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MICROBIOLOGY

Investigation of genotyping and phenotyping characteristics of carbapenem-resistant *Klebsiella pneumoniae* isolates

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Abstract: *Klebsiella pneumoniae* (*K. pneumoniae*) is a major cause of healthcare-associated infections and plays a prominent role in the widespread antibiotic resistance crisis. Accurate identification of carbapenemases is essential to facilitate effective antibiotic treatment and reduce transmission of *K. pneumoniae*. This study aimed to detect carbapenemase production in carbapenem-resistant *K. pneumoniae* strains using phenotypic and genotypic methods. A total of 67 carbapenem-resistant *K. pneumoniae* strains obtained from various clinical samples were utilized for identification and antimicrobial susceptibility by the Vitek 2 Compact system (Biomerieux, France). Carbapenemase production was determined by using the Polymerase chain reaction, Blue-carba test (BCT) and Carbapenem inactivation method (CIM). Out of the isolates, 59 (88.1%) were positive $bla_{\text{OXA-48}}$, 16 (23.9%) $bla_{\text{IMP,}}$ and five (7.5%) were positive bla_{NDM}. No bla_{KPC} genes were detected. The CIM identified 62 (92.5%), BCT identified 63 (94%) of PCR-positive isolates. The sensitivity and specificity of the BCT and the CIM were determined to be 96.7%, 40%, and 96.7%, 25% respectively. The $bla_{\text{OXA-48}}$ gene was found to be the most prevalent in *K. pneumoniae* isolates. Early identification of carbapenem resistance plays a vital role in designing effective infection control strategies and mitigating the emergence and transmission of carbapenem resistance, thus reducing healthcare-associated infections.

Key words: *Klebsiella pneumoniae,* polymerase chain reaction, $bla_{\text{OXA-48}}$, bla_{IMP}, bla_{NDM}, bla_{KPC}.

INTRODUCTION

Klebsiella pneumoniae (K. pneumoniae) is a Gram-negative bacterial pathogen that can cause invasive infections, particularly in immunocompromised individuals (Meatherall et al. 2009). This bacterium causes bloodstream, urinary tract, and respiratory tract infections with high mortality in hospitalized patients. With its ability to spread easily among hospitalize patients, it poses a significant risk for hospital-acquired outbreaks. Globally, *K. pneumoniae* is a major cause of healthcare-associated infections and plays a prominent role in the widespread antibiotic resistance crisis (Podschun & Ullmann 1998).

Carbapenem-resistant *K. pneumoniae* presents a grave public health concern and has been designated as a critically prioritized issue by the World Health Organization for the development of novel control strategies. The European Union/European Economic Area observes persistently high percentages of antimicrobial resistance in various bacterial species, including *K. pneumoniae*, with a notable increase in carbapenem resistance (WHO 2022).

The escalating issue of the dissemination of carbapenem-resistant *K. pneumoniae* has garnered global recognition. To facilitate effective antibiotic treatment and curtail the transmission of these bacteria, prompt detection of carbapenem-resistant *K. pneumoniae* and accurate identification of carbapenemases are imperative. Additional time and effort required in the absence of an accurate diagnosis, including strain identification, resistance profiling, and patient condition assessment, can potentially hinder the process of making informed therapeutic decisions (Reyes et al. 2019). Recent advancements in laboratory testing have introduced novel approaches for the detection and analysis of carbapenemase production, leading to changes in the strategies employed by hospitals for disease control. New phenotypic tests such as the Blue Carba test (BCT) and Carbapenem inactivation Method (CIM) have been reported to provide information about the detection and analysis especially of carbapenemases production, changing how hospitals prevent the spread of pathogens (Reyes et al. 2019, Miller & Humphries 2016).

The objective of this study was to evaluate the presence of bla_{NDM}, bla_{OXA-48}, bla_{IMP}, and bla_{KPC} genes, as well as to assess the efficacy of the Blue-carba test and CIM in carbapenem-resistant *K. pneumoniae* isolates.

