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NEÜ YAYINEVİ

Phenotypic and Genotypic Identification of Carbapenem-Resistant *Klebsiella Pneumoniae* and Determination of Antibiotic Susceptibility

Karbapenem Dirençli Klebsiella Pneumoniae'nin Fenotipik ve Genotipik Tanımlanması ve Antibiyotik Duyarlılığının Belirlenmesi

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Öz

Amaç: *K. pneumoniae* izolatlarında karbapenemaz genlerinin tanımlanması amaçlanmıştır.
Hastalar ve Yöntem: Bu çalışmaya Temmuz 2016-Aralık 2017 tarihleri arasında örneklerden izole edilen çok ilaca dirençli 95 *K. pneumoniae* suşu dahil edilmiştir. Meropenem, tigesiklin ve kolistinin MIC değerleri Vitek 2, Etest ve Sıvı Mikrodilüsyon Yöntemleri (SMD) ile belirlenmiştir. Kategorik uyum (KU), Çok Büyük Hata (ÇBH) ve Büyük Hata (BH) oranları hesaplanarak değerlendirilmiştir. blaOXA-48, blaOXA-181, blaNDM-1, blaVIM, blaIMP ve mcr-1 kolistin direnç geni araştırılmıştır.
Bulgular: SMD, Vitek 2 ve Etest ile izolatlarda belirlenen meropenem direnci sırasıyla; %70,5, %87,4, %81,1 olarak tespit edilmiştir. SMD yöntemine göre KU, ÇBH ve BH oranları Vitek 2 ve Etest için sırasıyla %69,5, %4,8 ve %20; %70,5, %1,5 ve %0 olarak belirlenmiştir. İzolatlarımızda en yüksek duyarlılık tigesikline karşı tespit edilmiştir. Tigesiklin için KU oranları Vitek 2 ve Etest ile %70,5 ve %95,8 olarak bulunmuştur. Vitek 2 tarafından belirlenen BH oranı, kabul edilebilir %7,6 (<%3) sınırının üzerinde olarak saptanmıştır. Kolistin direnci SMD ile %48,4 olarak tespit edilmiştir. Kolistinin belirlenen KU; ÇBH; BH oranları Vitek 2/Etest için sırasıyla %86,3/%72,6; %17,4/%50; %10,2/%6,1 olarak bulunmuştur. Çalışmamıza dâhil ettiğimiz izolatlarda ağırlıklı olarak blaOXA-48 (%93,7) saptanmıştır. blaOXA-48, izolatların 56'sında (%59) tek başına, 33 izolatta (%34,74) ise blaOXA-181 genleriyle birlikte tespit edilmiştir. İzole OXA-48 pozitif suşlarda, OXA-181 ile birliktelik gösteren izolatlara göre daha düşük kolistin MİK seviyelerine rastlanmıştır. İzolatların %3,2 'sinde blaNDM-1 geni tespit edilmiştir. PZR ile karbapenemaz kombine disk sonuçları izolatların %91,3'ünde uyumlu olarak bulunmuştur.
Sonuç: OXA-48 direnç geni bölgemizde yaygınlığını korumaktadır. Bunun yanında blaOXA-48-benzeri gen bölgelerinin gerçek prevalansının ve direnç dinamiklerinin kapsamlı olarak ortaya konması, ulusal karbapenemaz sürveyans politikalarının gelişimine katkı sağlayacaktır.

Anahtar Kelimeler: Karbapenemaz, *K. pneumoniae*, OXA-48

Abstract

Aim: Characterization of carbapenemase genes in *K. pneumoniae* isolates was aimed.
Patients and Methods: In this study, 95 multi-drug resistant *K. pneumoniae* from samples between July 2016 and December 2017, were included. The MIC of meropenem, tigecycline and colistin were determined by Vitek 2, Etest and Broth Microdilution Methods (BMD). The Categorical agreement (CA), Very Major Error (VME) and Major Error (ME) rates were calculated and evaluated. The mcr-1, blaOXA-48, blaOXA-181, blaNDM-1, blaVIM, blaIMP genes were detected.
Results: The meropenem resistance were determined in the isolates by BMD, Vitek 2 and Etest are respectively; as 70.5%, 87.4%, 81.1%. According to the BMD method, the rates of CA, VME and ME were determined by Vitek 2; Etest as 69.5%, 4.8% and 20 %; 70.5%, 1.5 % and 0 % respectively. It was determined that our isolates showed the highest sensitivity to tigecycline. The CA rates were determined by Vitek 2 and Etest for tigecycline as 70.5% and 95.8%. The ME rate determined by Vitek 2 was above the acceptable limit of 7.6%. The colistin resistance was 48.4% via BMD. The CA; VME; ME ratios determined by Vitek 2/Etest were 86.3%/72.6%; 17.4%/50%; 10.2%/6.1% respectively. In the isolates, predominantly blaOXA-48 (93.7%) was detected. blaOXA-48 was detected alone in 56. isolates and together with blaOXA-181 genes in 33 isolates. Lower colistin MIC levels were found in the only OXA-48 positive strains than in isolates together with OXA-181. The blaNDM-1 gene was investigated in 3.2% of the isolates. PCR and carbapenemase combined disc results were found to be compatible in 91.3% of the isolates.
Conclusion: The blaOXA-48 gene region remains prevalent. In addition, comprehensive identification of the real prevalence and resistance dynamics of blaOXA-48-like gene regions will contribute to the development of national carbapenemase surveillance policies.

