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Original Article

Investigation of Carbapenem resistance mechanisms in *Klebsiella pneumoniae* by using phenotypic tests and a molecular assay

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Abstract

Introduction: The aim of this study was to investigate the presence of carbapenemase production and carbapenem resistance mechanisms in 47 carbapenem resistant *Klebsiella pneumoniae* isolates by phenotypic confirmatory tests and molecular assay.

Methodology: Carbapenem resistance genes KPC, OXA-48 and NDM were investigated with the BD MAX CRE assay kit in the BD MAX real time PCR instrument. Modified Hodge test, MBL gradient strip test, D70C Carbapenemase Detection Set, Temocillin gradient strip test methods were used as phenotypic confirmatory tests. Clonal relationship between study isolates was investigated with pulsed-field gel electrophoresis.

Results: Analysis with BD MAX CRE assay revealed OXA-48 positivity in 17 (36%) strains, NDM positivity in 6 (13%) strains and coexistence of OXA-48 + NDM positivity in 8 (17%) strains. In 16 (34%) strains, none of the KPC, OXA-48 and NDM genes were detected. While MHT was the most sensitive phenotypic confirmatory test, D70C disc set had not been considered as a useful tool to assist the search for carbapenemase production. Temocillin gradient test alone could not be considered as sufficient to detect the presence of OXA-48. PFGE analyses revealed that 23 of 31 carbapenemase producing strains were in three major PFGE genotypes (A, B and C).

Conclusions: This study revealed that carbapenem resistance observed in *K. pneumoniae* isolates was mainly due to OXA-48 and NDM genes and the increase of carbapenem resistance among *K. pneumoniae* strains in our hospital was due to the interhospital spread of especially 3 epidemic clones.

Key words: carbapenemase*; Klebsiella pneumoniae;* KPC; NDM; OXA-48.

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Introduction

Klebsiella pneumoniae is a bacterium that can colonize the human intestinal tract, skin and nasopharynx flora. While it was well known as a cause of community-acquired bacterial pneumonia, the diversity and epidemiology of infections due to *K. pneumoniae* have dramatically changed at the beginning of 1970's and this bacterium became a major nosocomial infectious agent [1]. Their ability to spread in the hospital environment and widespread use of antimicrobial therapy promoted emergence of increased aquired antibiotic resistance among *K. pneumoniae* [1,2]. In the 1970s and 1980s aminoglycoside resistance encoded by plasmids was common. Subsequently, expanded spectrum β-lactamases (ESBL) have emerged. This has been followed by resistance to fluoroquinolones and subsequently by resistance to carbapenems [2].

Carbapenem resistance can be caused by the presence of reduced outer membrane permeability, overexpression of efflux pumps or carbapenemases. Carbapenemases are grouped as A, B and D according to the Ambler classification. Clinically important carbapenemases are *K. pneumoniae* carbapenemase (KPC) in class A; New Delhi Metallo β-lactamase (NDM), Active on imipenem (IMP) and Verona Integron-encoded Metallo β-lactamase (VIM) in class B; and oxacillinase (OXA) type enzymes in class D [3]. It has been reported that KPC enzymes have spread across countries and continents, and their distribution varies by geographical location. KPC was first reported in 1996 from USA, and within a few years it has caused epidemics [4]. OXA-48 was first reported from Turkey in 2003 and has been extensively identified in this country [5]. While VIM and IMP are the most frequently identified metallo β-lactamases worldwide,

NDM enzymes are the most recently recognized carbapenemases among Enterobacteriaceae [3,6].

Infections with carbapenemase producing strains cause prolonged hospitalization, high mortality and morbidity. Furthermore, carbapenemases are usually associated with many other resistance determinants, giving rise to multidrug resistance. So, if such strains are detected, infection control measures should be taken to prevent the horizontal spread of resistance genes [7-9].

It has been reported that if reduced susceptibility to carbapenems is detected in routine susceptibility tests, phenotypic methods should be applied to detect carbapenemases. modified Hodge test (MHT), combination disc methods (Mast discs, Rosco tablets), MBL gradient strip test and temocillin gradient strip tests are among the phenotypic verification methods used for the detection of carbapenemases [10]. Many clinical laboratories use in-house PCR-based methods to identify carbapenemase genes. There are also PCR and hybridization-based kits that determine the types of carbapenemase gene in the market [6]. However, there is no single method that has been put forward as ideal for the detection of all carbapenem resistance mechanisms.

