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Original Article

Investigation of carbapenemase and mcr-1 genes in carbapenem-resistant Klebsiella pneumoniae isolates

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Abstract

Introduction: Carbapenem-resistant *Klebsiella pneumoniae* are a major problem. We aimed to investigate carbapenemase-encoding genes and transferable *mcr*-1 genes among 57 carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates from hospitalized patients.
Methodology: Antibiotic susceptibility tests were performed by Phoenix (BD). Results for ertapenem and colistin were confirmed by gradient diffusion and microdilution methods. Carbapenemase and *mcr-1* genes were investigated by Polymerase Chain Reaction (PCR).
Results: Thirty-two (56.14%) isolates were from intensive care units (ICU). Antibiotic resistance rates by Phoenix: 52.63% for amikacin; 73.69% trimethoprim sulfamethoxazole; 91.23% cefepime; 82.46% tigecycline; 59.65% colistin. Carbapenemases positivity: 82.45% (47) for blaOXA-48, 40.35% (23) blaOXA-55, 3.50% (2) blaOXA-51, 1.75% (1) blaOXA-23, 1.75% (1) blaOXA-24, 1.75% (1) blaIMP. blaOXA-58, blaKPC, blaNDM-1, and blaVIM were not detected. Twenty (35.08%) isolates had both blaOXA-48 and blaOXA-55. Three isolates were *mcr-1* (+) and *bla*OXA-48 (+). One *mcr-1* (+) isolates was *bla*OXA-51 (+). One colistin sensitive isolate determined by Phoenix, was found colistin resistant by microdilution.
Conclusion: OXA-48 and OXA-55 co-harboring isolates and *mcr-1* gene (+) isolates were spreading. Automated colistin susceptibility results should be confirmed by microdilution method. Resistance mechanisms in *Enterobacteriaceae* should be determined and the isolates should be monitored by molecular epidemiological methods. Effective infection control measures will contribute to reduce risk of antibiotic resistant bacterial infections and dissemination of antibiotic resistance.

Key words: Carbapenem; colistin; resistance.

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Introduction

Klebsiella pneumoniae (KP) is a common cause of hospital-acquired infections including pneumoniae, bloodstream infections, and urinary tract infections.

Carbapenems belong to the group of beta-lactam antibiotics. They are considered to be an effective treatment of Gram-negative bacterial infections and confer exceptional stability against AmpC beta-lactamases and the extended spectrum beta-lactamases (ESBLs). However, due to a worldwide increase in the number of antibiotic resistant bacteria, carbapenem-resistant KP (CRKP) isolates have become a major problem. In clinical isolates, carbapenem resistance is most commonly caused by enzyme-mediated mechanisms . Carbapenemases encoded by horizontally transferable genes such as plasmids or transposons are able to inactivate carbapenems together with other beta-lactam antibiotics [1].

CRKP isolates are more resistant to antibiotics than carbapenem-susceptible KP (CSKP) isolates. Mortality rates are higher in CRKP infections [2].

Colistin belongs to the group of polymyxin antibiotics, and is considered as one of the last-resort treatments against infections caused by CRKP [3]. TheWorld Health Organization (WHO) classified polymyxins as one of the Highest Priority Critically Important Antimicrobials [3]. Colistin resistance is usually caused by lipid A modifications such as the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N), and phosphoethanolamine (pEtN). The lipopolysaccharide changes by *mgrB* gene mutations, responsible for the regulation of the PmrAB and PhoPQ two component systems, also can cause colistin resistance [4]. In addition to these mechanisms, the first plasmid-mediated colistin resistance gene, *mcr-1*, was identified on an IncI2 plasmid from *Escherichia coli* and *K. pneumoniae* in China [5]. Another plasmid-mediated colistin resistance gene *mcr-2* was found in 2016 [6]. It was suggested that dissemination of the *mcr* genes can lead to a serious escalation of the current antibiotic resistance crisis in the World [6]. Therefore, the characterization of carbepenem and colistin resistance mechanisms and understanding of the infection epidemiology are necessary for controlling dissemination of antibiotic resistant isolates. The aim of the present study was to evaluate the carbapenem resistance mechanisms and to investigate the frequency of the *mcr*-1 gene in CRKP isolates in a tertiary hospital in İstanbul.