Keywords: Carbapenemase, *K. pneumoniae*, OXA-48

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INTRODUCTION

Klebsiella pneumoniae, a member of the Enterobacterales family, is found in humans colonizing the skin, nasopharynx, and gastrointestinal flora (1,2). However, the diversity and epidemiology of infections caused by *K. pneumoniae* has changed recently and has become an important nosocomial agent (2,3). *K. pneumoniae* is an opportunistic pathogen that causes a variety of infections, often associated with risk factors such as mechanical ventilation, urinary catheterization and surgical interventions, and prolonged stay in intensive care unit (3). The increasing use of carbapenems in clinical practice has accelerated the emergence of Carbapenem Resistant *K. pneumoniae* (CRKP) worldwide (4-6). The most important well-known mechanism causing carbapenem resistance is carbapenemase enzymes (7). Carbapenemases are beta-lactamases reside belonging to various Ambler classes (A, B, D) that hydrolyze carbapenems (8). Although many of these enzymes have been reported in our country, OXA-48, which was first identified in our country in 2001, is the most common detected carbapenemase (3,9). Because of the ability of OXA-48 producing strains to transmit resistance genes to other bacteria, the prevalence and spread of this resistance pattern is increasing worldwide, particularly in the Mediterranean region. (4). However, there are studies reported from our country in which different enzymes have been identified, particularly metallo beta-lactamases (MBL) such as blaIMP, blaVIM, and blaNDM, and blaKPC, a class A carbapenemase (4,10). Most of the carbapenemase-producing Enterobacterales members are resistant to all agents except tigecycline and polymyxins; Since they pose a serious threat in hospitals, early detection of carbapenem resistance in *K. pneumoniae* will facilitate the rapid spread of these isolates under control (10-11). In microbiology laboratories that do not have the infrastructure and opportunity to show the presence of carbapenemase genes by molecular methods; Although the use of tests related to enzyme hydrolysis is considered phenotypically, it should be taken into account that the specificities and sensitivities differ (10-12). On the other hand, due to the regional variability of carbapenemase species and prevalence; regional surveillance has gained importance with the molecular characterization of these isolates and epidemic studies (4-6,10-11).

Since the options are limited in the treatment of infections with CRKP, it is important to accurately determine the susceptibility tests of last-resort antibiotics such as tigecycline and colistin. Available commercial automated systems and gradient tests greatly facilitate routine routine laboratory operation. However, error rates above the acceptable limits and categorical incompatibilities regarding these methods reduce the response to treatment (11-13).

The aim of this study was to demonstrate the presence of carbapenemase in CRKP strains isolated from various clinical samples by phenotypic and genotypic methods, to screen for plasmid-mediated colistin resistance gene mcr-1 and to evaluate susceptibility to some antibiotics.

MATERIALS AND METHODS

This study was carried out with the approval of the University of Necmettin Erbakan Non-Pharmaceutical and Medical Device Research Ethics Committee (Date: 17.06.2016 and Decision No: 2016/618).

Collecting Bacterial Isolates

In this study, 95 *K. pneumoniae* isolates, which were found to be resistant to at least one carbapenem, isolated from clinical samples (blood n=68, bronchoalveolar lavage n=13, urine n=4, wound n=3, drainage n=3, endotracheal aspirate n=3and catheter n=1) sent to the central microbiology laboratory of Necmettin Erbakan University Faculty of Medicine Hospital from the clinics and intensive care units between July 2016 and December 2017, were included. The strain isolated for the first time was included in the study only once from each patient.

