Molecular Epidemiology of Carbapenem-resistant *Klebsiella pneumoniae* Isolates

Karbapeneme Dirençli *Klebsiella pneumoniae* Suşlarının Moleküler Epidemiyolojisi

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Abstract

Aim: Carbapenem-resistant Klebsiella pneumoniae infection has become an important clinical problem with reduced therapeutic options. This study aimed to investigate the carbapenem resistance rates and responsible resistance genes in *K. pneumoniae* isolates derived from clinical samples collected in Istanbul.

Materials and Methods: This prospective study included a total of 1452 *K. pneumoniae* isolates from patients admitted to our hospital between July 2013 and July 2014. VITEK-2 (bioMérieux, Marcy-l'Ètoile, France) was used for microbial identification and antimicrobial susceptibility testing. The carbapenem-resistant isolates identified by VITEK-2 were also found to be resistant to ertapenem by the ertapenem gradient test. Resistance mechanisms of the carbapenem-resistant isolates were investigated using real time-polymerase chain reaction.

Results: Of the 1452 *K. pneumoniae* isolates, 45 (3.1%) were carbapenem-resistant. Of these, 32 (71.1%) were bla_{OXA-48}-positive, 9 (20%) bla_{NDM}-positive, and 1 (2.2%) bla_{VIM-1}-positive. None had the genes bla_{KPC} and bla_{IMP-1}. The greatest susceptibility by the isolated carbapenemase-producing *K. pneumoniae* was shown to the antimicrobials amikacin and gentamicin.

Discussion and Conclusion: In our hospital, there are several mechanisms causing carbapenem resistance, and the bla_{OXA-48} positivity rate of 71.1% is significant. This resistance may spread rapidly and, through enzymatic resistance gene transfer, lead to hospital epidemics difficult to manage. For this reason, accurate and rapid laboratory diagnosis is important in infection control. For faster results, molecular methods, as well as phenotypic methods, must be included in the hospital infrastructure.

Keywords: carbapenem resistance; *Klebsiella pneumoniae*; resistance gene

Öz

Amaç: Karbapeneme dirençli *Klebsiella pneumoniae* enfeksiyonları, azalan tedavi imkanlarıyla birlikte önemli bir klinik sorun haline gelmiştir. Bu çalışmada, İstanbul'da elde edilen *K. pneumoniae* izolatlarındaki karbapenem direnç oranlarını ve buna sebep olan direnç genlerini incelemek amaçlanmıştır.

Gereç ve Yöntemler: Bu prospektif çalışmaya, Temmuz 2013—Temmuz 2014 döneminde hastanemize kabul edilen hastalardan elde edilen toplam 1452 *K. pneumoniae* izolatı dahil edilmiştir. Mikroorganizmaların tanımlanması ve antimikrobiyal duyarlılık testi için VITEK-2 (bioMérieux, Marcy-l'Ètoile, Fransa) kullanılmıştır. VITEK-2 otomatize sistem ile karbapenem direnci saptanan suşların ertapenem gradient testi ile de ertapeneme dirençli olduğu bulunmuştur. Karbapenem direncine sebep olan genler *real time-polymerase chain reaction* ile araştırılmıştır.

Bulgular: 1452 *K. pneumoniae* izolatının 45'i (%3,1) karbapeneme dirençliydi. Bunların 32'si (%71,1) bla_{OXA-48}-pozitif, 9'u (%20) bla_{NDM}-pozitif, 1'i (%2,2) bla_{VIM-1}-pozitifti. Hiçbirinde bla_{KPC} ve bla_{IMP-1} geni mevcut değildi. Karbapenemaz üreten *K. pneumonae* izolatlarının en duyarlı olduğu antimikrobiyaller amikasin ve gentamisin idi.

Tartışma ve Sonuç: Hastanemizde karbapenem direncine sebep olan çeşitli mekanizmalar bulunmaktadır ve bla_{OXA-48} geninin %71,1 oranında görülmesi dikkat çekicidir. Bu direncin hızla yayılması ve enzimatik direnç genlerinin aktarımı yoluyla yönetimi zor hastane salgınlarına yol açması mümkündür. Bu nedenle enfeksiyon kontrolünde doğru ve hızlı laboratuvar tanı önemlidir. Daha hızlı sonuçlar elde etmek için moleküler yöntemler de fenotipik yöntemler gibi hastane altyapısına dahil edilmelidir.

