JOURNAL OF CLINICAL MEDICINE OF KAZAKHSTAN (E-ISSN 2313-1519)
Original Article
DOI: https://doi.org/10.23950/jcmk/12648

Molecular analysis of metallo-β-lactamase genes in some gram-negative bacteria and examination of the phylogenetic relationships of isolates

Farzad Heydari[1], Fatih Koksal[1], Cansu Onlen Guneri[2], Suna Kizilyildirim[3]

[1]Department of Medical Microbiology, Medical Faculty, Cukurova University, Adana, Turkey
[2]Department of Medical Laboratory Techniques, Gulhane Vocational School of Health Services, University of Health Sciences, Ankara, Turkey
[3]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Suleyman Demirel University, Isparta, Turkey

Received: 2022-08-26.
Accepted: 2022-10-21

J Clin Med Kaz 2022; 19(6):18-26

Corresponding author:
Cansu Onlen Guneri.
E-mail: cansuonlen.guneri@sbu.edu.tr;
ORCID: 0000-0002-6112-0693

Abstract

Aim: This study aimed to determine the susceptibility of carbapenem-resistant Gr (-) bacilli isolated from various clinical infections to various antibiotics and identify genes causing carbapenem resistance and their clonal relationships to elucidate the distribution of resistance in community and/or hospital-acquired strains.

Material and methods: In this study, antibiotic susceptibilities of 450 carbapenem-resistant Gr (-) bacilli isolated from clinical specimens at Cukurova University, Faculty of Medicine, Balcali Hospital, were investigated using phenotypic methods. The presence of carbapenems and □-lactamase genes were searched using polymerase chain reaction (PCR) and sequence analysis methods. Pulsed-field gel electrophoresis (PFGE) method was used to evaluate the phylogenetic relationship of the isolates.

Results: Based on the results, it was determined that 99.23% of the strains had gained resistance to meropenem, whereas 5.38% had developed resistance to colistin. The most dominant carbapenems genes in all isolates were OXA-51, OXA-23-like and OXA-24-like.

Conclusion: It was observed that the only antibiotic that could be used safely in carbapenem-resistant Gr (-) bacilli infections was colistin. In addition, when the clonal relationship of the strains was examined, it was found that the clones considered to be closely related persisted, and these clones settled in different clinics of our hospital.

Key words: Carbapenem, MBL, PFGE, CLSI

Introduction

Gr (-) bacteria are among the leading factors of hospital and community-acquired infections. Due to the outer membrane structure in the cell walls, it is resistant to many antibiotics compared to gram-positive bacteria, and it gains multiple resistance characteristics with the transfer of genetic material and/or the selective pressure of antibiotics in the hospital environment, creating problems for infections treatment caused by these bacteria. Resistant strains of these bacteria are more fatal when they cause infections, especially in patients who are observed in intensive care units. Enterobacteriaceae species and non-fermentative bacteria, such as *Acinetobacter and Pseudomonas,* are very important because they cause infections transmitted during hospital service and can easily transfer genes encoding resistance enzymes [1-3].

Enterobacteriaceae species, which are commensally found in flora, cause many infections as a primary or secondary pathogen in extraintestinal colonization, especially in people with diabetes, immunodeficiency, using immunosuppressive drugs, susceptible to infections, such as cancer patients, invasive instrument users and the elders. *Escherichia coli, Klebsiella spp.,*

Enterobacter spp., Proteus spp., Serratia spp., and Salmonella species are frequently isolated, especially in infections acquired during hospital service [4-7].

Acinetobacter spp and *P. aeruginosa species* are at the forefront among clinically important non-fermentative Gr (-) bacteria. Due to the increasing antibiotic resistance, there are challenges in the treatment of infections caused by these bacteria [8].