The aim of this study was to investigate the presence of carbapenemases and resistance mechanisms by phenotypic confirmatory tests and molecular assay in carbapenem resistant *K. pneumoniae*, which were isolated in our hospital since 2012. Clonal relationship between carbapenem resistant isolates and the sensitivities of carbapenem resistant strains to other antibiotics were also determined.

Methodology

This study included 47 *K. pneumoniae* strains (one isolate from each patient was evaluated) reported as resistant to one or more of the carbapenems by using the VITEK2 (BioMérieux, Inc., Marcy-l'Etoile, France) automated identification system in the Medical Microbiology Laboratory of Suleyman Demirel University Medical Faculty (Isparta, Turkey) between August 2012 and May 2014. Pulsed-field gel electrophoresis (PFGE) was performed to evaluate the clonal relationship between the study isolates.

Modified Hodge test (MHT) was performed as a phenotypic confirmatory test to investigate the presence of carbapenemase production and evaluated in accordance with the CLSI recommendations [11]. The MBL gradient strip test (MBL-GST) was performed using test strips with meropenem 0.125-8 mg/L on one side and meropenem + EDTA 0.032-2 mg/L on the other side (Liofilchem, Roseto degli Abruzzi, Italy). MEM/MEM + EDTA MIC ratio ≥ 8 or > 3 $\log^2$ dilution was interpreted as positive for MBL. Temocillin gradient test strips (TEM-GST) (0.064-1024 mg/L, Liofilchem, Roseto degli Abruzzi, Italy) were used for the phenotypic screening of OXA-48 production. Temocillin that confers high-level resistance (MIC > 32) was evaluated as a phenotypic marker to screen OXA-48-like determinants [10].

D70C Carbapenemase Detection Set (D70C) (Mast Diagnostics, Merseyside, UK) consists of four discs: A (10 μg carbapenem disc), B (carbapenem 10 μg + MBL inhibitor disc), C (carbapenem 10 μg + KPC inhibitor disc) and D (carbapenem 10 μg + AmpC inhibitor disc). The inhibition zone of disc A was compared to the inhibition zones of each of discs B, C, and D. If disc B showed a zone difference of ≥ 5 mm from disc A, the organism was recorded as demonstrating MBL activity. If disc C showed a zone difference of ≥ 4 mm from disc A, the organism was recorded as demonstrating KPC activity. If disc C and disc D both showed a zone difference of ≥ 5 mm from disc A, the organism was recorded as demonstrating AmpC activity coupled with porin loss.

D68C Disc Set (D68C) (Mast Diagnostics, Merseyside, UK) was used to evaluate the possibility of carbapenem resistance occuring as a result of porin loss in addition to the presence of AmpC and / or ESBL. This set consists of four discs: A (10 μg cefpodoxime), B (10 μg cefpodoxime + ESBL inhibitor disc), C (10 μg cefpodoxime + AmpC inhibitor disc) and D (10 μg cefpodoxime + ESBL inhibitor + AmpC inhibitor discs). If disc B showed a zone difference of ≥ 5 mm from disc A, and disc D showed a zone difference of ≥ 5 mm from disc C, the organism was recorded as only ESBL producer. If disc D showed a zone difference of ≥ 5 mm from disc B, and disc C showed a zone difference of ≥ 5 mm from disc A, the organism was recorded as only AmpC producer. If disc D showed a zone difference of ≥ 5 mm from disc C, the organism was recorded as ESBL + AmpC producer. It was reported that the absence of inhibition zone around any disc might be due to the fact that tested isolate produced MBL or KPC carbapenemase with ESBL or AmpC. The test result was considered as 'insufficient result' for such isolates. *E. coli* ATCC 25922 was used as negative control, *K. pneumoniae* NCTC 13438 (KPC), *K. pneumoniae* NCTC 13443 (NDM), *E. cloacae* NCTC 13406 (AmpC) and *K. pneumoniae* ATCC 700603 (ESBL) were used as positive controls for phenotypic confirmatory tests.