Anahtar Sözcükler: direnç geni; karbapenem direnci; *Klebsiella pneumoniae*

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INTRODUCTION

Healthcare-associated infections due to resistant *Enterobacteriaceae* have recently become a problem worldwide (1). As carbapenems are a major drug class used to treat serious community-onset or healthcare-associated infections caused by *Enterobacteriaceae*, resistance to these agents will continue to cause challenging clinical choices. Mechanisms of carbapenem resistance include carbapenemase production, a combination of AmpC hyperproduction and/or ESBL production, and a porin mutation (e.g., ESBLs of the SHV- and CTX-M types combined with a deficiency in OmpK35 or OmpK36 in *Klebsiella pneumoniae*, AmpC overexpression, and OmpC or OmpF in *Enterobacter cloacae*) (2–4). Carbapenemases have been classified as class A beta-lactamases (bla_{NMC}, bla_{IMI}, bla_{SME}, bla_{KPC}, and bla_{GES}), class B beta-lactamases (bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM}, and bla_{NDM}), and class D beta-lactamase (bla_{OXA-48}). Phenotypic detection of carbapenemase-producing *Enterobacteriaceae* may be difficult because carbapenem minimum inhibitory concentrations (MICs) may be low. Therefore, genotypic detection of carbapenemase genes is the gold standard. Here, we describe the detection of bla_{NDM}, bla_{KPC}, bla_{VIM-1}, bla_{IMP-1}, and bla_{OXA-48} genes, the most prevalent carbapenemases, using real-time polymerase chain reaction (RT-PCR) (2).

In this prospective study at our hospital, we evaluated the presence of carbapenem resistance in a number of *K. pneumoniae* isolates and the enzymatic mechanisms leading to carbapenem resistance in this species.

MATERIALS AND METHODS

Isolation of the *K. pneumoniae* strains and antimicrobial susceptibility testing

Patients hospitalized at our hospital, which has a 725-bed capacity, between July 2013 and July 2014 were prospectively followed up for healthcare-associated infections, and the results were evaluated. Surveillance work was conducted by the Infection Control Committee with daily service visits. Healthcare-associated infections were defined according to the diagnostic criteria issued by the Centers for Disease Control and Prevention (3).

All gram-negative isolates at the time of isolation were identified using the VITEK-2 automated system (bioMérieux) with an ID-GN card. Antimicrobial susceptibility testing was performed according to the Kirby–Bauer disk diffusion method and the microbroth dilution assay using VITEK-2 AST-GN cards (bioMérieux) according to the Clinical and Laboratory Standards Institute (CLSI) criteria (4).

The following antimicrobials were tested (Antimicrobial Susceptibility Disks, bioMérieux): meropenem, imipenem, ertapenem, piperacillin/tazobactam, ceftazidim, ceftriaxone, gentamicin, amikacin, ciprofloxacin, and levofloxacin. The isolates were classified as susceptible, intermediate, or resistant, according to the breakpoints established by the CLSI (4). Isolates were considered carbapenem-resistant if they were resistant or intermediate to one or more of the carbapenems tested (e.g., MEM, IMP, or ERT). Quality control was performed by testing *K. pneumoniae* ATCC 700603.

The carbapenem-resistant isolates confirmed by VITEK-2 were also found to be resistant to ertapenem by the ertapenem gradient test according to the CLSI criteria (4). Isolates were suspended in 1 mL 16% glycerol broth and stored at -20°C. One isolate from each patient was analyzed.

Screening of the carbapenemase genes

RT-PCR (Rotor-Gene 6000, Corbett Life Science, Mortlake, Australia) was used to investigate the genes causing carbapenemase production in the collected isolates. Analyses were performed by using the Rotor Gene software according to the company's instructions.

Statistical analysis

Statistical analysis was performed by using the hypothesis test with NCSS (Number Cruncher Statistical System) 2007 & PASS (Power Analysis and Sample Size) and 2008 Statistical Software (NCSS LLC, Kaysville, Utah, USA). Additionally, Fisher's exact test and the Fisher–Freeman–Halton test were used for qualitative data comparison with definitive statistical methods (e.g., average, standard deviation, median, frequency, and ratio). A 95% confidence interval was used and $p<0.05$ was considered statistically significant.

RESULTS

A total of 1452 *K. pneumoniae* strains were isolated from the clinical samples. Of these, 45 (3.1%) showed resistance or reduced susceptibility to at least one car-

bapenem. In total, 45 patients (55.6% female, 44.4% male) with a carbapenem-resistant *K. pneumoniae* (CRKp) infection were identified. The median age of these patients was 64 (range 20–87) years.

