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Investigation of phenotypic and genotypic characteristics of carbapenem resistant Klebsiella pneumoniae strains isolated from nosocomial infections

Hastane enfeksiyonlarından izole edilen karbapenem dirençli Klebsiella pneumonia suşlarının fenotipik ve genotipik özelliklerinin incelenmesi

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Abstract

Purpose: Carbapenem-resistant Klebsiella pneumoniae (CRKP) is an important nosocomial pathogen. The most common mechanism that causes carbapenem resistance in these bacteria is carbapenemase production. In this study, we aimed to investigate the molecular epidemiology of CRKP isolates and, their resistance to carbapenems and antibiotics other than carbapenems.

Materials and methods: The diagnosis and antibiotic susceptibility tests of 62 CRKP isolates were performed with Vitek-2 automated system and E test. Carbapenemase genes were determined by multiplex PCR. The genetic relationships were detected by rep-PCR.

Results: Eight clusters containing 25 strains with 95% or more similarity were identified by Rep-PCR method. On the other hand, 59.7% (37/62) of these strains were identified as "different" with similarities of less than 95% and more than two different bands. The blaOXA-48 gene was detected in 57 of the 62 isolates (91.9%) and the modified Hodge test was positive in all of the blaOXA-48 gene positive isolates. The resistance rates of carbapenems (imipenem, meropenem and ertapenem) were examined by Vitek-2 system and E-test. The lowest resistance rates were observed for meropenem (Vitek-2 35%, E-test 16%). Resistance rates in these isolates were 100% for ampicillin and amoxicillin-clavulanic acid, 90% for ceftriaxone.

Conclusion: We found that OXA-48 oxacillinase production was responsible for carbapenemase resistance in CRKP. The rep-PCR results suggested that the genetic similarities between CRKP isolates were not high. We suggest that this was due to the characteristic of blaOXA-48 gene which is horizontal trasnferred rather. than clonal spread among bacteria.

Key words: OXA-48, carbapenemase, rep-PCR, multiplex PCR.

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Özet

Amaç: Karbapenemlere dirençli Klebsiella pneumoniae (KDKP) önemli bir nozokomiyal patojendir. Bu bakterilerde karbapenem direncine neden olan en yaygın mekanizma karbapenemaz üretimidir. Bu çalışmada KDKP izolatlarının moleküler epidemiyolojisini, karbapenem ve karbapenem dışındaki antibiyotiklere direnç özelliklerini incelemeyi amaçladık.

Gereç ve yöntem: Toplam 62 KDKP izolatının tanımlanması ve antibiyotik duyarlılık testleri Vitek-2 otomatik sistem ve E testi ile yapıldı. Karbapenemaz genleri multipleks PCR ile belirlendi. Genetik benzerlikleri rep-PCR ile araştırıldı.

Bulgular: Rep-PCR yöntemi ile %95 veya daha fazla benzerliğe sahip 25 suş içeren sekiz küme tanımlandı. İzolatların %59,7'si (37/62), benzerlikleri %95'ten az ve ikiden fazla farklı bant içerenler, “farklı” olarak belirlendi. BlaOXA-48 geni, izolatların %91,9’unda (57/62) tespit edildi ve modifiye Hodge testi, blaOXA-48 geni pozitif izolatlarının tamamında pozitifti. Karbapenemlerin (imipenem, meropenem ve ertapenem) direnç oranları Vitek-2 sistemi ve E-testi ile incelendi. En düşük direnç oranları meropenem için belirlendi (Vitek-2 %35, E-test %16). Bu izolatlarda direnç oranları ampisilin ve amoksisilin-klavulanik asit için %100, seftriakson için %90 bulundu.

Sonuç: KDKP izolatlarında OXA-48 oksasilinaz üretiminin karbapenemaz direncinden sorumlu olduğunu belirledik. Rep-PCR sonuçları, KDKP izolatları arasındaki genetik benzerliklerin yüksek olmadığını göstermiştir. Bu verilere dayanarak, K. pneumoniae suşlarında blaOXA-48 geninin, klonal yayılımdan ziyade bakteriler arasında horizontal geçiş özelliğinden kaynaklandığını ileri sürmekteyiz.

Anahtar kelimeler: OXA-48, karbapenemaz, rep-PCR, multipleks PCR.