Methodology

Samples and isolates

A total of 57 CRKP strains isolated from 56 hospitalized patients between January and December 2017 at Okmeydani Training and Research Hospital, Istanbul were included in this study. Of these isolates, 21 were isolated from blood cultures, 2 were isolated from tissue samples, 13 from urine samples, 1 from a catheter sample, 2 from peritoneum samples, 1 from bronchoalveolar lavage samples. 17 isolates were also isolated from rectal swabs. The study isolates were identified by using BD Phoenix™ Automated Microbiology System (Becton-Dickinson, Sparks, Nevada USA).

Antibiotic susceptibility tests

The susceptibility of the isolates against amikacin, trimethoprim sulfamethoxazole, cefepime, tigecycline, meropenem, imipenem, ertapenem was determined by BD Phoenix™ Automated Microbiology System (bioMérieux, Nürtingen, Germany). The antibiogram results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria [7]. Additionally, ertapenem resistance was confirmed by the gradient diffusion method (E-test, Bio-Merieux, France) and interpreted according to the the Clinical and Laboratory Standards Institute (CLSI) criteria [7]. Minimal inhibitory concentrations (MIC) of colistin were confirmed by a microdilution method [8] and results were evaluated according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [9]. Isolates which were Resistant (R) or Intermediate resistant (I) against antibiotics were considered as resistant (R) in this study.

Polymerase Chain Reaction (PCR)

All CRKP isolates were tested for the presence of carbapenemase-encoding genes including *bla*OXA-23, *bla*OXA-24, *bla*OXA-48, *bla*OXA-51, *bla*OXA-55, *bla*OXA-58, *bla*KPC, *bla*NDM-1, *bla*VIM, *bla*IMP by multiplex PCR. Then all CRKP isolates were tested for the presence of the *mcr-1* gene. The primers used in this study are shown in Table 1.

Table1. Primers used in the study.

Oligo name	Base sequence 5’-3’	Amplicon size (bp)	Reference
CLR5	F: CGGTCAGTCCGTTTGTTC R: CTTGGTCGGTCTGTAGGG	305	[1]
OXA-23	F:GATCGGATTGGAGAACCAGA R: ATTTCTGACCGCATTTCCAT	501	[11]
OXA-24	F: GGTTAGTTGGCCCCCTTAAA R: AGTTGAGCGAAAAGGGGATT	246	[12]
OXA-48	F: TTGGTGGCATCGATTATCGG R: GAGCACTTCTTTTGTGATGGC	743	[13]
OXA-51	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353	[11]
OXA-55	F: CATCTACCTTTAAAATTCCC R: AGCTGTTCCTGCTTGAGCAC	975	[14]
OXA-58	F: AAGTAT TGGGGCTTGTGCTG R: CCCCTCTGCGCTCTACATAC	599	[11]
IMP	F: CATGGTTTGGTGGTTCTTGT R: ATAATTTGGCGGACTTTGGC	488	[11]
VIM	F: ATTGGTCTATTTGACCGCGTC R: TGCTACTCAACGACTGAGCG	780	[11]
NDM-1	F: GAGATTGCCGAGCGACTTG R: CGAATGTCTGGCAGCACACTT	497	[11]
KPC	F: ATGTCACTGTATCGCCGTCT R: TTTTCAGAGCCTTACTGCCC	893	[11]

A bacterial suspension equal to four McFarland turbidity was prepared in Phosphate Buffered Saline (PBS, Sigma, Germany) and boiled for 10 minutes. Then, it was centrifuged for 2 minutes at 13000 g and the supernatant was used as the DNA source [10]. PCR reaction was established by using 10 pmol of each primer, and 2.5 U of Taq DNA polymerase (Thermo Scientific-Fermantas Corporation, Vilnius, Lithuania) in a final reaction volume of 50 µL. The following amplification conditions were used: initial denaturation at 94°C for 4 minutes, followed by 40 cycles of 94°C for 30 seconds, 52°C for 40 seconds, and 72°C for 50 seconds, with a final extension step at 72°C for 10 minutes. The amplified DNA products were analyzed by electrophoresis on a 1.5% agarose gel and the results were evaluated according to the size of each amplicon.