Table 1. Comparative evaluation of phenotypic test findings with BD Max CRE assay findings of the study strains.

BD MAX (n)	Positive Strain Number (%)			
	MHT	D70C/MBL	MBL - GST	TEM-GST
NDM (6)	6 (100)	2 (33.3)	2 (33.3)	6 (100)
OXA (17)	16 (94.1)	-	4 (23.5)	16 (94.1)
OXA+NDM (8)	7 (87.5)	1 (12.5)	4 (50)	7 (87.5)
Negative (16)	15 (93.8)	0	5 (31.2)	15 (93.8)
Total number (47)	44 (93.6)	3 (6.4)	15 (31.9)	44 (93.6)

The presence of carbapenem resistance genes KPC, OXA-48 and NDM, were investigated with BD MAX CRE assay kit in the BD MAX real time PCR, a fully automated system (Becton Dickinson and Company, USA).

Pulsed field gel electrophoresis method was applied by modifying the PFGE protocol of *Escherichia coli* O157: H7, *Escherichia coli* non-O157 (STEC), Salmonella serotypes, *Shigella sonnei* and *Shigella flexneri* recommended by PulseNet [12,13]. Lambda Ladder PFG Marker (New England Biolabs, UK) was used as the molecular size standard. Band profiles were analyzed both visually, as described previously by Tenover *et al*. [14] and using the GelCompar II (Applied Systems, Belgium) program. Dendograms of PFGE profiles were generated and cluster analysis was performed using the 'Unweighted pair group method with mathematical averaging' method and Dice similarity coefficient.

Results

Analysis with BD MAX CRE assay revealed OXA-48 positivity in 17 (36%) strains, NDM positivity in 6 (13%) strains and coexistence of OXA-48 + NDM positivity in 8 (17%) strains, while none of the KPC, OXA-48 and NDM genes were detected in 16 (34%) strains (Table 1).

MHT was positive among 29 of 31 strains which have been detected as carbapenemase producers with BD Max CRE assay, whereas it was also positive among 15 of 16 strains of which none of the carbapenemase genes have been detected. MBL-GST was positive among 6 of 14 (42.9%) strains which have been detected as MBL producers with molecular assay while it was negative among 24 of 33 (72.7%) strains which were detected as MBL-negative with molecular assay.

Figure 1. The strain (No. 19) which was detected as ESBL and AmpC positive with D68C disc set.

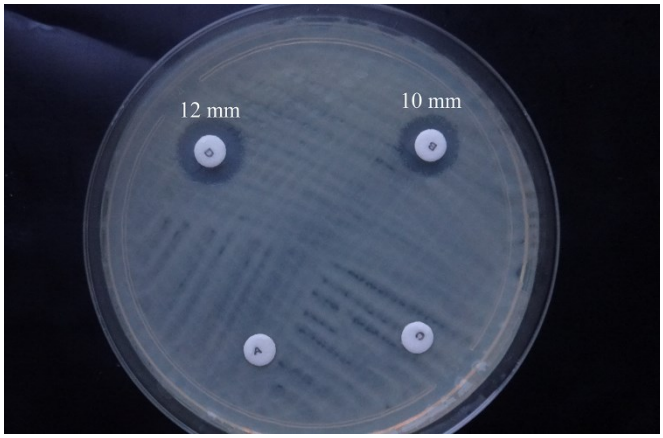


Figure 2. The strain (No. 16) which was detected as MBL producer with D70C disc set.

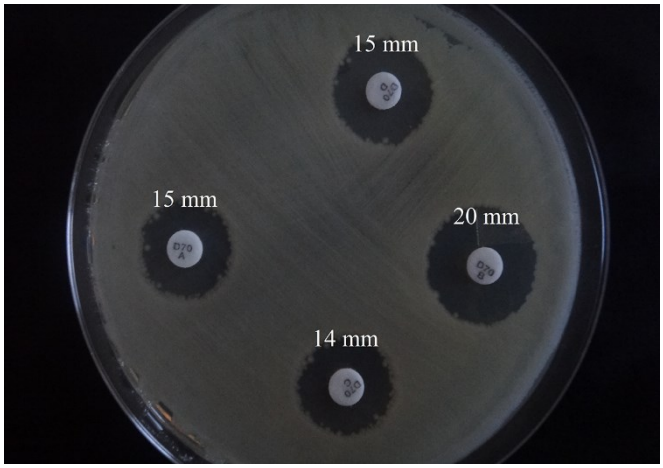


Table 2. The sensitivity, specificity and positive / negative predictive values of the phenotypic methods by taking BD MAX CRE assay as the reference method.