MATERIALS AND METHODS

This study was a cross-sectional study approved by the Ethics Committee of Tokat Gaziosmanpaşa University (Number: 22/KAEK/180, on October 20, 2023). A total of 67 strains of carbapenem-resistant *K. pneumoniae* were isolated from diverse clinical samples obtained at the Microbiology Laboratory of Tokat Gaziosmanpaşa University Research and Application Hospital were included in this study. The identification of the strains was accomplished using conventional methods and the Vitek 2 Compact system (Biomerieux, France). Antibiotic susceptibility testing was conducted employing the Vitek 2 Compact system (bioMerieux, France) and evaluated according to the EUCAST criteria (EUCAST 2022). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 strains were utilized as quality control measures. Strains exhibiting resistance to imipenem or meropenem were classified as carbapenem-resistant and were subsequently incorporated into the study. The strains were preserved at a temperature of -20°C until further analysis was performed. The investigation included samples sent from intensive care units as well as clinics. This analysis removed repetitive isolates from identical patient tissues.

Blue carba method

Mueller-Hinton agar was employed for the cultivation of the isolates. A 5 µL loop containing a pure bacterial culture was suspended in a test solution consisting of a 0.04% aqueous solution of bromothymol blue (Sigma) and 0.1 mmol/liter of ZnSO4 (Sigma), supplemented with 6 mg of Tienam (MSD) (equivalent to 3 mg of imipenem), and adjusted to a final pH of 7. The mixture was then incubated at a temperature of 37°C for a duration of two hours. The presence of carbapenemase activity was considered positive if the test solution and negative-control solution exhibited color changes, such as yellow or blue, yellow versus green, or green versus blue. Carbapenem-resistant bacteria appeared as blue or green in both solutions. The test was repeated for each isolate (Pires et al. 2013). (Figure 1)

Carbapenem inactivation method

For each isolate, 10 µL loopfuls culture and 10 µg meropenem (Oxoid Ltd, Hampshire, United

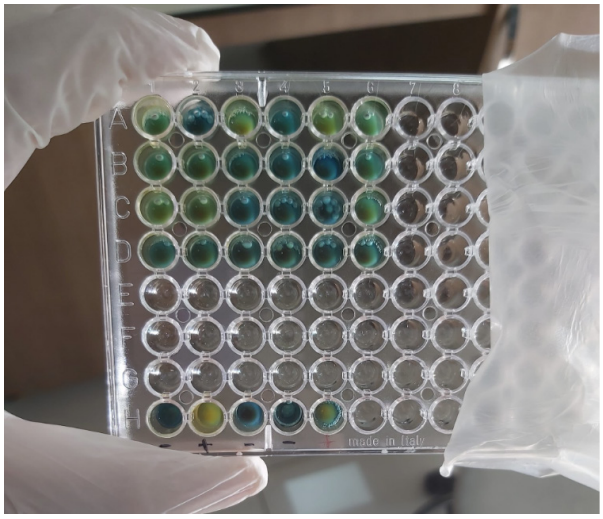

Figure 1. Carbapenem-resistant bacteria appeared as blue or green and carbapenem-sensitive bacteria appeared as yellow with the Blue-Carba test.

Kingdom) disk were suspended in 400 µL of distilled water and incubated at 35°C for four hours. Simultaneously, a 0.5% McFarland suspension of *Escherichia coli* ATCC was spread onto Mueller-Hinton agar and allowed to dry for 3-10 minutes at room temperature. After the incubation, the meropenem disc was withdrawn from the solution and streaked onto Mueller-Hinton agar along with *Escherichia coli* ATCC. The plates were then incubated at 37°C for 18–24 hours. Results were considered positive if inhibitory zone diameters ranged from 0 to 16 mm or if there was satellite expansion of colonies measuring 16-18 mm. Negative results were indicated by an inhibitory zone diameter of 19 mm (van der Zwaluw et al. 2015). (Figure 2)

Polymerase chain reaction

To detect specific genes (bla_{NDM}, bla_{IMP}, bla_{KPC}, and bla_{OXA-48}), DNA extraction was performed using the boiling method (Dashti et al. 2009). The polymerase chain reaction (PCR) was carried out using specific primers to determine the presence of these genes (Table I) (Sievert et al. 2013, Erdem et al. 2017, Mostachio et al. 2009). Positive control strains for bla_{NDM}, bla_{IMP}, bla_{KPC}, and bla_{OXA-48} obtained from the national quality control program, were included in the analysis. *E. coli* ATCC 25922 was used as a negative control. A 1.5% agarose gel (Sigma, USA) was prepared for gel electrophoresis to detect PCR products. The bands of the gen were shown with UV light (Figure 3).