Statistical Analysis

The data were analyzed with SPSS software version 18. The Pearson chi-square test was used to analyze the data and statistically significant level was accepted as less than 0.05.

Results

Out of the 57 patients, 30 were female, 27 were male. The female patients' age ranged from 1 to 93 years old (The average age: 60.9). The male patients' age ranged from 37 to 87 years old (The average age: 65.1). There was no significance in terms of gender among CRKP isolates. Among the isolates, 32 (56.14%) were isolated from intensive care units (ICUs) ($p < 0.05$). The others were isolated from the patients hospitalized in various wards including hematology (n = 13), internal medicine (n = 5), infectious disease (n = 4), urology (n = 1), oncology (n = 1), general surgery (n = 1).

The antibiotic susceptibility results determined by Phoenix System, for amikacin 47.37% (n = 27) S (susceptible), 52.63% (n = 30) R; for trimethoprim sulfamethoxazole 26.31% (n = 15) S, 73.69% (n = 42) R; for cefepime 8.77% (n = 5) S, 91.23% (n = 52) R; for tigecycline 17.54% S (n = 10), 82.46% (n = 47) R; for colistin 40.35% S (n = 23), 59,65% R (n = 34). MIC values for colistin and ertapenem ranged from 0.25 to 64 µg/mL and from 16 to 32 µg/mL, respectively.

Table 2. Distribution of carbapenemases genes and *mcr*-1 gene among 57 CRKP isolates.

Gene	Positivity rate (n/57)
*bla*OXA-48	82,45% (47/57)
*bla*OXA-55	40.35% (23/57)
*bla*OXA-51	3.50% (2/57)
*bla*OXA-23	1.75% (1/57)
*bla*OXA-24	1.75% (1/57)
*bla*IMP	1.75% (1/57)
mcr-1	5,26% (3/57)

Out of the 57 CRKP isolates, positivity rates of carbapenemases detected by PCR were as follows: 82.45% (n = 47) for blaOXA-48, 40.35% (n = 23) for blaOXA-55, 3.50% (n = 2) for blaOXA-51, 1.75% (n = 1) for blaOXA-23, 1.75% (n = 1) for blaOXA-24, 1.75% (n = 1) for blaIMP (Table 2). Among the isolates, blaOXA-58, blaKPC, blaNDM-1, and blaVIM were not detected. A total of 20 (35.08%) isolates had both blaOXA-48 and blaOXA-55. One isolate was harboring three of the carbapenemases OXA-48, OXA-55, and IMP. Out of the 57 CRKP isolates, three isolates (5,26%) were positive for the *mcr-1* gene (Table 2).

The characteristics of *mcr-1* gene positive isolates are shown in Table 3. All *mcr-1* gene positive isolates harbored *bla*OXA-48, one of the *mcr-1* gene positive isolates was also positive for *bla*OXA-51. All of the m*cr-1* gene positive isolates were isolated from intensive care unit patients. The hospital records indicated that two of the three patients with *mcr*-1 positive *K. pneumoniae* isolates had been transfered from Emergency Service; one patient had been transfered from Infectious Disease Service to ICUs. All of the *mcr-1* positive isolates were resistant against colistin with MICs ranging from 32 to 64 µg/mL. It was shown that a patient's first isolate, in September was *mcr-1* negative, while a second isolate from the same patient in December, was *mcr-1* positive.

Table 3. The characteristics and antibiotic susceptibilities of three *mcr*-1 positive isolates.