	MHT	D70C/MBL	MBL-GST	TEM
Sensitivity (%)	93.5	21.4	42.9	92
Specificity (%)	6.3	100	72.7	4.5
PPV (%)	66	100	40	52.3
NPV (%)	33.3	75	75	33.3

PPV: Positive predictive value, NPV: Negative predictive value.

Table 3. PFGE types, strain numbers, clinics in which strains are isolated, specimen types, isolation dates and results obtained by genotypic and phenotypic methods.

PFGE Type	Strain No	Clinic	Specimen Type	Date	BD MAX	MHT	D68C	D70C	MBL-GST	TEM-GST
A	12	ICU	Blood	May 2014	OXA	+	IR	-	-	R
	14	IM	Wound	May 2014	OXA	+	IR	-	-	R
	17	Surgery	Urine	Jan 2014	-	+	-	-	-	R
	19	Urology	Urine	Jan 2014	OXA	-	+/+	-	-	S
	20	ICU	Blood	Apr 2014	OXA+NDM	+	IR	-	+	R
	22	ICU	Urine	Oct 2013	OXA	+	IR	-	+	R
	26	Surgery	Blood	May 2014	-	+	IR	-	-	R
	28	ICU	TA	Sep 2013	-	+	IR	-	+	R
	29	IM	Wound	Sep 2013	-	+	IR	-	+	R
	30	ICU	TA	Apr 2014	NDM	+	IR	-	+	R
	31	NS	Urine	Dec 2013	OXA	+	IR	-	-	R
	33	IM	Wound	Mar 2014	OXA+NDM	+	IR	-	+	R
	35	NS	Urine	May 2014	OXA+NDM	+	IR	-	+	R
	36	ICU	TA	Oct 2013	OXA	+	IR	-	+	R
	39	ICU	TA	Oct 2013	-	+	IR	-	+	R
	40	ICU	TA	Nov 2013	-	+	IR	-	+	R
	43	ICU	Blood	Dec 2013	OXA	+	IR	-	+	R
	45	IM	urine	Jul 2013	-	+	IR	-	+	R
	46	ICU	TA	Aug 2013	OXA	+	IR	-	+	R
B	1	NS	Blood	Aug 2013	OXA	+	+/+	-	-	R
	4	NS	TA	Sep 2013	-	+	-/+	-	-	R
	9	ICU	Blood	Dec 2012	-	+	IR	-	-	R
	11	ICU	TA	Oct 2013	-	+	-/+	-	-	R
	16	Surgery	Blood	Apr 2014	NDM	+	IR	-/+	-	R
	24	ICU	TA	Oct 2013	OXA	+	-/+	-	-	R
	27	ICU	Blood	Sep 2012	-	+	-/+	-	-	R
	32	NS	Blood	Dec 2013	OXA	+	-/+	-	-	R
	37	ICU	TA	Mar 2014	OXA	+	+/+	-	-	R
	38	Surgery	Blood	Nov 2013	OXA+NDM	+	IR	-/+	+	R
	42	ICU	Wound	Mar 2014	-	-	-/+	-	-	S
C	2	ICU	Blood	Nov 2013	-	+	IR	-	-	R
	15	ICU	TASP	Sep 2013	OXA	+	IR	-	-	R
	18	PS	Wound	Oct 2013	OXA+NDM	+	IR	IR	-	R
	21	Pediatrics	Urine	Dec 2013	OXA+NDM	+	-/+	-	-	R
	34	IM	TA	Nov 2013	OXA	+	IR	-	-	R
	48	ICU	Blood	Nov 2013	OXA	+	IR	-	-	R
D	3	NS	TA	Aug 2012	-	+	-	-	-	R
	10	IM	Wound	Aug 2012	-	+	IR	-	-	R
	25	ICU	TA	Aug 2012	-	+	-	-	-	R
E	6	NS	Urine	Jan 2014	OXA+NDM	+	-/+	-	-	R
	23	Oncology	Wound	Jan 2014	NDM	+	-/+	-	-	R
F	5	Surgery	Urine	May 2014	OXA	+	IR	-	-	R
G	7	ICU	Blood	Nov 2013	NDM	+	-	-	-	R
H	8	Oncology	Urine	Aug 2013	OXA+NDM	-	-/+	-	-	S
I	41	IM	Blood	Sep 2013	OXA	+	-	-	-	R
J	44	Orthopedic	Wound	Aug 2012	NDM	+	+/-	-	-	R
K	47	ICU	Urine	Jan 2014	NDM	+	-/+	-/+	+	R