2.2. Identification of Bacterial Strains and Antibiotic

Susceptibility Tests

Vitek-2 automated system (bioMérieux, France) was used to identify bacteria and detect antibiotic susceptibility. Strains were confirmed at species level simultaneously with the MALDI-TOF MS (bioMérieux, France) system. Meropenem (0.002-32 mg/L), tigecycline (0.016-256 mg/L), fosfomycin (0.016-226 mg/L), and colistin (0.016- 256 mg/L) (HiMedia, Mumbai, India) Ezy MIC™ commercial The minimum inhibitory concentration values of these antibiotics were determined by running the strip tests in accordance with the manufacturer's recommendations. For the in house broth microdilution (BMD) antibiotic susceptibility test, powdered active substances (Sigma-Aldirich, USA) of meropenem, tigecycline and colistin antimicrobials were dissolved in accordance with CLSI (standard M100-27; CLSI 2017) (12) document and stock solutions were prepared. The stock solutions were serially diluted (32-0.032 mg/L) in cation-adjusted Mueller-Hinton

broth (Oxoid Ltd., Basingstoke, UK) prepared daily in 96 microplates. After the suspension of all isolates was prepared with a turbidity of 0.5 Mc Farland standard, it was added to the microdilution plates at a final bacterial concentration of 5X105 cfu/ml. The microplates were incubated for 18-24 hours at 35±2 °C. The meropenem and colistin were evaluated according to EUCAST clinical breakpoints (14,15). The tigecycline was interpreted by US Food and Drug Administration (FDA) criteria (Sensitive ≤2, Intermediate sensitive= 4, and Resistant≥8 mg/L) (16,17). In the study, *K. pneumoniae* NCTC 13443 (NDM-1), *E. coli* NCTC 13846 (mcr-1) isolates were positive control; *E. coli* ATCC 25922 was used as negative control. The evaluation was performed using the double disc synergy method (DDST) and E test methods to confirm the ESBL producing strains

Investigation of Carbapenemase Activity Phenotypically

Isolate suspensions with 0.5 McFarland turbidity were prepared for carbapenemase typing in carbapenem resistant isolates. Inoculation was made on Mueller Hinton Agar (MHA) medium (BioMérieux, France) with the help of a sterile swab. Commercial discs of “MASTDISCSTM ID carbapenemase” were placed in the medium using the combined disc method. The isolates were evaluated after 18-24 hours of incubation in accordance with the manufacturer's recommendations.

Polymerase Chain Reaction

DNA extraction from bacterial suspensions for molecular assay was applied with the “GF-1 Bacterial DNA Extraction Kit” (Vivantis brand, Vivantis company, Malaysia). Purified DNA samples of all samples were prepared in house in LightCycler® system (Roche Applied Science, Switzerland) in accordance with the manufacturer's instructions, using the consensus (18-20) primer sequences in Table 1. Real-time PCR method was studied and proliferation curves were evaluated. PCR protocol first denaturation 10 min at 95ºC; denaturation was carried out at 95ºC for 20 seconds, primer bonding at 60ºC for 20 seconds, and elongation at 72ºC for 20 seconds as 40 cycles.

Klebsiella pneumoniae NCTC 13442 (blaOXA-48), *K. pneumoniae* NCTC 13438 *K. pneumoniae* Carbapenemase (blaKPC), *K. pneumoniae* NCTC 13443 Metallo-β-lactamase (blaNDM-1), *E. coli* NCTC 13476 Metallo-β-lactamase (blaIMP), *K. pneumoniae* NCTC 13440 Metallo-β-lactamase (blaVIM) (KWIK-STIK™, MicroBiologics®) and *E. coli* NCTC 13846 (mcr-1) reference strains were used as positive control.

Statistical analysis

ISO 20776-1 standards (21) were used in calculating the categorical agreement (CA), Very Major Errors (VME), Major Errors (ME), and minor error rates of the tests. According to this, a VME is resistant in the reference antibiotic susceptibility test (AST) while giving a sensitive result in the other antibiotic susceptibility method. Major Errors (ME) reference AST is sensitive while the other AST method gives a resistant result; minor error, reference AST is intermediate sensitive; other AST was defined as a sensitive or resistant result.

RESULTS

All strains were found to be MDR. The clinical specimens were obtained from reanimation intensive care unit (n=26), internal intensive care unit (n=15), other medical units (emergency unit, neurosurgery unit, chest diseases unit, thoracic surgery unit, neurology wards (n=9), pediatric intensive wards

Table 1. Primary sequences used in real-time PCR process

Target gen	Primary direction	Primer sequence (5’-3’)	Band size (bp)	Reference
blaOXA-48	OXA-48-F	TGTTTTTGGTGGCATCGAT	177	18
	OXA-48-R	GTAAMRATGCTTGGTTCGC		
blaKPC	KPC-F	TCGCTAAACTCGAACAGG	785	18
	KPC-R	TTACTGCCCGTTGACGCCCAATCC		
blaNDM-1	NDM-F	TTGGCCTTGCTGTCCTTG	82	18
	NDM-R	ACACCAGTGACAATATCACCG		
blaIMP	IMP-F	GAGTGGCTTAATTCTCRATC	120	18
	IMP-R	AACTAYCCAATAYRTAAC		
blaVIM	VIM-F	GTTTGGTCGCATATCGCAAC	382	18
	VIM-R	AATGCGCAGCACCAGGATAG		
blamcr-1	mcr-F	CGGTCAGTCCGTTTGTTC	35-343	19
	mcr-R	CTTGGTCGGTCTGTAGGG		
blaOXA-181	OXA-181-F	ATGCGTGTATTAGCCTTATCG	798	20
	OXA-181-R	AACTACAAGCGCATCGAGCA		