Statistical analysis

The data was statistically analyzed using SPSS Statistical Program Version 21.0. (SPSS Inc., Chicago, Illinois, USA). Mean and standard deviation were used to describe quantitative variables with a normal distribution. Mean and range were used to characterize non-normally distributed data. The qualitative characteristics were described using numbers and percentages.

RESULTS

Study population

A total of 67 carbapenem-resistant *K. pneumoniae* isolates were examined in this study. Among the isolates, 51 (76.1%) were found to be resistant to

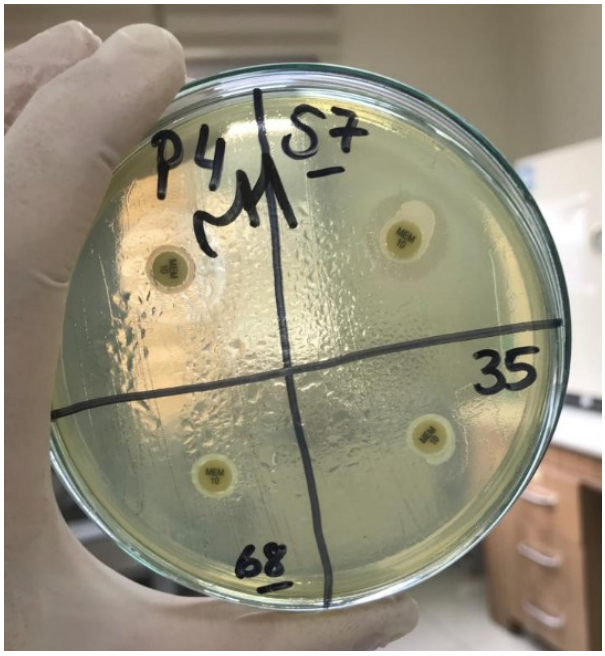

Figure 2. Carbapenem-resistant bacteria appeared in P4 with Carbapenem inactivation method results.

imipenem, and 66 (98.5%) exhibited resistance to this antibiotic. The isolates were identified from samples sent from intensive care units (35/52.2%), internal polyclinics (21/31.3%), and surgical departments (11/16.4%). These isolates were obtained from various clinical specimens, including urine (23/34.3%), blood (20/29.9%), tracheal aspirate (13/19.4%), wound (9/13.4%), cerebrospinal fluid (1/1.5%), and sterile body fluid (1/1.5%) specimens.

Data of PCR, CIM and BCT positivity

Additionally, among the isolates, 59 (88.1%) tested positive for bla_{OXA-48}, 5 (7.5%) were identified as bla_{NDM} positive, and 16 (23.9%) showed positivity for bla_{IMP}. No bla_{KPC} genes were detected. In this study, 59 (88.1%) of the tested isolates were positive by all three methods, while one (1.5%) isolate was negative. For bla_{OXA-48}, 56 (98.2%) isolates were positive by the BCT and CIM methods, five (100%) for bla_{NDM}, and 14 (93.3%) for bla_{IMP}. Out of the PCR-positive isolates, 62 (92.5%) were identified as positive by CIM, and 63 (94%) were determined as positive by the BCT. The positivity rates of BCT and CIM positivity in PCR-positive and PCR-negative isolates are presented in Table II.

However, three PCR-positive isolates (two bla_{OXA-48}, one bla_{IMP}) were negative with BCT, and five PCR-positive isolates (three bla_{OXA-48}, two IMP) were negative with CIM. Three PCR-negative isolates were found to be positive by both the BCT and CIM tests. The sensitivity and specificity of the BCT were determined as 96.7% and 40%, respectively, while the sensitivity and specificity of CIM were found to be 96.7% and 25%, respectively.