ICU: Intensive care unit, IM: Internal medicine, NS: Neurosurgery, TA: Tracheal aspirate, IR: Insufficient result, (-): Negative, (+): Positive, S: Sensitive, R: Resistant.

Table 4. Antibiotic susceptibility patterns of *K. pneumoniae* strains with respect to 5 major PFGE types.

Antibiotic (R/S)	PFGE Type (n)				
	A (19)	B (11)	C (6)	D (3)	E (2)
Amikacin	**17/2**	**3/8**	0/6	0/3	0/2
Gentamicin	**18/1**	**9/2**	6/0	0/3	0/2
Ertapenem	19/0	11/0	6/0	3/0	2/0
Imipenem	19/0	**10/1**	6/0	3/0	2/0
Meropenem	**18/1**	**5/6**	6/0	**1/2**	0/2
Ceftazidime	9/10	11/0	6/0	0/3	2/0
Ceftriaxone	19/0	11/0	6/0	0/3	2/0
Cefepime	**18/1**	**6/5**	**3/3**	0/3	0/2
Aztreonam	19/0	11/0	6/0	0/3	2/0
Ampicillin-sulbactam	19/0	11/0	6/0	3/0	2/0
Ticarcillin-clavulanic acid	19/0	11/0	6/0	3/0	2/0
Piperacillin-tazobactam	19/0	**10/1**	6/0	3/0	2/0
Colistin	**7/12**	**4/7**	**4/2**	0/3	**1/1**
Trimethoprim-sulfamethoxazole	**17/2**	**9/2**	**5/1**	0/3	2/0
Ciprofloxacin	**18/1**	11/0	6/0	0/3	2/0
Levofloxacin	**18/1**	11/0	**5/1**	0/3	2/0
Tigecycline	**11/8**	**8/3**	**4/2**	0/3	**1/1**

R: Resistant**,** S: Susceptible.

Table 5. Antibiotic resistance rates of 47 *K. pneumoniae* strains in respect of their resistance genes detected with BD Max CRE assay (n/%).

Antibiotic	BD MAX (n/%)				
	OXA 17 (36)	NDM 6 (13)	OXA+NDM 8 (17)	Negative 16 (34)	Total 47
AK	**7 (41)**	**3 (50)**	**4 (50)**	**7 (44)**	**21 (45)**
GN	15 (88)	**3 (50)**	7 (88)	11 (69)	36 (77)
ETP	17 (100)	6 (100)	8 (100)	16 (100)	47 (100)
IMP	16 (94)	5 (83)	7 (88)	15 (94)	43 (92)
MEM	14(82)	4 (67)	6 (75)	9 (56)	33 (70)
CAZ	13 (76)	4 (67)	6 (75)	9 (56)	32 (68)
CRO	16 (94)	5 (83)	8 (100)	13 (81)	42 (89)
FEP	**11 (65)**	**3 (50)**	**5 (63)**	**9 (56)**	**28 (60)**
AZT	16 (94)	5 (83)	7 (88)	13 (81)	41 (87)
SAM	17 (100)	6 (100)	8 (100)	16 (100)	47 (100)
TIC	17 (100)	6 (100)	8 (100)	16 (100)	47 (100)
TPZ	17 (100)	6 (100)	8 (100)	15 (94)	46 (98)
CL	**11 (65)**	**0**	**4 (50)**	**2 (13)**	**17 (36)**
SXT	15 (88)	**3 (50)**	7 (88)	11 (69)	36 (77)
CIP	16 (94)	4 (66.6)	8 (100)	12 (75)	40 (85)
LEV	16 (94)	4 (66.6)	7 (87.5)	12 (75)	39 (83)
TGC	12 (71)	**3 (50)**	6 (75)	**6 (38)**	**27 (58)**