Table 2. Vitek 2 Automated System antibiotic susceptibility results of *K. pneumoniae* isolates (n=95)

Antibiotics	Numbers of Resistant isolates (%)
Ampicillin	95 (100)
Amoxicillin clavulanic acid	95 (100)
Amikacin	72 (75.8)
Ceftazidime	91 (95.8)
Ciprofloxacin	83 (87.4)
Ceftriaxone	95 (100)
Colistin	43 (45.3)
Cefuroxime	95 (100)
Cefuroxime-axetil	95 (100)
Cefazolin	95 (100)
Ertapenem	90 (94.7)
Cefepim	94 (99)
Cefoxitin	89 (93.7)
Gentamicin	87 (91.6)
Meropenem	83 (87.4)
Trimethoprim-sulfamethoxazole	71 (74.7)
Tigecycline	0 (0)
Piperacillin-Tazobactam	87 (91.6)

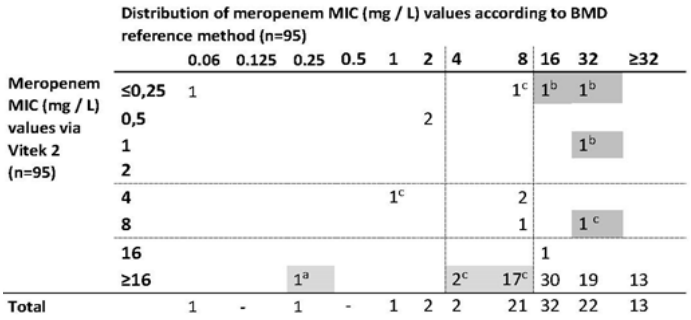

Figure 1.1. Comparison of MIC Values of *K. pneumoniae* strains determined by reference BMD method and Vitek 2 Automated System

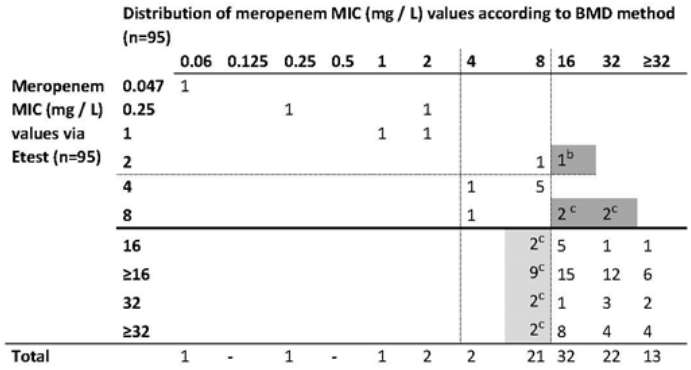

Figure 1.2. Comparison of reference BMD method and Etest Meropenem MIC Values of *K.pneumoniae* strains
a Numbers of isolates with major error,
b Numbers of isolates with very major error,
c Numbers of isolates with minor error
(Meropenem MIC was interpreted as <=2mg /L sensitive and >8mg/L resistant according to EUCAST clinical breakpoints www.eucast.org)

(n=14), internal wards (n=15), pediatric wards (n=15), and surgical wards (n=15) were included. These samples were blood (71.6%), bronchoalveolar lavage (13.7%), urine (4.2%), wound (3.16%), drainage (3.16%), endotracheal aspirate (3.16%) and catheter (1.1%), respectively. Multidrug resistant (MDR) was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories. All tested isolates were multidrug resistant. The Vitek 2 results of the isolates are given in Table II. In addition that the AST of meropenem, tigecycline and colistin were also tested by Etest and BMD methods. The MIC distributions of the isolates according to the BMD method are given (Table III.). Of all isolates, 74 (77.9%) were isolated from patients in intensive care units. The MIC50 and MIC90 value were given in Table IV. Meropenem resistance was detected with BMD in 67 (70.5%) of 95 patients. The numbers of these resistant isolates for the Vitek 2 automated system and the Etest, respectively; 83 (87.4%) and 77 (81.1%) were determined (Figure 1.1 and Figure 1.2). According to Meropenem AST; VME, ME and minor error ratios of the Vitek 2 system are determined respectively; as 4.8%, 20% and 21.1%. In reference to Meropenem BMD; the VME, ME and minor error ratios of the Etest were 1.5%, 0%, 20%. The categorical agreements were observed for meropenem with Vitek 2 and Etest 69.5% and 70.5%, respectively.