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Introduction

Outbreaks of carbapenemase-producing Enterobacteriaceae are increasing worldwide and are mainly due to the occurrence and spread of carbapenemase-producing strains [1]. Carbapenem-resistant Enterobacteriaceae, in particular *Klebsiella pneumoniae*, is an important cause of nosocomial infections and is typically resistant to many classes of antibiotics that limit all beta-lactam antibiotics and treatment options. This has led to a growing preference for carbapenems in the treatment of infections caused by microorganisms [2, 3]. Previous studies have reported that mortality rates associated with carbapenem-resistant *K. pneumoniae* (CRKP) infections are 3.7-6.5 times higher than those caused by carbapenem-sensitive *K. pneumoniae* [4].

Carbapenems are considered to be the preferred antibiotics for Gram-negative bacteria that produce plasmid-encoded extended-spectrum β-lactamase (ESBL) or chromosomal cephalosporinase [5]. The most common mechanism that causes carbapenem resistance in these bacteria is carbapenemase production [1]. Carbapenemases are often expressed by mobile genetic elements, such as plasmids or transposons, which contain multi drug resistance genes and have the potential to transfer to other bacteria through horizontal gene transfer. Thus, bacterial populations susceptible to antibiotics gain resistance in remote or closely related [1, 6]. Transferable carbapenem resistance is a major concern, especially among hospital strains [2, 3, 7].

The most important reasons responsible for the development of resistance to carbapenems include the production of enzymes that hydrolyze carbapenems such as class A KPC, class B VIM, IMP, NDM-1 or class D oxacillinase [8, 9]. An other cause of carbapenem resistance in *K. pneumoniae* is accused of excessive production of class A ESBL or AmpC enzymes and a decrease in membrane permeability due to mutations in porin channels. Active drug efflux is also known as another cause [10].

Carbapenem-resistant Enterobacteriaceae, which cause nosocomial infections, are resistant to the majority of existing antibiotics, so patients with serious underlying diseases, long hospitalizations, and immunosuppressed patients are particularly susceptible to infections with these pathogens. In order to prevent hospital outbreaks, it is important to reveal the resistance mechanisms and propagation pathways in these microorganisms [11-15]. In this study, the presence of carbapenemases in CRKP strains isolated from inpatients was investigated by both phenotypic and genotypic methods. It was also aimed to investigate the clonal relationships of these strains and therefore their genetic similarities by repetitive extragenic palindromic sequence based-Polymerase Chain Reaction (rep-PCR).

Materials and methods

Bacterial isolates

This study included 62 CRKP strains isolated from patients diagnosed with nosocomial infection, that were sent to the Clinical Microbiology Laboratory of our hospital between May 2011 and April 2012. Only one strain from each patient was included in the study. Isolates were considered resistant to carbapenems if they were found resistant or intermediate to one or more of the three carbapenem antibiotics tested which were imipenem (IPM), meropenem (MEM), and ertapenem (ERT). Environmental samples were taken from the hospital wards where patients infected with CRKP resided and cultured for investigation of CRKP presence.

Bacterial identification, susceptibility testing and phenotypic determination of carbapenemase production

Species identification was done with Vitek 2 automated identification system (bioMérieux, France). Vitek MS MALDI-TOF system (bioMérieux, France) was used for confirmation of identification. The susceptibility tests (minimum inhibitory concentration; MIC) of carbapenems and other antimicrobial agents were determined with the Vitek 2 system with AST-261 cards. Also MICs of IPM, MEM and

ERT were evaluated by E-test (bioMérieux, France). The modified Hodge test (MHT) was performed as previously described in CLSI guidelines for preliminary screening of presence of carbapenemases [7]. Metallo-beta-lactamase production was investigated with E-test MBL strips (bioMérieux, France) which contain increasing concentrations of IPM on one end and IPM overlaid with EDTA on the other end [8]. Production of extended spectrum beta-lactamases was tested using CLSI ESBL initial screen test and confirmatory method [7].

Detection of carbapenemase genes

For investigation of genes that cause the production of carbapenemases in the collected CRKP strains a commercial multiplex PCR (hyplex® SuperBug ID, Amplex, Germany) was used. With this system metallo-beta-lactamase genes such as blaVIM, blaIMP and blaNDM-1, oxacillinase genes such as blaOXA-48 encoding genes and all variants of blaKPC genes can be identified.