AK: Amikacin, GN: Gentamicin, ETP: Ertapenem, IMP: Imipenem, MEM: Meropenem, CAZ: Ceftazidim, CRO: Ceftriaxon, FEP: Cefepime, AZT: Aztreonam, SAM: Ampicillin-sulbactam, TIC: Ticarcillin-klavulanic asit, TPZ: Piperacillin tazobactam, CL: Colistin, SXT: Trimethoprim sulfamethoxazole, CIP: Ciprofloxacin, LEV: Levofloxacin, TGC: Tigecycline.

Only 3 of the 47 (6.4%) strains were positive for MBL with D70C, and no other resistance mechanisms were detected in any of the strains, with this set (Table 1). D68C test results revealed only ESBL positivity in 11 (23.4%) strains, ESBL and AmpC positivity in 3 (6.4%) and only Amp C positivity in 1 (2.1%) strain, while 6 (12.8%) strains were detected as ESBL and AmpC negative and 'insufficient results' were obtained in 26 (55.3%) strains Photos showing results from D70C and D68C sets have been presented in Figures 1 and 2. Among 16 strains which were KPC, NDM and OXA-48 negative, 4 were ESBL positive, 4 were ESBL and AmpC negative and 8 gave 'insufficient results'.

On the other hand, at least one of the KPC, NDM or OXA-48 genes has been detected with BD Max CRE assay, in 18 of 26 isolates that revealed 'insufficient result' with D68C. Although high-level temocillin resistance was detected in 44 (93.6%) strains, OXA-48 positivity was not detected with BD Max CRE assay among 21 (47.7%) of these strains (Table 1). The sensitivity, specificity and positive/negative predictive values of the phenotypic methods are given on Table 2.

PFGE typing of the 47 *K. pneumoniae* strains revealed 5 major PFGE patterns (A-E) and six independent patterns (F-K). Of the 17 strains which were only OXA-48 positive, 8 were in genotype A, 4 in genotype B, 3 in genotype C, 1 in genotype F and 1 in genotype I. Of the 8 OXA-48 + NDM positive strains, 3 were in genotype A, 1 in genotype B, 2 in genotype C, 1 in genotype E and 1 in genotype H. Each of the 6 strains which were only NDM positive revealed different PFGE patterns. PFGE types and dendogram of clonal relationships of study strains are given in Figure 3. PFGE types, clinics in which strains are isolated, specimen types, isolation dates and results obtained by phenotypic and genotypic methods are given on Table 3.

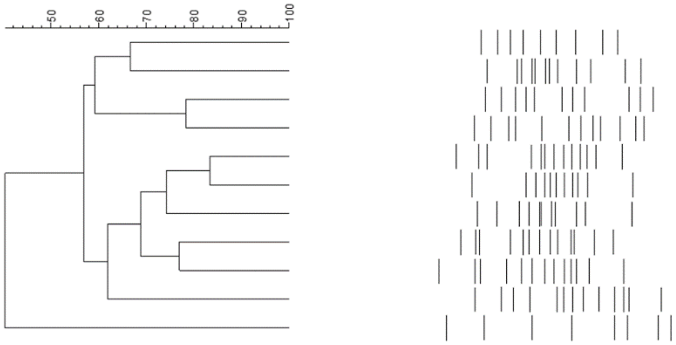
Different susceptibility results were obtained with one or more strains for amikacin (AK), gentamicin (GN), meropenem (MEM), ceftazidime (CAZ), cefepime (FEP), colistin (CL), trimethoprim sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LEV) and tigecycline (TGC) in genotype A; for AK, GN, imipenem (IMP), MEM, FEP, piperacillin-tazobactam (TPZ), CL, SXT and TGC in genotype B; for FEP, CL, SXT, LEV, TGC in genotype C; for MEM in genotype D and finally for CL, TGC in genotype E. (Table 4). Antibiotic resistance profiles of 47 *K. pneumoniae* strains in respect of their resistance genes detected with BD Max CRE assay are given on Table 5. The lowest resistance rates were detected against CL, AK and FEP in all strains with any type of β-lactamase encoding gene.

