was reported in 2011 (26). In two studies conducted in Istanbul in 2014-2016 and 2015-2016 years,

NDM-type carbapenemase was found to be 22.6% and 27%, respectively (27,28). In a study conducted on *Escherichia coli* and *Klebsiella pneumoniae* strains in Turkey in 2019, the rate of NDM-1 alone was found to be 15% (21).

In our study, 20 (22.9%) out of 87 carbapenem-resistant *K. pneumoniae* strains were found NDM positive. The NDM rate was 19.4% in 2015, 19.2% in 2016, and 33.3% in 2017. Changes in NDM rates by years were not found statistically significant. In two different studies conducted in our hospital in the following years, NDM rates were found to be 36% and 27.7% (24,25). Our NDM-type carbapenemase ratio in these two studies seems to be consistent with the data from 2017.

The co-existence of OXA-48 and NDM-1 was first reported in 2013 in Istanbul (29). In the same year, this association rate was found to be 1% in Kayseri (19). It was found to be 2.1% in a study conducted in 2014 with *E.coli* and *K. pneumoniae* strains, and 6.7% in another study conducted in 2016-2017 (18,23). In a prospective, multicentre observational cohort study conducted in Turkey in 2018-2019, the association of OXA-48-like and NDM was found to be 16% (30). In two studies conducted in our hospital in 2021 and 2021-2022, this rate was found to be 36% and 27.7% (24,25). The co-existence of OXA and NDM was found in five (5.7%) strains in our study. The evaluation of the distribution within years showed that no OXA + NDM co-existence was detected in 2015. The coexistence of OXA + NDM was detected in three (11.5%) out of 26 samples in 2016 and in two (8.3%) out of 26 samples in 2017. This result was consistent with the Turkish data. When evaluated together with the studies conducted in the following years, it is seen that the association of OXA + NDM has increased over the years.

The most common type of class A carbapenemases is KPC which was first detected in 1996 in a *Klebsiella pneumoniae* strain isolated in the United States (31). KPC is endemic in the United States, Greece, and Italy (32,33) KPC could not be identified in most studies conducted in Turkey (18,23,34-37). The first KPC enzyme was reported by Labarca et al. (26) in a KPC-2 expressing *Klebsiella pneumoniae* strain in Turkey in 2014.

In a study conducted in 2015-2016, KPC was found to be 1.1%, and in another study in 2019, it was found to be 16% (21,28). We detected KPC in 2017 in an isolate obtained from a clinical sample of a 62-year-old woman who underwent renal transplantation ten years ago. KPC was shown to become sensitive after gene losses following the repeat culture passages of the carbapenemase-carrying strains. Negative results may be detected if these strains are overlooked (38).

Since KPC has not been previously reported, its spread should be carefully monitored in our hospital. The most common responsible mechanism for the carbapenem resistance of Enterobacteriaceae species is the production of carbapenemase enzymes. The decrease in carbapenem susceptibility may also be detected in the association of GSBL or AmpC enzyme production and porin loss (39). We found no such isolation in our study. In two studies in 2018 and 2019, KPC and NDM associations were detected, but in our study, we did not detect KPC and NDM associations (21,40).

Class D carbapenems hydrolyze penicillins and carbapenems, however, their ability to hydrolyze the extended-spectrum cephalosporins such as cefotaxime, ceftazidime, ceftriaxone, and cefepime may be limited (13). Besides, these strains are rarely susceptible to broad-spectrum cephalosporins owing to the mostly co-existence of OXA-48, and ESBL (41,42).

The evaluation of the antibiotic susceptibility test results showed that 3 (5%) strains were susceptible to cefotaxime, 6 (10.7%) strains were susceptible to ceftazidime, 7 (10.6%) strains were susceptible to cefepime, and 1 (1.5%) strain was intermediately susceptible to cefepime. The strain with intermediate cefepime susceptibility was found susceptible to imipenem and meropenem, and resistant to ertapenem, and no carbapenemase was detected in the conducted tests. OXA-type carbapenemase was detected in all strains that were found susceptible to one or more of cefepime, cefotaxime, and ceftazidime.

The clinical efficacy of the aminoglycosides in carbapenemase-producing strains was reported insufficient, even if they found susceptible in vitro (33). Most strains producing NDM-type carbapenemase are resistant to aminoglycosides because they also include the aminoglycosides inactivating 16S rRNA methylases (43). In our study, the aminoglycoside resistance was found higher in the strains with NDM compared to the strains with only OXA.

CONCLUSION

Although the combined disc method is practical and cost-effective, it is insufficient in strains containing more than one carbapenemase and in the presence of OXA-48 because temocillin resistance is not specific to OXA-48 type carbapenemases. In this case, EUCAST recommends verification by other methods (44). Genotypic methods can be used for confirmation testing. The detection of strains with NDM, multiple carbapenemases, and the first detection of KPC were striking. Monitoring the spread of these strains in the hospital will be necessary for infection control.

Ethics Committee Approval: The study was approved by the Clinical Research Ethics Committee of İstanbul University (25.11.2016, 1355).

Conflict of Interest: None declared by the authors.

Financial Disclosure: None declared by the authors.

Acknowledgments: This study was supported by the Research Fund of İstanbul University. (Project No. 24626).

Author Contributions: Idea/Concept: MAD, MDA; Design: MAD, MDA; Data Collection/Processing: MAD, MDA; Analysis/Interpretation: MAD, MDA; Literature Review: MAD, MDA; Drafting/Writing: MAD, MDA; Critical Review: MAD, MDA.

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