DISCUSSION

The global concern regarding the increasing prevalence of carbapenemase-producing bacteria has garnered significant attention. To facilitate appropriate antibiotic treatment and mitigate the dissemination of these bacteria, it is crucial to rapidly diagnose carbapenemase-producing bacteria and accurately identify the specific carbapenemases involved (Khalifa et al. 2020). According to the World Health Organization, in 2020, approximately 30% of nations reported antimicrobial resistance rates of 25% or higher, with 15% of 41 countries or areas recording rates of 50% or higher. Among Gram-negative pathogens, *K. pneumoniae* demonstrated a higher prevalence of carbapenem resistance

Table I. The specific primers utilized in the Polymerase Chain Reaction.

Gene	Primer	Sequence	Amplicon size (bp)	Reference
bla_{OXA-48}	$bla_{OXA-48-F}$	TTG GTG GCA TCG ATT ATC GG	438	(Sievert DM et al. 2013)
	$bla_{OXA-48-R}$	GAG CAC TTC TTT TGT GAT GGC		
bla_{NDM}	bla_{NDM-F}	GCA GCT TGT CGG CCA TGC GGG C	782	(Erdem F et al. 2017)
	bla_{NDM-R}	GGT CGC GAA GCT GAG CAC CGC AT		
bla_{KPC}	bla_{KPC-F}	TGT CAC TGT ATC GCC GTC	900	(Erdem F et al. 2017)
	bla_{KPC-R}	CTC AGT GCT CTA CAG AAA ACC		
bla_{IMP}	bla_{IMP-F}	GGA ATA GAG TGG CTT AAT TC	188	(Mostachio AK et al. 2009)
	bla_{IMP-R}	CCA AAC CAC TAC GTT ATC		

compared to *Enterobacterales* isolates (WHO 2022).

Carbapenemases are classified into different classes according to the Ambler classification system. Class A carbapenemases include *K. pneumoniae* carbapenemase (bla_{KPC}), while class B or metallo-beta-lactamases encompass New Delhi metallo-beta-lactamases (bla_{NDM}) and Imipenemase (bla_{IMP}). Class D carbapenemases are represented by bla_{OXA-48}-like carbapenemases (Ambler 1980). Significant proportions of bla_{KPC} -positive *K. pneumoniae* among carbapenem-resistance isolates were identified in Italy, with 187 (96%) out of 195 isolates being positive, Israel with 31 (80%) out of 39, Greece with 56 (65%) out of 86 isolates and Portugal with 36 (59%) out of 61 isolates (Grundmann et al. 2017). In China, among the 121 carbapenem-resistant *K.pneumoniae* strains the bla_{KPC} gene was detected in 78.26%, the $bla_{NDM\ -1}$ gene was found in 47.83%, and the $bla_{NDM\ -5}$ gene was found in 17.39% of the strains in 2022. However, bla_{OXA-48}, bla_{VIM}, and bla_{IMP} genes were not detected (Chen et al. 2022). David et al. (2019) analyzed genome sequence data of the 684 carbapenem-resistant *K. pneumoniae* isolates obtained from 32 countries during the European Survey of Carbapenemase-Producing Enterobacterales isolataes. Among these *K. pneumoniae* isolates, 311 contained $bla_{KPC\text{-}like}$ genes, 248 contained $bla_{OXA\text{-}48\text{-}like}$ genes, 79 contained $bla_{NDM\text{-}like}$ genes, 56 contained $bla_{VIM\text{-}like}$ genes, and 3 contained $bla_{IMP\text{-}like}$ genes. Although there are regional differences, $bla_{OXA\text{-}48\text{-}like}$ genes and $bla_{KPC\text{-}like}$ genes are most frequently present in carbapenem resistant *K. pneumoniae* isolates.