When the antimicrobial susceptibilities of the 45 CRKp isolates were evaluated, all were nonsusceptible (NS) (i.e., either intermediate or resistant) to ertapenem; 97.8% were NS to piperacillin/tazobactam; 93.3% were NS to ceftazidime, ceftriaxone, and meropenem; 91.1% were NS to imipenem; 86.7% were NS to ciprofloxacin; 77.8% were NS to levofloxacin; 62.2% were NS to gentamicin; 28.9% were NS to amikacin. Of all isolates, 8.9% were NS to colistin by VITEK-2 according to the 2013 CLSI standards (7).

The bla_{OXA-48} gene was detected in 32 (71.1%) of the isolates by RT-PCR. Using the same method, 9 (20%) were found bla_{NDM}-positive and 1 (2.2%) bla_{VIM-1}-positive. All isolates were bla_{KPC}- and bla_{IMP-1}-negative (Table 1).

The median age of the 32 bla_{OXA-48}-positive patients (65.6% female, 34.4% male) was 62 years. No significant difference was observed in terms of age or sex.

The resistance rate of the bla_{OXA-48}-positive CRKp isolates was 100% for piperacillin/tazobactam and ertapenem, 96.9% for meropenem and imipenem, 93.8% for ceftazidime and ceftriaxone, 87.5% for ciprofloxacin and levofloxacin, 53.1% for gentamicin, 31.3% for amikacin, and 9.4% for colistin.

There was no statistically significant difference between bla_{OXA-48}-, bla_{KPC}-, bla_{NDM}-, bla_{VIM-1}-, and bla_{IMP-1}-positive and -negative isolates with respect to the antibiogram results for these antimicrobials ($p>0.05$).

DISCUSSION AND CONCLUSION

In recent years, there has been an increased frequency of gram-negative healthcare-associated bacterial infection (1). Isolation rates of *K. pneumoniae*, an etiological agent of healthcare-associated infection, have reached remarkable proportions in Turkey as well as throughout the world. However, the uncontrolled use of meropenem and imipenem, effective antimicrobials used therapeutically, has caused a resistance problem (5).

CRKp was first isolated in America in 1997 and in Turkey in 2001 (1,9). Subsequently, CRKp epidemics have been reported throughout the world (1). According to the EARRS data, all European countries, except for those with high levels of bla_{KPC}-mediated carbapenem resistance, such as Greece and Italy, and those where hospital epidemics have been identified, the rate of carbapenem resistance is 0.6% in *Klebsiella spp.* isolates (6). The rate of imipenem resistance in *K. pneumoniae* isolates is 3.2% according to the results from the multicenter HITIT-2 study conducted in 2007 in Turkey (7). In our study, the rate of carbapenem resistance in *K. pneumoniae* isolates was determined to be 3.1%, consistent with other studies in Turkey.

In a study by Lascols et al. (8), all 110 CRKp isolates examined were NS to ertapenem, imipenem, and ciprofloxacin; 95% were NS to amikacin; and 32% were NS to colistin. In another study, Baran et al. (1) reported that all 69 CRKp isolates were NS to ertapenem and piperacillin/tazobactam, 92.75% were NS to imipenem, 78.26% NS to meropenem, 86.96% NS to ceftazidime and ceftriaxone, 78.26% NS to ciprofloxacin, 63.77% NS to gentamicin, 46.38% NS to amikacin, and 14.49% NS to colistin. In accordance with both studies, in our study, there was 100% resistance to ertapenem and the greatest susceptibility was shown to amikacin. These results are consistent with the order of susceptible antimicrobials, although the percentages differ from those in the literature. We explain the lower rate of colistin resistance found in our study by the susceptibility method we used.

Although several studies have investigated carbapenem resistance in many countries, molecular studies of resistance mechanisms are limited (7). However, in regional studies, bla_{OXA-48} carbapenemase activity associated with carbapenem resistance has been shown in enteric gram-negative bacteria (9,13).

The first bla_{OXA-48}-positive *Klebsiella spp.* strain was isolated in Turkey in 2001 from a patient's urine (9). In the following years, bla_{OXA-48} producers have become an increasingly important cause of nosocomial infection in Turkey (1). A multicenter study in Europe in 2012 found 2% bla_{OXA-48} positivity in *K. pneumoniae* with a reduced susceptibility to carbapenems (10). Demir et al. (11) analyzed 95 *Enterobacteriaceae spp*. isolates by multiplex PCR and found bla_{OXA-48} in 49 isolates. In another multicenter study, 79% bla_{OXA-48} gene positivity was found in CRKp isolates in Turkey (12). We detected 71% bla_{OXA-48} gene positivity in our study.