Discussion

It has been determined in this study that, carbapenem resistance which is being detected among *K. pneumoniae* isolates in Suleyman Demirel University Hospital in recent years is mainly due to OXA-48 (53.2%) and NDM (29.8%) genes. Carbapenem resistance due to the KPC gene has not been detected in our hospital. In the literature, there are only two studies reporting the isolation of KPC gene positive carbapenem resistant *K. pneumoniae* from Turkey [15,16]. Azap *et al*. [17], investigated sixteen carbapenem-resistant strains of *K. pneumoniae* at a University Hospital in Turkey between the years 2007 and 2011 and reported that OXA-48 was found in all isolates, whereas KPC, NDM, GES, IMP and VIM genes were not detected. In addition, Genc *et al*. [18], reported that they found OXA-48 in 129 strains, NDM in 10 strains and VIM in 1 strain among 140 Enterobacteriaceae isolates.

In this study, the sensitivity and specificity of MHT for the detection of carbapenemase production were 93.5% and 6.2%, respectively by taking BD-MAX CRE assay as the reference method. The MHT showed high sensitivity in identifying the presence of MBL (92.8%)

Figure 3. Dendogram obtained by PFGE analysis of 47 *K. pneumoniae* strains.



NO	PFGE TYPE
8	H
3,10,25	D
12,14,17,19,20,22,26,28,29,30,31,33,35,36,39,40,43,45,46	A
47	K
2,15,18,21,34,48	C
5	F
7	G
1,4,9,11,16,24,27,32,37,38,42	B
6,23	E
41	I
44	J

and OXA-48 (92%). Since none of the isolates tested in the study were positive for KPC, the performance of MHT in determining KPC production couldn't be assessed. In the study of Doyle *et al*. [19], the sensitivity of MHT was reported as 61% and the specificity as 93%. Birgy *et al*. [20], reported a sensitivity of 95% for MHT and pointed out that the test was false positive in 2 strains of AmpC producing *Enterobacter cloacae*. Unlike Birgy and colleagues [20], Carvalhaes *et al*. [21], and Pasteran *et al*. [22], reported that despite high carbapenem MICs, ESBL positive strains did not show false positivity in MHT. However, they have also mentioned that, weakly positive and false-negative MHT results have been reported for Enterobacteriaceae, producing MBLs, and especially NDM-1 [20]. In our study, MHT revealed false positive results in 15 of 16 strains, and false negative results in 2 strains. Although these findings indicate that the specificity of MHT is very low, the fact that other carbapenemase types which have not been investigated in this study might be present in BD MAX negative isolates should not be ruled out. On the other hand, the presence of ESBL positivity in 3 strains with false positive MHT results might be observed as a result of ESBL production coupled with porin loss [21,22].

Since the presence of carbapenemase genes has been detected with the BD MAX CRE assay in 18 of 26 isolates which gave 'insufficient result' with the D68C, it was thought that the 'insufficient results' in these isolates were caused by the coexistence of ESBL or AmpC with carbapenemase positivity. On the other hand, carbapenemase activity could not be demonstrated with BD MAX in 8 isolates which had insufficient results with D68C, while positive results were obtained by at least one of the phenotypic tests. This finding suggests that there may be other carbapenemase resistance mechanisms which were not investigated with the BD MAX system in these isolates.

The sensitivity of MBL-GST was lower than MHT, higher than D70C for detection of MBL. It was considered that false positivity of MBL-GST detected in 9 strains may have been caused by other MBL enzymes which were not investigated by BD MAX CRE assay. On the other hand, the sensitivity and specificity of the D70C were 21.4% and 100% in detecting MBL positivity. Considering these findings of our study, it is thought that although the D70C is easy to apply, it will not be able to assist the routine laboratory in the search for carbapenemase production.

In our study, the temocillin-GST has not been considered alone as a useful test to detect the presence of OXA-48. However, Huang *et al*. [23], suggested that the presence of carbapenemase production may be ruled out by the combined use of modified-zone diameter cut-off values for piperacillin-tazobactam (≥ 16 mm) and temocillin (≥ 12 mm) in geographical areas with a high prevalence of OXA-48 producers. Woodford *et al.* [24] have also reported that the detection of high-level temocillin resistance was insufficiently robust as a sole diagnostic marker for OXA-48 carbapenemase, although its absence effectively ruled out this carbapenemase. The authors have suggested its use as part of an algorithm to identify carbapenemases, rather than as a single test. However, findings of our study did not support the view that the presence of OXA-48 could be ruled out in the absence of high-level temocillin resistance.