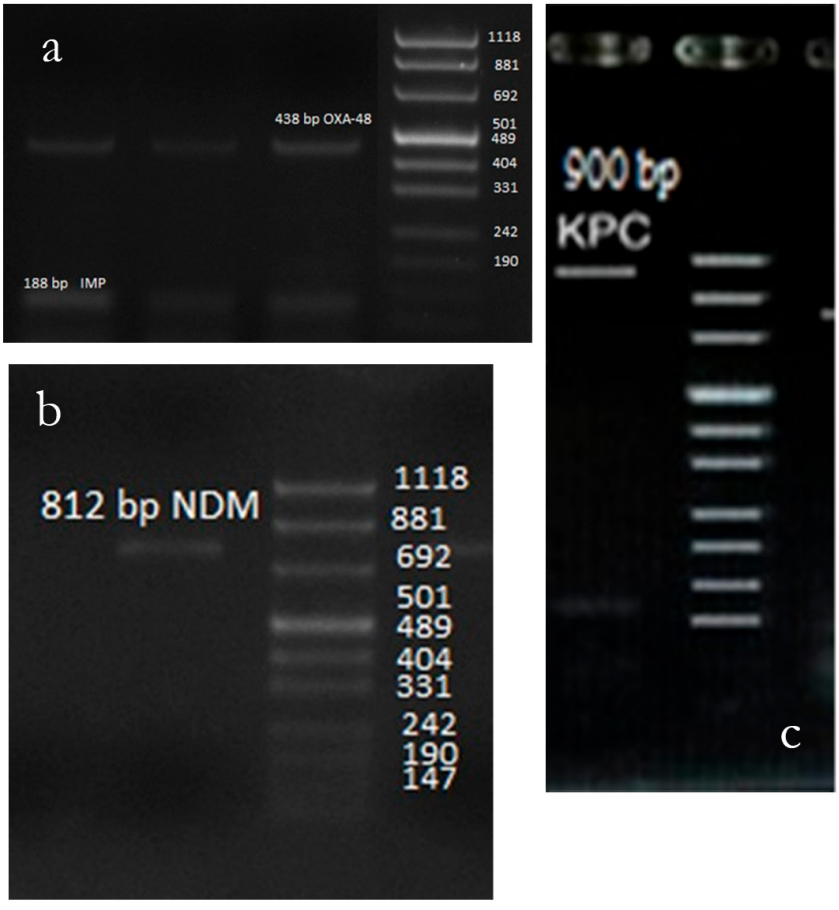

Figure 3. Bands of the genes under UV light.

Meier & Hamprecht (2019) evaluated 81 carbapenem-negative and 104 carbapenem-positive isolates, among them, 25 were bla_{OXA-48} positive, 20 were bla_{NDM} -positive, 18 were bla_{KPC} -positive, 25 were bla_{VIM}-positive, 5 were bla_{GIM}-positive, nine were $bla_{OXA-48\text{-like}}$ positive, and two were positive for both $bla_{OXA-48\text{-like}}$ and $bla_{NDM.}$ The sensitivity and specificity rates for Carba NP, Carba NeoRapid Carba, and CIM were determined as 99% and 95%, 99% and 91%, and 100% and 95%, respectively. Abulaila et al. (2021) indicated that among 91 *K. pneumoniae* isolates, 49 were positive for bla_{OXA-48}, 34 were positive for both $bla_{OXA-48,}$ and bla_{NDM-1}, seven were positive for bla_{NDM-1}, and one was positive for bla_{KPC}. The sensitivity rates for CIM, and BCT were found to be 100%, and 90.8%, respectively. Tanriverdi Cayci et al. (2021) reported the positivity rates for $bla_{OXA-48}$and bla_{NDM} were reported as 71.4% and 1.3%, respectively153 in *Enterobacterales* isolates. No isolates were positive for bla_{KPC} or bla_{VIM} by PCR. The sensitivity and specificity rates for the BCT and CIM were determined 89.4%, 52.5%, and 98.2%, 77.5%, respectively.

Table II. The positivity rates of Blue-carba test and Carbapenem Inactivation Method in PCR-positive and PCR-negative isolates.