Molecular epidemiology

Clonal relationship between all CRKP isolates was determined by rep-PCR using the semi-automated system Diversilab (bioMérieux, France). Data analysis was performed with the web-based DiversiLab software version 3.4 (bioMérieux). Pearson correlation coefficient was used to determine distance matrices and UPGMA (Unweighted Pair Group Method with Arithmetic averages) method was used to create dendrograms. In the evaluations, strains with more than 97% similarity and no band difference were identified as “clone”. Strains with similarities of 95-97% and those with one or two band differences were defined as “similar” group, strains with similarities smaller than 95% and strains with more than two band differences were defined as “different” group. Ethics committee approval was taken from Ankara Numune Training and Research Hospital. 15.02.2012; 2012-328.

Statistical analysis

Data were analyzed by SPSS 15 package program. Descriptive statistics were evaluated with mean ± standard deviation for normal variables, median (minimum-maximum) for non-normal variables, and nominal variables for Pearson Chi-Square or Fisher Exact tests. Kappa test was used to compare the fit of the variables. The state of the variables and their distinctive features were made by Roc curve analysis and their superiority to each other was made by comparison analysis of Roc curves in medcalc program. Results were considered statistically significant for $p<0.05$.

Results

Clinical isolates and patients

This study included 62 patients, 44% female and 56% male, who were mainly hospitalized in intensive care units (ICU) (66%). The mean age of the patients was 68.58±16.08 years (range 20-94 years). The majority of 62 CRKP strains 45% (n=28) were from surgical ICU. This was followed by reanimation 15% (n=9), burn unit 10% (n=6), emergency medicine ICU 6% (n=4), orthopedics 5% (n=3), and neurology 5% (n=3). From other clinics approximately 14% (n=9) of the strains isolated. Any K. pneumonia was not isolated from simultaneous environmental cultures.

Molecular tests

Genetic relatedness among all *K. pneumoniae* isolates was determined by Diversilab rep-PCR. Six “clones” (A-F) with a similarity of more than 97% and no band difference were found in 33.9% (21/62) of all CRKP strains. Two “similar” groups (G1, G2) were identified at a rate of 6.5% (4/62) with similarities of 95-97% and one or two band differences (Figure 1). All of these 25 isolates were OXA-48 positive. “Different” strains with similarities of less than 95% and band differences of more than two were found to be 59.7% (37/62).

In this study, blaOXA-48 gene was detected in 91.9% (57/62) of *K. pneumoniae* isolates by Multiplex PCR. However, blaVIM, blaIMP, blaKPC, blaNDM-1 genes could not be detected in any of the isolates.