Isolate	Service	Sample	A	S	C	T	Co	OXA-23	OXA-24	OXA-48	OXA-51	OXA-55	OXA-58	KPC	NDM	VIM	IMP	Co MIC (µg/mL)
1	General ICU	urine	I	S	R	R	R	(-)	(-)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	32
2	General ICU	blood	S	S	R	I	R	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	64
3	Urology ICU	urine	S	S	R	R	R	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	64

A: Amikacin, S: Trimethoprim sulfamethoxazole, C: Cefepime, T: Tigecycline, Co: Colistin, ICU: Intensive care unit, S: Susceptible, I: Intermediate, R: Resistant, (-): Negative, (+): Positive.

Of the 34 colistin-resistant isolates as detected by the Phoenix System, one (2,9%) was identified as colistin sensitive by the microdilution method. On the other hand, among the 23 colistin-sensitive isolates as detected by the Phoenix System, one (4,3%) was found to be colistin resistant by the microdilution method. Both of these two isolates were *mcr-1* gene negative by PCR.

Discussion

The rapid global spread of carbapenem resistance is a threat for the health system. Carbapenem-resistant *Enterobacteriaceae* (CRE) isolates cause infections especially in patients hospitalized in ICUs with long-term antibiotic treatment and indwelling urethral catheters. The independent risk factors for CRKP infections include admission to ICU, use of beta-lactams and beta-lactamase inhibitor combination antibiotics, cephalosporins, fluoroquinolones, and indwelling urethral catheter. Delayed start of proper antibiotics can lengthen hospital stay and increase mortality [11-15]. In our study the majority of the CRKB isolates were isolated from patients hospitalized in ICUs, hematology clinics, and oncology clinics in accordance with the literature data.

Among *Enterobacteriaceae* species carbapenem resistance is a big problem especially in *K. pneumoniae*. A study performed on 70 CRE isolates at a tertiary hospital in Brazil in 2015 showed that the most prevalent microorganism was *K. pneumoniae* (95.7%) with a high-level resistance to carbapenems (> 98%) [16]. In a study from Turkey evaluating 181 CRE isolates, about half of the isolates displayed multidrug-resistance [17]. In our study, 52.63% of the isolates were resistant to amikacin; 73.69% of isolates were resistant to trimethoprim sulfamethoxazole; 82.46% of isolates were resistant to tigecycline; 91.23 % were resistant to cefepime by Phoenix System and 59.65% of isolates were resistant to colistin. In accordance with the literature [1,17], these data indicate that CRKP isolates commonly display multiple resistances against antimicrobial drugs other than carbepenems.

A study from Turkey found that the carbapenem resistance rate was 5.8% in 1605 *K. pneumoniae* isolates and 71% of the carbapenem-resistant isolates were recovered from ICU patients. In this study, *bla*OXA-48 and *bla*NDM-1 were detected in 90.3% and 6.5% of the carbapenem-resistant isolates, respectively. Due to the high rate of carbapenem resistance and 80.6% clonal relationship by pulsed-field gel electrophoresis (PFGE) in a 3-years period, the authors suggested that CRKP isolates pose a potential threat for patients to get hospital-acquired infections [10]. In Turkey, the bla_{OXA-48} genes were identified previously in *K. pneumoniae*, *E. aerogenes*, *E. coli*, *C. freundii*, *E. cloacae*, *S. marcescens*, *P. rettgeri*, *K. oxytoca*, *P. mirabilis*, *M. morganii*, *P. stuartii*, *R. planticola*, and *A.baumanii*. The OXA-48 enzyme can hydrolyze penicillins but has poor or no activity against extended-spectrum cephalosporins and aztreonam. The OXA-48 enzyme has a weak carbapenemase activity. However, in isolates with ESBLs or cell wall permeability defects, it can cause increased level of cephalosporin and carbapenem resistance. Furthermore, co-expression of OXA-48 and ESBLs (CTX-M-15, SHV) and co-expression of OXA-48 and AmpC enzymes in CRE were reported [11,16,17]. In the presented study, out of the 57 CRKP isolates, the frequency of carbapenemases determined were blaOXA-48 (82.45%), blaOXA-55 (40.35%), blaOXA-51 (3.50%), blaOXA-23 (1.75%), blaOXA-24 (1.75%), blaIMP (1.75%). We suggest that spreading of blaOXA-48 and OXA-55 co-harboring isolates were problematic in our hospital and our country.