Many β-lactamase enzymes that can hydrolyze penicillins, cephalosporins, monobactams, and carbapenems have been found in the majority of Gr (-) bacteria. [9]. Metallo-β-lactamases (MBL) plays a critical role in developing resistance against carbapenem group antibiotics, which is a good option in treating severe infections caused by resistant bacteria against most antibiotic groups. Carbapenems are a type of β-lactamase that causes a broader spectrum of antibiotic resistance [10]. Carbapenems, also known as MBL, are quite common, especially among *E. coli* and *Klebsiella* strains. In hospitals, it is essential to know the type of β-lactamase in the causative pathogen to optimize the treatment protocols for the patients infected with Expanded Spectrum β-lactamase, induced β-lactamases and MBL-producing strains. This study aimed to enlighten the regional epidemiology of MBL positive isolates that cause major problems in treatment and to type the carbapenemase enzymes. Thus, the presence, types, and frequencies of MBLs in Gr (-) bacilli isolated from clinical samples of patients treated at Cukurova University, Balcali Hospital were investigated in this study. Phylogenetic analysis was performed to elucidate the distribution of the clonal association and the resistance of the community or the hospital-acquired strains.

Material and methods

A total of 450 Gr (-) bacilli isolates were isolated from various clinical materials at Cukurova University, Faculty of Medicine, Balcali Hospital and identified with the VITEK-II device, and were discussed to shed light on the epidemiology of nosocomial infections. Isolates were verified with conventional culture methods, biochemical tests, and the BD-Crystal Enteric/Nonfermented Identification kit. Phenotypic Carbapenems production was performed by Modified Hodge Test (MHT) according to Clinical Laboratory Standards Institute (CLSI) criteria. MHT is considered the gold standard for carbapenem resistance. *Escherichia coli* ATCC 25922 was selected as a reference strain. Imipenem-EDTA double-disk synergy test was used to detect MBL production in the isolates included in the study. To determine the susceptibility of the isolates included in the study against various antibiotics, the Kirby-Bauer Disk Diffusion test was performed according to CLSI recommendations [11]. Sequence analysis studies were conducted for the presence of carbapenemase and β-lactamase genes in isolates, using the automated system of "ABI Prism 310 DNA sequencer (Applied Biosystems)" with PCR-multiplex-PCR [12-14]. The data obtained by sequence analysis were compared with the gene bank database using the BLAST program on the "National Center for Biotechnology Information" (NCBI) web page (htpp: //www.ncbi.nlm.nih.gov/BLAST/). The pulsed-field gel electrophoresis method, which is the gold standard, was used to evaluate the phylogenetic relationship between isolates [15, 16]. This study was approved by the Non-Interventional Clinical Research Ethics Committee of the Medical Faculty of Cukurova University (Date: 06.12.2013 and Decision No: 19).

Results

In the current study, 450 isolates of MBL-resistant Gr (-) bacilli (*A. baumannii* (n=290), *P. aeruginosa* (n=75), *K. pneumoniae* (n=40), *E. coli* (n=20), *P. mirabilis* (n=10), *Enterobacter cloacae* (n=13) and *Chryseobacterium indologenes* (n=2)) were subjected to double-disk synergy and MHT tests; 130 of these isolates were found to be MBL positive. A. baumannii (n=88), P. aeruginosa (n=26), K. pneumoniae (n=9), E. coli (n=3), P. mirabilis (n=1), E. cloacae (n=1), and C. indologenes (n=2) were among the isolates. (Table 1).

Table 1 Species distribution of 130 isolates included in the study

Isolates	No	Percentage %
A. baumannii	88	67.69
P. aeruginosa	26	20
K. peumoniae	9	6.92
E. coli	3	2.30
C. indologenes	2	1.53
P. mirabilis	1	0.76
E. cloacae	1	0.76
Total	130	

Table 2 Antibiotic susceptibility test result of *A. baumannii* isolates

ANTIBIOTIC	RESISTANT		INTERMEDIATE		SUSCEPTIBLE	
	Number	Percentage %	Number	Percentage %	Number	Percentage %
AMIKACIN	51	57.95	3	3.4	34	38.63
AMPICILLIN SULBACTAM	85	96.59	3	3.40	-	-
CEFEPIME	13	97.72	1	2.27	-	-
CEFTAZIDIME	87	98.86	1	1.13	-	-
CIPROFLOXACIN	85	96.59	1	1.13	2	2.27
COLISTINE	1	1.13	-	-	87	98.86
GENTAMICIN	79	89.77	-	-	9	10.22
IMIPENEM	87	98.86	1	1.13	-	
LEVOFLOXACIN	77	87.5	11	12.5	-	
MEROPENEM	88	100	-	-	-	
PIPERACILLIN	88	100	-	-	-	
TETRACYCLINE	67	76.13	1	1.13	20	22.72
TIGECYCLINE	12	13.63	18	20.45	58	65.90
TRIMETOPRIM SULFAMETOXAZOL	82	93.18	-	-	6	6.81
PIPERACILLIN/TAZOBACTAM	88	100	-	-	-	