PCR			BCT	
			Positive	**Negative**
bla_{OXA-48} **Positive**	**CIM**	Positive	56	0
		Negative	1	2
	Total		57	2
bla_{OXA-48} **Negative**	**CIM**	Positive	6	0
		Negative	0	2
	Total		6	2
bla_{NDM} **Positive**	**CIM**	Positive	5	-
		Negative	0	-
	Total		5	2
bla_{NDM} **Negative**	**CIM**	Positive	57	0
		Negative	1	4
	Total		58	4
bla_{IMP} **Positive**	**CIM**	Positive	14	0
		Negative	1	1
	Total		15	1
bla_{IMP} **Negative**	**CIM**	Positive	48	0
		Negative	0	3
	Total		48	3
bla_{KPC} **Negative**	**CIM**	Positive	62	0
		Negative	1	4
	Total		63	4

CIM: Carbapenem inactivation method.
BCT: Blue-Carba test.
PCR: Polymerase chain reaction.

According to Kamel et al. (2022) the sensitivity and specificity of CIM and BCT were reported as 51.7%, 100%, and 82.7% and 100% respectively. The BCT demonstrated high sensitivity and rapid detection of carbapenemase-producing isolates. In Brazil, the sensitivity and specificity rates for BCT, and CIM were found to be 95%, 100%, and 74.4%, 97.5%, respectively (Pancotto et al. 2018).

In this study, 59 (88.1%) isolates were positive for bla_{OXA-48}, 16 (23.9%) were positive for bla_{IMP}, and five (7.5%) were positive for bla_{NDM}. While bla_{KPC} is the most common gene in carbapenem-resistant *K. pneumoniae* isolates in worldwide, no bla_{KPC} gene was detected in this study. In Turkey, bla_{KPC} was not detected in a study conducted in a localization close to our region (Tanriverdi Cayci et al. 2021). Previous studies in which bla_{KPC} was not detected emphasized this may be due to combination of ESBL or AmpC-type enzyme with porin loss (Bina et al. 2015, Crowley et al. 2002). However, the study conducted in 2020 indicates that the lack of restriction-modification (R-M) systems could increase bla_{KPC} production. According to this study the type I R-M systems could attack most invading DNA elements, such as bla_{KPC} genes, and impact the acquisition of bla_{KPC} genes in *K. pneumoniae* (Zhou et al. 2020). The absence of bla_{KPC} may be related to the type I R-M systems.

Out of the PCR-positive isolates, 62 (92.5%) were determined to be positive by CIM, and 63 (94%) of them were positive by the BCT. A total of 59 (88%) of the isolates were determined to be positive, while one (1.5%) isolate tested negative using all three methods. However, three PCR-positive isolates (two bla_{OXA-48}, one bla_{IMP}) were negative with the BCT, and five PCR-positive isolates (three bla_{OXA-48}, two bla_{IMP}) were negative with CIM. On the other hand, the three PCR-negative isolates were determined to be positive with both the BCT and CIM methods. AMPC production or porin loss may be involved in these three isolates. The sensitivity and specificity rates for the BCT and CIM were 96.7%, 40%, and 96.7%, 25%, respectively. Consistent with the previous studies, both the Blue-carba and CIM demonstrated high sensitivity in the current study. The lower specificity rates in the current study can be attributed to the absence of carbapenem-susceptible isolates and the relatively low number of strains.

CONCLUSION

To control the spread of carbapenem-resistant *K. pneumoniae*, it is crucial to have a comprehensive understanding of its development and spread across different settings. The results of this study highlight the predominance of the bla_{OXA-48} gene, which was identified as the most prevalent gene in carbapenem-resistant *K. pneumoniae* isolates. It is also important that bla_{KPC} genes may be associated with type I R-M. Future studies on this subject will be more revealing. In any case, early identification of carbapenem resistance plays a vital role in designing effective infection control strategies and mitigating the emergence and transmission of carbapenem resistance, thus reducing healthcare-associated infections. The use of the BCT and CIM tests can aid in the detection and characterization of carbapenem-resistant *K. pneumoniae*, providing valuable tools for the surveillance and management of these strains.

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UMUT SAFIYE ŞAY COŞKUN: conceptualization, data curation, writing – original draft, formal analysis, methodology, project administration, writing – review & editing. YELDA DAGCIOĞLU: methodology, validation, software, visualization, writing – review & editing.