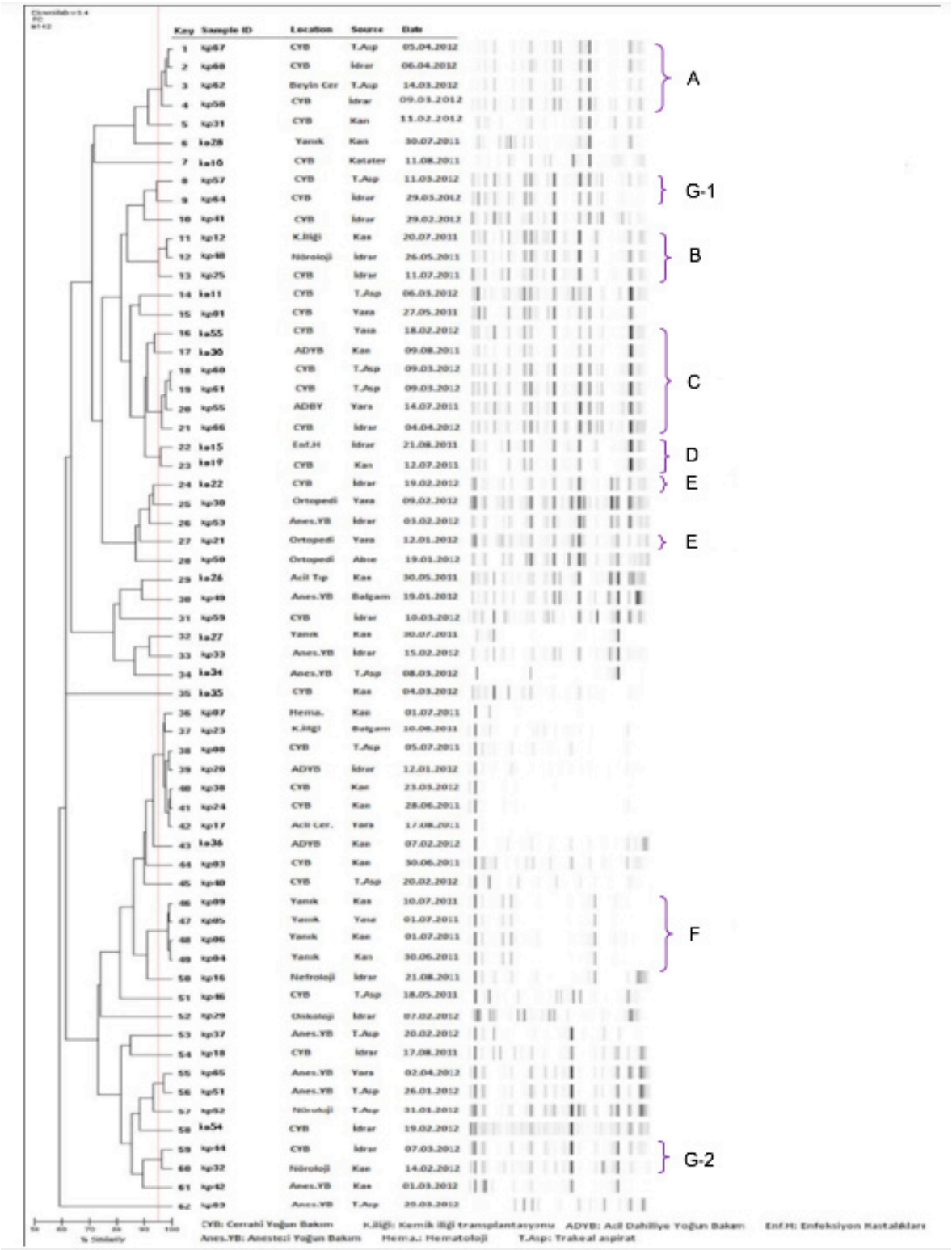

Figure 1. Dendrogram of the similarities of the 62 carbapenem-resistant K. pneumoniae isolates (with similarity percentages) as analysed with DiversiLab virtual-gel bands. Clusters (A-F clons and G1, G2 similar groups) are formed by the isolates with ≥95% similarity.

Phenotypic tests

IPM, MEM and ERT were studied by Vitek-2 system and E-test. Resistance rates were 84%, 35%, 100% by Vitek-2 and 48%, 16% and 94% by E-test, respectively. The lowest resistance ratio was determined for MEM. Resistance rates to carbapenems are shown in Table 1. When the MIC values of IPM, MEM, ERT E-test were evaluated by Roc curve analysis, it was determined that all carbapenems were discriminative in terms of "discrimination of OXA-48 positivity" (AUC=0.818, p=0.004; AUC=0.812, p=0.004; AUC=0.723, p=0.007, respectively). The IPM E-test was found to be the most distinctive for OXA-48, because the AUC value was highest. However, it was found that carbapenems were not statistically superior to each other (p>0.05).

Vitek-2 and E-test carbapenem MIC values (median) of OXA-48 positive strains were compared with OXA-48 negative strains (Table 2). Only IPM E-test, MEM E-test and IPM-Vitek median values were higher and statistically significant in OXA-48 positive strains compared to negative strains (p=0.016, p=0.018, p=0.012, respectively).

E-test resistance ratios of OXA-48 positive strains for carbapenems were higher for IPM and ERT than OXA-48 negative strains and this difference was statistically significant (p=0.003, p=0.001, respectively). Similarly, carbapenem resistance rates determined by Vitek-2 were evaluated. IPM-Vitek resistance rates were significantly higher in OXA-48 positive strains than OXA-48 negative strains (p<0.001). When ERT-Vitek results were evaluated, resistance was determined in all isolates (Table 1).