Of the isolates, it was not detected resistant to

Table 3. Distribution of Isolates by MIC Values of Meropem, Tigecycline and Colistin Detected by Broth Microdilution Method

Type of antibiotic	Distribution of Isolates by MIC Values (mg/L)										
	0,064	0,125	0,25	0,5	1	2	4	8	16	32	>=32
Meropenem (n=95)	1	-	1	-	1	2	2	21	32	22	13
Tigecycline (n=95)	-	10	18	40	19	5	3	-	-	-	-
Colistin (n=95)	4	9	16	14	3	3	3	5	12	19	7

Table 4. Comparative Activity of Meropenem, Tigecycline, and Colistin Against *K. pneumoniae* isolates in Patients with and without Intensive Care Unit

	ICU			Non-ICU	
	n	MIC50	MIC90	n	MIC50
Total isolate	74			21	
Meropenem		16	>=32		16
Tigecycline		0,5	1		0,5
Colistin		4	32		0,5

ICU: intensive care unit
MIC: Minimum inhibitor concentration, n:number

tigecycline, only 3.2% of them were "intermediate catagory". According to tigecycline BMD, the detected ME and minor errors rates by Vitek 2 were 7.6 % and 22.1 %, respectively. (Figure 2.1.). VME and ME were not detected by Tigecycline Etest and the minor errors rate was 4.2 % (Figure 2. 2.). The categorical agreements were observed for tigecycline with Vitek 2 and Etest as 70.5% and 95.8%, respectively.

In this study, colistin resistance was found to be 48.4% with BMD. The VME rates were determined as 17.4% and 50% for Vitek 2 and Etest, respectively. The ME rates of Vitek 2 and Etest were found to be 10.2 % and 6.1 %, respectively. The CA between the BMD-Vitek 2 system and BMD-Etest methods for colistin was determined as 86.3% and 72.6%, respectively. (Figure 3.1 ve Figure 3.2).

The resistance rates to fosfomycin, which is an alternative for the treatment of CRE infections, was observed as 16.8%. The MIC50 and MIC90 values for fosfomycin were interpreted as 6 mg/L and 24 mg/L, respectively.

Expanded Spectrum Beta-Lactamase (ESBL) production was observed in 20 (21.05%) of the strains by the combined disc method (Oxoid, Thermo Fisher Scientific, Basingstoke, UK) and only 9 (9.47%) isolates were found to be ESBL positive by the gradient test (HiMedia, India).

According to the PCR results, at least one resistance gene was detected in 92 (96.8%) of the isolates, while no carbapenemase resistance gene was detected in 3 of them. The most common carbapenemase detected in isolates was OXA-48 (93.7%). blaOXA-48 was shown alone in 56 (59%) isolates and together with blaOXA-181 genes in 33 (34.7%) isolates. Although the MIC50 values of colistin were 0.5mg/L in OXA-48 positive isolates (n=56), the colistin MIC50 was determined as 16mg/L coexistence of OXA-48+OXA-181 (n=33). The blaNDM-1 gene was detected in 3 (3.2%) of the isolates. The colistin plasmid-mediated resistance gene (mcr-1), IMP, VIM and KPC carbapenemase resistance genes were also not detected in any of the isolates. In this study, the presence of carbapenemase enzyme in 88 isolates and ESBL+porin loss in 3 isolates were determined by the phenotypic carbapenemase combination disc test. No carbapenemase enzymes were detected with combined discs in 4 isolates. However, the presence of OXA-48 gene was determined by PCR in 2 of these isolates. In 2 of them, no carbapenemase

Distribution of tigecycline MIC (mg / L) values according to BMD method (n=95)

Tigecycline MIC (mg / L) values via Vitek 2 (n=95)	0,06	0,125	0,25	0,5	1	2	4	8	16	32	≥32
≤0,5		2	2	9	3		1[c]				
1		2	3	12	4	1	1[c]				
2		3	8	12	5	1	1[c]				
4		3[c]	4[c]	2[c]	6[c]	3[c]					
≥8			1[a]	5[a]	1[a]						
Total	-	10	18	40	19	5	3				

Figure 2.1. Comparison of tigecycline MIC Values of *K.pneumoniae* strains determined by BMD method and Vitek 2

Distribution of tigecycline MIC (mg / L) values according to BMD method (n=95)