PFGE analyses of this study revealed that 23 of 31 *K. pneumoniae* strains which were determined as carbapenemase producers were in major PFGE genotypes A, B and C. Six carbapenemase producing strains showed independent patterns (F-K). It was determined that all isolates in the genotype C were isolated in 2013, isolates in the genotype D were isolated in 2012, and isolates in the genotype E were isolated in 2014, and the spread of these isolates in ICU and surgical units was noticed. Indeed, admission to intensive care and surgical services has been identified as a risc factor for these resistance patterns in the previous studies [25,26]. The first NDM positive strain was isolated in 2012 and the first OXA-48 positive strain was isolated in 2013. In addition, the fact that each of the six NDM positive strains exhibited different PFGE patterns let us make a consideration that these cases observed in our hospital were sporadic. NDM positivity is a resistance pattern requiring a variety of infection control measures when detected, as it is very threatening due to its epidemic potential [3]. Since solely NDM positive strains appeared in our hospital as sporadic cases, we could make a positive assessment that infection control measures have been applied sufficiently in these cases. On the other hand, a significant portion of the isolates with OXA-48 and OXA-48 + NDM positivity were in the 3 major PFGE patterns (PFGE types A, B and C). Thus, we can suggest that the increase in the number of carbapenem resistant *K. pneumoniae* infections in our hospital was caused by the interhospital spread of especially 3 epidemic clones, which coexisted with epidemiologically unrelated sporadic strains. These data has once again demonstrated the necessity to be constantly awake for these resistance genes, in terms of their potential for spreading within the hospital.

While strains in the same genotype gave similar results to the majority of the tested antibiotics, strain dependent different susceptibility results were found especially for MEM, CL and TGC. It has been suggested that the presence of different resistance profiles in the same PFGE patterns may be due to the fact that some features of the bacteria had changed as they spread and replicated, or that these bacteria could easily exchange mobile genetic elements [27]. In a study conducted by Hammoudi *et al*. OXA-48 gene was detected in 31 of 102 Enterobacteriaceae isolates. In the OXA-48 negative strains, carbapenem resistance was attributed to the production of acquired AmpC cephalosporinases, outer membrane impermeability, and/or efflux pump overproduction [28]. OXA-48 was the most frequently detected carbapenemase resistance gene in the isolates of our hospital, and ESBL + AmpC positivity was detected with D68C disc set in only one of the 16 strains of which no carbapenem resistance gene was identified with BD Max CRE assay. A limited number of carbapenemase genes have been searched for in a limited number of strains within the possibilities of our study. So, the fact that other carbapenemase resistance genes (VIM, IMP, etc.) have not been investigated in these strains have emerged as the weak point of our study.

We have also determined remarkable resistant rates against antibiotics other than carbapenems, so other antibiotic resistance mechanisms besides carbapenemase production were considered to be present in our study strains. Since we have detected the lowest resistance rates against CL (36%), AK (45%) and TGC (58%), as has been reported in previous studies [29,30], we have concluded that the combination of these agents might be putforward as an option in treatment protocols for carbapenem resistant *K. pneumoniae* infections.

Conclusion

This study revealed that carbapenem resistance among *K. pneumoniae* isolates in our hospital is mainly due to OXA-48 and NDM genes and the increase of carbapenem resistance among *K. pneumoniae* strains is due to the interhospital spread of especially 3 epidemic clones. In addition, none of the phenotypic confirmatory tests has been determined as the most useful method for detecting the presence of carbapenemase activity. These data have once again demonstrated the necessity to be constantly awake for these resistance genes, in terms of their potential for spreading within the hospital. The lack of any resistance genes in some of the strains that have been found to be resistant to any of the carbapenems has led to the need for further studies including other carbapenem resistance genes that have not been investigated in this study. In this regard, the diagnostic efficiency of phenotypic confirmatory tests might be more accurately demonstrated.

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