Table 1. Susceptibility and carbapenemase results of *Klebsiella pneumoniae* isolates

Isolate	Age	Sample	Clinic	MEM	IMP	ETP	OXA-48	NDM	VIM-1	KPC	IMP-1
K4	65	Tracheal a.	ICU	R	R	R	(+)	(+)	(-)	(-)	(-)
K36	29	Tracheal a.	ICU	S	S	R	(+)	(+)	(-)	(-)	(-)
K34	80	Tracheal a.	ICU	I	R	R	(+)	(-)	(+)	(-)	(-)
K11	45	Tracheal a.	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K15	86	Tracheal a.	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K16	72	Tracheal a.	ICU	I	I	R	(+)	(-)	(-)	(-)	(-)
K17	75	Tracheal a.	ICU	I	I	R	(+)	(-)	(-)	(-)	(-)
K18	74	Tracheal a.	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K20	66	Tracheal a.	ICU	R	I	R	(+)	(-)	(-)	(-)	(-)
K27	68	Tracheal a.	ICU	I	R	R	(+)	(-)	(-)	(-)	(-)
K30	68	Tracheal a.	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K33	65	Tracheal a.	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K38	84	Tracheal a.	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K19	61	Tracheal a.	NS	R	R	R	(+)	(-)	(-)	(-)	(-)
K12	79	Tracheal a.	ICU	R	R	R	(-)	(+)	(-)	(-)	(-)
K40	79	Tracheal a.	ICU	S	S	R	(-)	(+)	(-)	(-)	(-)
K23	85	Tracheal a.	ICU	R	R	R	(-)	(-)	(-)	(-)	(-)
K29	60	Tracheal a.	NE	R	R	R	(-)	(-)	(-)	(-)	(-)
K22	40	Deep tissue a.	GS	R	R	R	(+)	(-)	(-)	(-)	(-)
K39	83	Deep tissue a.	GS	R	R	R	(+)	(-)	(-)	(-)	(-)
K41	76	Deep tissue a.	GS	I	I	R	(+)	(-)	(-)	(-)	(-)
K31	47	Deep tissue a.	NS	R	R	R	(+)	(-)	(-)	(-)	(-)
K9	52	Deep tissue a.	URO	R	I	R	(+)	(-)	(-)	(-)	(-)
K35	67	Deep tissue a.	ICU	R	S	R	(-)	(+)	(-)	(-)	(-)
K37	47	Deep tissue a.	ICU	R	R	R	(-)	(+)	(-)	(-)	(-)
K3	61	Deep tissue a.	GS	R	R	R	(-)	(+)	(-)	(-)	(-)
K32	68	Deep tissue a.	GS	R	R	R	(-)	(-)	(-)	(-)	(-)
K44	87	Deep tissue a.	GS	R	I	R	(-)	(-)	(-)	(-)	(-)
K14	74	Urine	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K21	57	Urine	EM	R	R	R	(+)	(-)	(-)	(-)	(-)
K26	66	Urine	ID	R	R	R	(+)	(-)	(-)	(-)	(-)
K28	84	Urine	NE	R	R	R	(+)	(-)	(-)	(-)	(-)
K43	51	Urine	NEF	I	R	R	(+)	(-)	(-)	(-)	(-)
K45	75	Urine	GS	I	I	R	(+)	(-)	(-)	(-)	(-)
K10	49	Urine	ICU	R	R	R	(-)	(+)	(-)	(-)	(-)
K42	67	Urine	URO	S	S	R	(-)	(-)	(-)	(-)	(-)
K6	20	Blood	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K7	45	Blood	ICU	R	R	R	(+)	(-)	(-)	(-)	(-)
K1	52	Blood	GS	R	R	R	(+)	(-)	(-)	(-)	(-)
K13	39	Blood	NE	R	R	R	(+)	(-)	(-)	(-)	(-)
K24	41	Blood	IM	R	R	R	(+)	(-)	(-)	(-)	(-)
K25	75	Blood	URO	R	R	R	(+)	(-)	(-)	(-)	(-)
K8	77	Blood	ICU	R	R	R	(-)	(+)	(-)	(-)	(-)
K5	59	Sputum	NE	R	R	R	(+)	(-)	(-)	(-)	(-)
K2	78	Sputum	ICU	R	R	R	(-)	(-)	(-)	(-)	(-)

Deep tissue a.: deep tissue aspirate; EM: emergency; ERT: ertapenem; GS: general surgery; I: intermediate; ICU: intensive care unit; ID: infection disease; IMP: imipenem; IM: internal medicine; MEM: meropenem; NE: neurology; NEF: nephrology; NS: neurosurgery; R: resistive; Tracheal a.: tracheal aspirate; URO: urology

The limited studies on this subject in Turkey generally have found higher rates of resistance compared to those reported in other countries. This was considered an expected result because the bla_{OXA-48} resistance gene was first found in Turkey.