Tigecycline MIC (mg / L) values via Etest (n=95)	0,06	0,125	0,25	0,5	1	2	4	8	16	32	≥32
0,12											
5				1							
0,25			1								
0,5		1	2	8	4						
0,75		4	4	13	5						
1		3	2	9	2	2					
1,5		2	5	7	5	2					
2			4	2	2	1	1[c]				
4					1[c]						
≥8							2[c]				
Total	-	10	18	40	19	5	3				

Figure 2.2. Comparison of tigecycline MIC Values of *K.pneumoniae* strains determined by reference BMD method and Etest

a Numbers of isolates with major error, b Numbers of isolates with very major error, c Numbers of isolates with minor error

(Tigecycline MIC was interpreted accordid to FDA criteria as Sensitive ≤ 2 mg /L, Intermediate sensitive = 4 mg/L, and Resistant ≥ 8 mg/L)

Distribution of colistin MIC (mg / L) values according to BMD method (n=95)

		0,06	0,125	0,25	0,5	1	2	4	8	16	32	≥ 32
Colistin MIC (mg / L) values via Vitek 2 (n=95)	≤ 0,5	4	8	15	12	3	1	1[b]		1[b]	1[b]	1[b]
	1						1	1[b]				
	2									1[b]	2[b]	
	4							1	1	1		
	8				1[a]		1[a]		1	3	1	
	16											1
	≥ 16		1[a]	1[a]	1[a]			3	6	15	5	
Total		4	9	16	14	3	3	3	5	12	19	7

Figure 3.1. Comparison of colistin MIC Values of *K.pneumoniae* strains determined by reference BMD method and Vitek 2 system

Distribution of colistin MIC (mg / L) values according to BMD method (n=95)

		0,06	0,125	0,25	0,5	1	2	4	8	16	32	≥32
Colistin MIC (mg / L) values via Etest (n=95)	0,064	1										
	0,25			1								
	0,5			2	1							
	0,75		1	3	1							
	1	1	4	4	2	2			1[b]	1[b]		2[b]
	1,5	2	3	4	10	1	2	2[b]	3[b]	5[b]	6[b]	
	2			1						2[b]	1[b]	
	4		1[a]						1		2	3
	8			1[a]						1	5	2
	16						1[a]	1		2	3	
	≥ 32									1	2	
Total		4	9	16	14	3	3	3	5	12	19	7

Figure 3.2. Comparison of colistin MIC Values of *K.pneumoniae* strains determined by reference BMD method and Etest
a Numbers of isolates with major error, b Numbers of isolates with very major error, c Numbers of isolates with minor error
(Colistin MIC was interpreted <=2mg/L sensitive, >2mg/L resistant according to
EUCAST clinical break points www.eucast.org)

gene was detected by PCR. In combination tests, it was determined that 83 of the carbapenemase types carried OXA-48, 2 had KPC and 3 had MBL resistance gene.

Comparing the carbapenemase combined disc and PCR results, the agreement was found at the rate of 91.3% (n=84). OXA-48 enzyme was also detected with carbapenemase combined disc in 82 of 89 isolates whose presence of OXA-48 gene was detected by PCR, and the sensitivity of the combined discs for OXA-48 enzyme was found to be 92.1%. Combination disc tests were detected as 'metallo-betalactamase enzyme positive' in 2 of 3 isolates with positive NDM-1 gene by PCR. In one isolate, the OXA-48 enzyme was positive. The sensitivity of

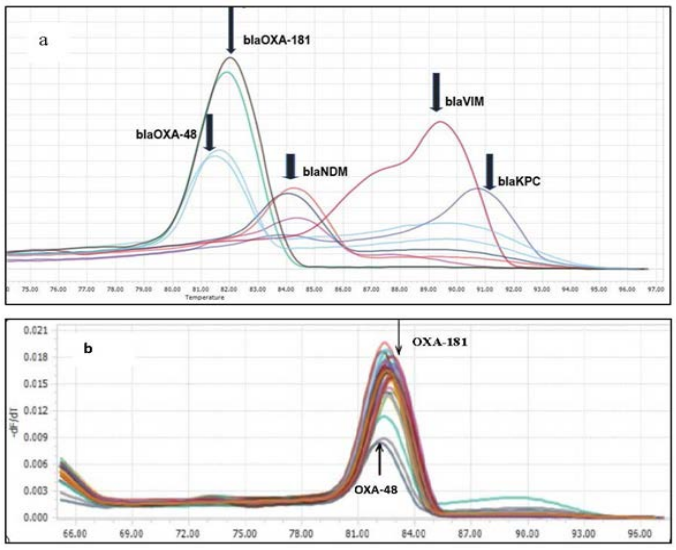

Figure 4a. Evaluation of melting curves by in-house real-time PCR. The detected Tm values of blaOXA-48 (81.65 °C), blaOXA-181 (82.04 °C), blaNDM (84.07 °C), blaVIM (89.410C) and blaKPC (90.69 °C) primers are shown. **Figure 4b.** There are fluctuations that deviate from the clustering. The Tm degree of OXA-181 variants was higher than that of OXA-48 containing strains.

combined discs in detecting the presence of NDM-1 was 66.7%. The real PCR melting temperature (Tm) is specific for each product, so the method can be used reliably when two unknown DNA sequences are compared with this technique (22). The specific Tm grades detected in the primers are given in Figure 4a. In our study, the Tm degree of the OXA-48 variant OXA-181 was found to be higher than the strains containing the OXA-48 gene in PCR melting curve analysis (Figure 4a-b).