Kirby-Bauer Disk Diffusion test was performed according to CLSI recommendations to determine the sensitivity of 130 isolates included in the present study against various antibiotics. According to these results, more than 50% resistance was observed in *A. baummanii* isolates against other tested antibiotics except tigecycline and colistin antibiotics. Moreover, *Pseudomonas* isolates were more than 50% resistant to all tested antibiotics. In K. pneumoniae, high resistance rates were found against antibiotics other than colistin and amikacin. Also, in E. coli high resistance rates were found against antibiotics other than colistin and fosfomycin. The results of Kirby-Bauer Disk Diffusion antibiotic susceptibility tests are given in Tables 2-7. Considering all the isolates included in our study, the highest resistance developed against meropenem with 99.23%, and the lowest resistance against colistin with 5.38%.

Table 3 Antibiotic susceptibility test result of *P. aeruginosa* isolates

ANTIBIOTIC	RESISTANT		INTERMEDIATE		SUSCEPTIBLE	
	Number	Percentage %	Number	Percentage %	Number	Percentage %
AMIKACIN	19	73.07	-	-	7	26.92
AMPICILLIN SULBACTAM	26	100	-	-	-	-
CEFEPIME	16	61.53	7	26.92	3	38.46
CEFOPERAZONE SULBACTAM	18	69.23	7	26.92	1	3.84
CEFTAZIDIME	16	61.53	6	23.07	4	15.38
CIPROFLOXACIN	18	69.23	-	-	8	30.76
COLISTINE	-		-	-	26	100
GENTAMICIN	17	65.38	-	-	9	34.61
NETILMICIN	16	61.53	6	23.07	4	15.38
IMIPENEM	26	100	-		-	-
LEVOFLOXACIN	18	69.23	-	-	8-	30.76
MEROPENEM	25	96.15	1	3.84	-	-
PIPERACILLIN	2	76.92	6	23.07	-	-
TETRACYCLINE	26	100	-		-	-
TIGECYCLINE	25	96.15	1	3.84	-	-
TRIMETOPRIM SULFAMETOXAZOL	26	100	-	-	-	-
PIPERACILLIN/TAZOBACTAM	26-100	-	-	-	-	-

Table 4 Antibiotic susceptibility test result of *K. peumoniae* isolates

ANTIBIOTIC	RESISTANT		INTERMEDIATE		SUSCEPTIBLE	
	Number	Percentage %	Number	Percentage %	Number	Percentage %
AMIKACIN	4	44.44	5	55.55	-	-
AMPICILLIN	9	100	-	-	-	-
AMOXICILLIN-CLAVULANATE	9	100	-	-	-	-
CEFEPIME	8	88.88	1	11.11	-	-
CEFOXITINE	9	100	-	-	-	-
CIPROFLOXACIN	9	100	-	-	-	-
COLISTINE	3	33.33	-	-	6	66.66
ERTAPENEME	9	100	-	-	-	
GENTAMICIN	7	77.77	-	-	2	22.22
IMIPENEM	8	88.88	1	11.11	-	-
MEROPENEM	9	100	-	-	-	-
TRIMETOPRIM SULFAMETOXAZOL	8	88.88	-	-	1	11.11
PIPERACILLIN/TAZOBACTAM	9	100	-	-	-	-