MHT was positive in 57 of 62 CRKP (91.9%). MHT positive isolates were the same isolates that were blaOXA-48 gene positive. MHT showed perfect concordance with blaOXA-48 gene detection with multiplex PCR (kappa number=1). Metallo-beta-lactamase production was investigated by IPM/IPM+EDTA and 60 of the isolates (96.8%) were found negative, and two were positive.

Antibiotics other than carbapenem were also studied by Vitek-2. All isolates were found to be 100% resistant to ampicillin and amoxicillin-clavulanic acid. Resistance rates of the isolates for other antibiotics were 90% to ceftriaxone, 87% to cefepime, 87% to cefoxitin, 64% trimethoprim-sulfamethoxazole, 66% amikacin, 47% to gentamicin, 84% to levofloxacin, 15% to tigecycline. Fifty-nine of 62 CRKP strains were found to be ESBL positive (95.2%). All CRKP were found to be susceptible to colistin.

Table 1. Resistance percent of carbapenem resistant *Klebsiella pneumoniae* isolates for carbapenems by Vitek-2 and E-test isolates.

	IMIPENEM			MEROPENEM			ERTAPENEM		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Vitek-2	84	6	10	35	8	57	100	0	0
E-test	48	31	21	16	27	57	94	0	6

R: resistance, I: intermediate, S: susceptible

Table 2. Median MIC values of OXA-48 negative and OXA-48 positive carbapenem resistant *Klebsiella pneumoniae* isolates for carbapenems.

	Vitek-2 Median MIC values			E-test Median MIC values		
	IPM	MEM	ERT	IPM	MEM	ERT
OXA-48 (+)	4	1	8	3	1	32
OXA-48 (-)	1	2	4	0.19	0.016	0.25
Total	4	1	8	2.5	1	32

MIC: minimum inhibitory concentration, IPM: imipenem, MEM: meropenem, ERT: ertapenem

Discussion

Since carbapenem antibiotics are the last choice for serious infections caused by life-threatening and multi-drug resistant Gram-negative bacteria, determination of carbapenem resistance is very important [16]. Carbapenem resistance is a rapidly increasing globally due to the rapid spread of multi-drug resistant *K. pneumoniae* strains producing carbapenemase [15]. In this study, rep-PCR analysis was performed to investigate the genetic relatedness of 62 *K. pneumoniae* isolates which were found to be resistant to carbapenems obtained from different clinical samples. Eight clusters were identified containing six clones (21 isolates) and two similar (4 isolates) groups. Among the 62 isolates, small clusters of strains with ≥95% similarity and isolation dates close to each other were identified. All of these 25 isolates were OXA-48 positive. This can be explained by the fact that the blaOXA-48 gene was detected in the majority of the isolates (91.9%). Because, in previous studies, it has been reported that the spread of blaOXA-48 gene is not from a single clone, and that these genes can be transferred horizontally from bacteria to bacteria via insertion sequences (IS1999) [17, 18]. Poirel et al. [19] first reported that IS1999 adjacent to the plasmid-encoded blaOXA-48 gene was involved in the formation of promoter sequences and the mobilization function of this beta-lactamase gene and was associated with the oxacillinase gene. Carrer et al. [17] found that the spread of OXA-48 gene did not originate from a single clone, and that the IS-associated OXA-48 gene was spread by gene transfer among existing bacteria. The results of these studies support the cause of genetic differences between the isolates detected by rep-PCR. In this study, it was determined that 59.7% (37/62) of the isolates had three or more band differences with other isolates. This shows that genetic similarity is not very high in these CRKP isolates in general.

Us et al. [2] in a study conducted with 26 CRKP strains in 2011, reported that the majority of CRKP isolates originated from the ICU and that the spread was from patient to patient and this spread may have occurred during patient transfer. Similarly, in our study, it was found that the samples belonging to the clones were sent most frequently from the surgical ICU (47.6%; 10/21). In addition to the horizontal spread, a clonal spread to other services is also possible, suggesting that isolation measures should be reconsidered, especially in ICU.