DISCUSSION

In this study, 77.9% of all strains were isolated from patients in ICUs where colonization and infections due to resistant pathogens are common. OXA-48 carbapenemase was detected most frequently. However, OXA-181 was also determinated as remarkable. High colistin MIC value detected in the coexistence of OXA-48 and OXA-181 were noteworthy.

Carbapenem susceptibility may vary in isolates containing carbapenemase. Although the isolate is a carbapenemase producer, it can be detected within the carbapenem sensitivity limits. In this study, isolates with reduced carbapenem susceptibility with meropenem MIC>0.12mg/L were included. In

one isolate meropenem and ertapenem MICs were 0.06mg/L. However, it was included in the study because of the detected high level the colistin MIC (32mg/L). Only by PCR could it be determined that it carried the blaOXA-48 carbapenemase gene. It was thought that the presence of carbapenemase could not be detected completely phenotypical methods due to the weak carbapenemase expression of the related strain. There are inadequate data on the response of patients infected with such a strain to carbapenem therapy. It should be kept in mind that in our country, where OXA-48 type carbapenemase production is common, such isolates may be encountered in patients who do not respond to treatment (23).

Broth microdilution is a reference AST methods for many antibiotic groups. In addition, it is required intensive labor and experience. Thus, alternative commercial methods are being researched. Morover there is an increasing need for studies comparing performances between methods in order to ensure the necessary standardization.

In this study, the susceptibilities of meropenem, tigecycline and colistin were tested by Vitek 2, Etest and BMD methods. The detected meropenem resistance by BMD, Vitek 2 and Etest was 70.5%, 87.4% and 81.1%, respectively. The determined CA rates were below the acceptable limit, 69.5% and 70.5% for Vitek 2 and Etest, respectively. The VME ratios for meropenem were detected by Vitek 2 and Etest as 4.8%, 1.5%, respectively. The ME rates were observed almost 20% by Vitek 2. None major error was not detected via Etest. Haldorsen et al. (24) found meropenem MICs via BMD for 50%, 61% and 25% higher than >2 fold dilution levels in Vitek 2 for class A, B, D carbapenemase producing bacteria, respectively. In the study, the rates of CA, ME and minor error between BMD-Vitek2 methods were determined as 56%, 26% and 18%, respectively. The CA between BMD-Etest was 73%; ME and minor error rates were reported as 7% and 20%, respectively. Similar to our results, high ME rates detected with Vitek 2 is a major problem, that limits the use of meropenem, which is still an important agent in combination therapies in CRE infections (25).

Tigecycline shows promise in the treatment of infections caused by MDR gram-negative bacteria, including CRKP (26). Previous studies have shown that tigecycline results can be affected by the method used (27). In addition, the lack of clinical breakpoints and a recommended method for Enterobacterales including CLSI and the limited number of data from literature studies reveal the need for accurate and reliable tigecycline AST (17, 26). In the study of Yin et al. (27), in which they compared the effectiveness of tigecycline on 372 CRKP strains with disc diffusion, modified disc diffusion, Vitek 2 system, Etest methods and BMD method. The CA between these methods were found 78.5%, 96.5%, 69.9% and 96.7%, respectively. These investigators found that 96.8% of strains with BMD were susceptible to tigecycline. Li et al. (17) reported that the CA between agar dilution method, disk diffusion method, Etest, MicroScan, Vitek 2 Compact, BD Phoenix 100 methods and SMD method were determined as 96%, 53%, 88%, 92%, 74%, and 93%, respectively. The rate of ME detected only with Vitek 2 (9%) was above the acceptable limit of 3%. Etest has been reported to have a higher BMD agreement compared to Vitek 2.