Table 5 Antibiotic susceptibility test result of *E. coli* isolates

ANTIBIOTIC	RESISTANT		SUSCEPTIBLE	
	Number	Percentage %	Number	Percentage %
AMIKACIN	2	66.67	1	33.33
AMOXICILLIN-CLAVULANATE	3	100	-	-
AMPICILLIN	3	100	-	-
CEFOXIDINE	3	100	-	-
CEFTRIAXONE	3	100	-	-
CEFUROXIM	3	100	-	-
CYPROFLOXACIN	3	100	-	-
ERTAPENEM	3	100	-	-
FOSFOMYCIN	-	-	3	100
GENTAMICIN	2	66.67	1	33.33
IMIPENEM	3	100	-	-
MEROPENEM	3	100	-	-
NITROFURANTOIN	3	100	-	-
PIPERACILLIN/TAZOBACTAM	3	100	-	-
TRIMETHOPRIM/SULFAMETHOXAZOLE	3	100	-	-
COLISTIN	-	-	3	100

Table 6 Antibiotic susceptibility test result of *Chryseobacterium indologenes* isolates

ANTIBIOTIC	RESISTANT		SUSCEPTIBLE	
	Number	Percentage %	Number	Percentage %
AMIKACIN	2	100		
TICARCILLIN/CLAVULANIC ACID	2	100		
CEFTAZIDIME	2	100		
CEFOPERAZONE/SULBACTAM	2	100		
CIPROFLOXACIN	-	-	2	100
COLISTIN	2	100		
CEFAZOLIN	2	100		
ERTAPENEM	2	100		
CEFEPIME	2	100		
CEFOXITINE	2	100		
GENTAMICIN	2	100		
IMIPENEM	2	100		
LEVOFLOXACIN	-	-	2	100
MEROPENEM	2	100		
PIPERACILLIN-TAZOBACTAM	2	100		
TRIMETHOPRIM/SULFAMETHOXAZOLE	-	-	2	100
AZTREONAM	2	100		

Table 7 Antibiotic susceptibility test result of *Proteus mirabilis* and *Enterobacter cloacae* isolate

ANTIBIOTIC	Resistant (R)- Intermediate (I)- Susceptible (S)	
	Proteus mirabilis	*Enterobacter cloacae*
AMIKACIN	S	R
AMPICILLIN SULBACTAM	R	R
CEFEPIME	I	R
CEFOPERAZONE/SULBACTAM	R	R
CEFTAZIDIME	I	R
CIPROFLOXACIN	S	S
COLISTIN	R	S
GENTAMICIN	S	R
IMIPENEM	R	R
LEVOFLOXACIN	S	S
MEROPENEM	R	R
NETILMICIN	S	R
PIPERACILLIN	R	R
PIPERACILLIN-TAZOBACTAM	R	R
TETRACYCLINE	R	R
TIGECYCLINE	R	R
TRIMETHOPRIM/SULFAMETHOXAZOLE	R	R

Table 8 PCR result amplified β-lactamase genes in *A. baumanii* (88) isolates

β-lactamase genes	Number of isolates	Percentage%
OXA 51 LIKE	88	100
OXA 23 LIKE	54	61.36
OXA 24 LIKE	15	17.04
NDM-1	2	2.28
TEM	20	22.73
OXA 48 LIKE	3	3.40
GES	5	5.69
CTX-M	6	6.82
CTX-M 1	1	1.14
CTX-M 2	1	1.14
CTX-M 9	2	2.28

Table 9 β-lactamase genes amplified by PCR in *P. aeruginosa* (26) isolates

β-lactamase genes	Number	Percentage%
oxa 23 like	4	15.38
oxa 48 like	4	15.38
GES	2	7.70
CTX-M	1	3.85
VIM 1	1	3.85

Table 10 β-lactamase genes amplified by PCR in *K. pneumonia* (9) isolates.

β-laktamaz genleri	Number	Percentage%
oxa 48like	4	44.44
oxa 24 like	2	22.22
CTX-M	4	44.44
CTX-M 9	4	44.44

In-house PCR and Multiplex-PCR techniques targeting frequently seen β-lactamase genes were amplified in order to characterize carbapenemase genes.The most dominant carbapenems genes in all isolates had been found as OXA-51, OXA-23-like and OXA-24-like in Tables 8-11.

Sequence analyzes were performed by amplifying the detected MBL genes with specific primers. Also, it was defined at the sub-type of level. PCR results defining β-lactamase gene profiles within the species and the profiles of the gene sequenced are given in Table 12. Carbapenemase genes determined by sequence analysis were OXA-48, GES-11, VIM-1, NDM-1.