Patients in intensive care units (ICUs) are sometimes put on mechanical ventilation and undergo intensive antimicrobial treatment, both of which are considered risk factors for healthcare-associated infection. Small-scale outbreaks have been reported in the literature. In our study also, bla_{OXA-48}-positive CRKp isolates were isolated primarily in the samples obtained from ICU patients, consistently with the previous studies.

The bla_{OXA-48} enzyme can successfully hydrolyze penicillins, but exhibits poor or no activity against extended-spectrum cephalosporins. It also has weak carbapenemase activity and does not show a high level of carbapenem resistance. ESBL production or a cell wall permeability defect is necessary for an increased level of resistance to cephalosporins and carbapenems (17–22). However, the bla_{OXA-48}-positive CRKp isolates examined in our study showed a high level of resistance to extended-spectrum cephalosporins and carbapenems. No significant difference was observed between bla_{OXA-48}-positive and -negative CRKp isolates in terms of antimicrobial susceptibility. These findings suggest that other mechanisms of resistance were present in our isolates.

New Delhi metallo-β-lactamase-1 (bla_{NDM-1}) was first discovered in a *K. pneumoniae* strain isolated from the urine sample of a patient who was previously hospitalized in India in 2009 (13). Later it spread to the Balkans and the Middle East (1,24). The first report of bla_{NDM-1} from Turkey was made in 2011 by Poirel et al. based on a *K. pneumoniae* strain isolated from an allogenic stem cell-transplanted leukemia patient who was previously hospitalized in Iraq (1,25). In a multicenter study, Grundmann et al. (12) investigated 124 CRKp isolates and found bla_{NDM} genes in 9 CRKp isolates from Turkey. In the present study, we found the bla_{NDM} gene in 9 CRKp isolates, and only 2 were also positive for the bla_{OXA-48} gene.

Another plasmid-mediated metallo-β-lactamase, Verona integron-encoded metallo-β-lactamase (bla_{VIM}), was first reported in Italy in 1997 (15). In Turkey, bla_{VIM-1} was initially detected by Yildirim et al. in a *K. pneumoniae* isolate from the urine of 3-year-old girl in 2005 (16). In a multinational study, bla_{VIM} positivity was found in 4% of the CRKp isolates from Turkey (12). In our study, we identified the bla_{VIM-1} gene in 1 *K. pneumoniae* isolate that also contained the bla_{OXA-48} gene, which is consistent with a previous study in Turkey (1).

bla_{KPC}-producing isolates were mostly found in Greece, the US, and Israel (8). The first bla_{KPC}-producing isolate identified was a *K. pneumoniae* isolate; in a second report the bla_{KPC}-producing isolate was *Klebsiella oxytoca* (27–29). In a US study, bla_{KPC} positivity was found in 90% of the CRKp isolates, and no other carbapenemase gene was identified (17). In a multicenter study on 124 samples from 17 centers in Turkey, bla_{KPC} gene was not detected (12). Consistently with this, we made no detection of bla_{KPC} gene either.

The bla_{IMP}-type enzymes have become widespread in *Pseudomonas spp*., *Acinetobacter spp*., and *Enterobacteriaceae* members in Japan (31,32). In Turkey, bla_{IMP} was first detected in a *K. pneumoniae* isolate from the blood of a 1-year-old girl in 2005 by Aktas et al. (18). However, we made no detection of bla_{IMP-1} gene.

Considering the international data, a very high level of carbapenem resistance has not been reported in *Enterobacteriaceae* in Turkey (10,11). However, CRKp can spread clonally from person to person, or genes that encode carbapenemases can spread horizontally between isolates. This is important because plasmid-encoded and easily transferable carbapenemases are involved. CRKp infections are associated with high mortality and reduced treatment options. For this reason, accurate and rapid laboratory diagnosis is important for infection control. For faster results, molecular methods, as well as phenotypic methods, must be included in the hospital infrastructure (17,25,34).

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Statement of Conflict of Interest

The authors have no conflict of interest to declare.

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