In recent years, colistin resistance reduced the available therapeutic options. Risk factors for colistin-resistant Gram-negative infections are neurologic diseases, residence in a skilled nursing facility, methicilin-resistant *Staphylococcus aureus* (MRSA) antimicrobials or carbapenem use in the last 90 days, and prior infection with carbapenem-resistant microorganism [6]. Plasmid-mediated colistin resistance encoded by *mcr-1*, has been identified in isolates from humans, animals, and the environment in an increasing number of countries including Algeria, Belgium, China, Egypt, UK, Germany, Portugal, South Africa, USA, France [18]. Until now there have been no reports of *mcr-1* harboring *Enterobacteriaceae* human isolates in Turkey. Sarı *et al.* from Turkey, screened a total of 329 *Enterobacteriaceae* isolates from 22 laboratories between 2015 and 2016 by PCR but they did not detect *mcr-1/mcr-2* genes [18]. Recently, Kurekci C *et al.* detected *E. coli* isolates carrying *mcr-1* in chicken meats [19]. In the presented study, we detected that colistin resistance was problematic due to a resistance rate of 60% and *mcr-1* positivity in three isolates. Due to the common use of colistin in CRE infections, in animals [20], the food industry [20], and dissemination of plasmid-mediated *mcr-1* gene, colistin-resistant Gram-negative isolates can spread in our country. We suggest that all *mcr-1* positive samples should be confirmed by sequencing of the amplicons.

Early and approriate therapy of patients with colistin-resistant Gram-negative bacterial infections has been associated with reduced mortality [20]. In our study, in one patient, three months after the isolation of *mcr-1* negative CRKP, *mcr-1* positive CRKP was isolated. In addition to this, patients with *mcr-1* positive isolates had been transferred to ICU from various clinics. We suggest that monitoring the colistin resistance during therapy, investigating the cross-contamination between isolates, and effective infection control measures such as ensuring hand hygiene compliance, reduction of the duration of hospital stay of patients, and prevention of improper and over use of antibiotics [21] are necessary to reduce the risk of colistin-resistant bacterial infections and dissemination of resistance.

In our study, we observed discrepancies in the detection of colistin resistance between Phoenix System and microdilution methods in two isolates. We suggest that colistin susceptibility results obtained from automated antibiotic susceptibility systems should be confirmed by microdilution method in accordance with the recommendations of EUCAST [9]. On the other hand, the Gram-negative bacteria which were susceptible to antibiotics other than colistin can harbor *mcr-1* [20,22]. *mcr-1* positive isolates can be colistin sensitive due to a non-functional *mcr-1* gene [21]. We suggested screening of *mcr-1* gene in both colistin susceptible and resistant Gram-negative strains isolated from ICU patients.

Conclusion

In conclusion, carbapenem and/or colistin resistance in *K. pneumoniae* was a significant public health problem in ICU and hospital settings in Turkey. Tle blaOXA-48 and blaOXA-55 were the most common carbapenemases in the study population and spreading of blaOXA-48 and OXA-55 co-harboring isolates was problematic. This is the first study showing the presence of *mcr-1* gene among CRKB isolates in Turkey. Colistin susceptibility results obtained from automated antibiotic susceptibility systems should be confirmed by microdilution method. Colistin and carbapenem resistance mechanisms in *Enterobacteriaceae* should be rapidly determined and the isolates should be monitored by molecular epidemiological methods. Monitoring colistin resistance during therapy and effective infection control measures will contribute to reduce the risk of antibiotic resistant bacterial infections and dissemination of resistance.

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