In recent years, class D OXA carbapenemase-mediated carbapenem resistance has become widespread among Enterobacteriaceae strains that cause nosocomial infections [20]. In our country, the first OXA-48 oxacillinase-positive *K. pneumoniae* isolate was isolated from the urine culture of a patient hospitalized in Istanbul University, Istanbul Medical Faculty Hospital in 2001 [19]. A short time later, a number of other major cities in Turkey sporadic cases and outbreaks have been reported in hospitals [17, 20, 21]. The blaOXA-48 gene has also been described in other Middle East and North African countries, mostly in *K. pneumoniae* isolates [22, 23]. All these countries can be considered as important reservoirs of OXA-48 enzyme producing isolates [24]. Thereafter, outbreaks of OXA-48 enzyme-producing strains from other European countries and all over the world (Lebanon, Turkey, Greece, Ireland, France) have been reported [10, 14, 25-27]. In France, Cuzon et al. [23] first isolated 17 *K. pneumoniae* strains producing OXA-48 from 10 patients. In our study, OXA-48 producing strains were detected in 91.9% of the CRKP isolates by multiplex PCR, while NDM-1, VIM, IMP and KPC producing strains could not be detected. OXA-48 was negative in 5 of 62 isolates. Carbapenem resistance in these isolates may be due to decreased outer membrane permeability via porin mutations or expression of efflux pumps. Unfortunately, in recent years, in studies conducted in Turkey, NDM-1, VIM, IMP and KPC-producing strains have been reported [20, 28].

In several studies, the catalytic activity of OXA-48 enzymes was reported to be high for ERT and IPM but low for MEM [19, 29]. In a study conducted in Lebanon, ERT resistance was found to be higher in OXA-48 positive isolates than other carbapenems. The researchers in this study suggested that carbapenem resistance may be due to loss of porine. In addition, according to RAPD analysis results, it was reported that resistance to these drugs is not only caused by a single clone, but may also be caused by selective pressure, prolonged treatment, disruption of membrane permeability

and differences in resistance gene levels [10]. Similarly, in our study, ERT resistance rate was the highest and MEM resistance rate was the lowest in carbapenems resistant strains. MBL production in CRKP isolates was investigated by E-test method. MBL production was not detected in 60 (96.8%) of the isolates. MBL genes were not detected in any strains by multiplex PCR method. We think that the reason for the presence of MBL positivity in the two isolates by e-test may be false positivity due to the possible increase of the outer membrane permeability of the EDTA used in the test [30]. It has been reported that the sensitivity of MHT to investigate carbapenemase enzyme varies between 95-100% [31]. Although this test cannot differentiate between KPC, MBL, OXA enzymes, it is an easy and valuable test used to determine carbapenemase activity [13, 32). Sahin et al. [20] reported a significant relationship between MHT results and OXA-48 positivity (p=0.004). Raghunathan et al. [33] found a 96% correlation between PCR positivity and MHT positivity in Enterobacteriaceae strains. In our study, 57 (91.9%) of 62 *K. pneumoniae* isolates were found to be positive with MHT. The blaOXA-48 gene was also detected in these MHT positive isolates. Five MHT negative isolates were negative for blaOXA-48 gene. This showed that the PCR results were 100% compatible with MHT. Because of the frequent detection of blaOXA-48 gene in the Enterobacteriaceae family in our country [34, 35], we believe that MHT may be useful in determining carbapenem resistance when molecular studies are not possible. Resistance development in microorganisms that cause nosocomial infections is associated with length of hospital stay, follow-up in ICU, advanced age, invasive intervention, immunosuppressive therapy and antibiotic use [12]. Klebsiella strains have been reported to have high levels of carbapenem resistance due to OXA enzymes, as well as resistance to aminoglycosides, quinolones and broad-spectrum cephalosporins. In our study, CRKP isolates were found to be highly resistant to quinolone and aminoglycosides in accordance with the literature [36]. Tigecyclin was found to be the most effective antibiotic.

As a conclusion blaOXA-48 genes responsible for oxacillinase production can be transferred horizontally between bacteria. Thus, a non-clonal spread can occur. Such spread can be considered in terms of the choice of infection control measures to be taken. Determination of carbapenem resistance in *K. pneumoniae* isolates is important in terms of selection of appropriate antimicrobial treatment and taking necessary infection control measures in outbreaks.

Conflict of interest: No conflict of interest was declared by the authors.

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