In this study, the tigecycline resistance was not detected, similar to the study of Yin et al. (27). Tigecycline was determined to be the most effective antibiotic against MDR *K. pneumoniae* among the antibiotics in our study. The CA rates for tigecycline BMD-Vitek 2 and BMD-Etest were determined as 70.5% and 95.8%, respectively. These results are in agreement with the data of Yin et al. (27). The CA rate (95.8%) determined for Etest was higher than the study of Li et al. (17) (88%). In our study, VME was not found in any of the tigecycline AST methods. Consistent with previous studies, the rate of ME via Vitek 2 was determined to be 7.6% above the acceptable limit (<3%). This result suggests that the isolates found resistant with Vitek 2 should be confirmed with an alternative method. In line with our data, it was determined that tigecycline Etest had higher compatibility with BMD and lower error rates compared to Vitek 2.

In the study evaluating Sensitire, Vitek 2, Etest, MicroScan and BMD methods on 76 multi-resistant Enterobacterales, 21 of which were mcr-1 positive, by Chew et al. (28). It has been reported that colistin MIC values determined by Sensititre and Vitek 2 have a higher agreement with BMD compared to Etest. Despite the high CA detected in Vitek2-BMD and Etest-BMD, it was reported that the VME rates were also found to be above acceptable limits (12-36%). In our study, colistin resistance was 48.4% with BMD. The CA, VME, ME ratios for Vitek 2/Etest were determined as 86.3%/72.6%, 17.4%/50%, 10.2%/6.1%, respectively. Although Vitek 2 showed higher CA ratio than Etest, the detected VME rates for both tests were highly above the acceptable limit.

In our isolates, resistance to fosfomycin was found to be 16.8%, For this reason, it should be considered as one of the alternative options in combination protocols with other drugs in the treatment of CRE.

Although it is known that various carbapenemases are encountered in different parts of the world, it is known that the blaOXA-48 gene is mainly responsible for resistance in our country. This enzyme (4), which was reported in our country for the first time in the world, was predominantly detected (93.7%) in our study. blaOXA-48 was detected alone in 56 (59%) isolates and together with blaOXA-181 genes in 33 (34.74%) isolates. blaOXA-181 is a variant carbapenemase strain derived from blaOXA-48 with similar carbapenemase activity, first identified in Enterobacterales isolates in India. Colistin resistance in *K. pneumoniae* isolates producing blaOXA-181 has been reported worldwide (29-31). It is currently known that inactivation of mgrB gene is associated with colistin resistance and is the most common mechanism responsible for polymyxin resistance in *K.pneumoniae*. It has been reported in previous studies that the mobile genetic element, which becomes functional with the insertion of the blaOXA-181 carbapenemase gene, causes colistin resistance by inactivating the mgrB gene (29). This study finding were found compatible with previous data. Although colistin MIC values were found as MIC50:0.5mg/L only in OXA-48 positive isolates (n=56); in isolates with OXA-48+OXA-181 association (n=33), colistin MIC50 was determined as 16mg/L. This data suggests that revealing the resistance genetics carried by the blaOXA-181 gene in detail will be useful in elucidating the factors that facilitate the development of resistance to existing antibiotics. In this study, the blaNDM-1 gene was detected in 3.2% of the isolates. In a multicenter study reported from our country, it was stated that the predominant carbapenemase in the country was blaOXA-48, but the increase in blaNDM-1 was remarkable (10). blaIMP, blaVIM and blaKPC carbapenemase resistance genes were not detected in any of the isolates. The mcr-1 could not be determined. Moreover, there is also a need to investigate other mechanisms and genes in colistin-resistant strains. The agreement between our carbapenemase combined disc and PCR results was found in 91.3% of the isolates. The sensitivity of the combined discs in detecting the presence of blaOXA-48 and blaNDM-1 was as 92.1% and 66.7% respectively. The sensitivities in phenotypic methods may differ according to the type of carbapenemase carried by the predominantly isolates in the investigated region. Considering the relatively low sensitivity of the combined disk test for the blaNDM-1 enzyme and the low number of samples. It is highly recommended that optimized studies involving more bacterial numbers and species are needed.

Parlak et al. (32) found the sensitivity of the temocillin disc to detect the presence of OXA-48 as 88%, and the specificity as 89%. In the present study, temocillin disc and Etest sensitivities were 95.6% / 94.4% respectively. The specificities were determined as 66.7% for both methods. Temocillin Etest and discs are considered to be strong indicators for detecting the presence of OXA-48, but the tests are not sufficient to exclude it alone. In addition, since our sensitivity and specificity results with temosilin disc and Etest are similar; cost-effective disk method can be recommended.

According to the data obtained from our study, blaOXA-48 producing isolates maintain their frequency in our region. Among the OXA-48 variants, the prevalence of the blaOXA-181 gene, its association with the bla-OXA-48 gene, and the high colistin MIC values detected in these isolates are noteworthy. Although the sensitivity of phenotypic tests varies according to the carbapenase type, our results indicated that the OXA-48 producing isolates are still dominant in our country, these tests can be used for screening purposes in routine laboratories but it should be supported by molecular methods.

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