Other PCR analyses also revealed the presence of NDM-1 in one of three E. coli isolates, OXA-48 like genes in two isolates, and NDM-1, OXA-48 like genes, and CTX-M genes in one E. cloacae isolate. OXA-48, GES, and NDM-1 genes are given in Figure 1.

When the phylogenetic relationships of MBL resistant *A. baumannii* isolates (n: 88) were examined, it was determined that they were distributed in 17 clusters in a close relationship.

Table 11 Distribution of β-lactamase genes determined by multiplex-PCR in all isolates

Code	Species	β-lactamases	Code	Species	β-lactamases
1	*A. baumannii*	OXA-51-like + OXA-24-like	66	*A. baumannii*	OXA-51-like+OXA-23-like
2	*A. baumannii*	OXA-51-like+ OXA-23-like+ OXA-24-like	67	*A. baumannii*	OXA-51-like+NDM-1
3	*A. baumannii*	OXA-51-like+OXA-24-like	68	*A. baumannii*	OXA-51-like+TEM+ OXA-23-like
4	*A. baumannii*	OXA-51-like+ OXA-24-like	69	*A. baumannii*	OXA-51-like+GES+ OXA-23-like
5	*A. baumannii*	OXA-51-like+ OXA-23-like+ OXA-24-like	70	*A. baumannii*	OXA-51-like+ OXA-23-like
6	*A. baumannii*	OXA-51-like+ OXA-23-like+ OXA-24-like	71	*A. baumannii*	OXA-51-like+ OXA-23-like
7	*A. baumannii*	OXA-51-like+ OXA-23-like+OXA-24-like	72	*A. baumannii*	OXA-51-like+CTX-M9
8	*A. baumannii*	OXA-51-like+ OXA-23-like+ OXA-24-like	73	*A. baumannii*	OXA-51-like+ OXA-23-like
9	*A. baumannii*	OXA-51-like+ OXA-23-like+ OXA-24-like	74	*A. baumannii*	OXA-51- like +TEM+ GES +OXA-23-like
10	*A. baumannii*	OXA-51-like+ OXA-23-like+ OXA-24-like	75	*A. baumannii*	OXA-51-like+CTX-M+ OXA-23-like
11	*A. baumannii*	OXA-51-like+CTX-M2	76	*A. baumannii*	OXA-51-like+TEM+OXA-23-like+ OXA-48-like
12	*A. baumannii*	OXA-51-like+ OXA-24-like	77	*A. baumannii*	OXA-51-like+ OXA-23-like
13	*A. baumannii*	OXA-51-like	78	*A. baumannii*	OXA-51-like+ OXA-23-like
14	*A. baumannii*	OXA-51-like	79	*A. baumannii*	OXA-51-like+OXA-23-like
15	*A. baumannii*	OXA-51-like	80	*A. baumannii*	OXA-51-like+ OXA-23-like +OXA-24-like
16	*A. baumannii*	OXA-51-like+ OXA-23-like	81	*A. baumannii*	OXA-51-like+OXA-23-like+ OXA-24-like
17	*A. baumannii*	OXA-51-like	82	*A. baumannii*	OXA-51-like+ OXA-24-like
18	*A. baumannii*	OXA-51-like	83	*A. baumannii*	OXA-51-like
19	*A. baumannii*	OXA-51-like	84	*A. baumannii*	OXA-51-like+ CTX-M9+ OXA-23-like+ OXA-24-like
20	*A. baumannii*	OXA-51-like+ OXA-23-like+ OXA-48-like	85	*A. baumannii*	OXA-51-like
21	*A. baumannii*	OXA-51-like	86	*A. baumannii*	OXA-51-like
22	*A. baumannii*	OXA-51-like	87	*A. baumannii*	OXA-51-like+GES+ OXA-23-like
23	*A. baumannii*	OXA-51-like	88	*A. baumannii*	OXA-51-like+CTX-M1
24	*A. baumannii*	OXA-51-like	89	*P. aeruginosa*	-
25	*A. baumannii*	OXA-51-like+GES	90	*P. aeruginosa*	-
26	*A. baumannii*	OXA-51-like+TEM+OXA-23-like	91	*P. aeruginosa*	OXA-23-like
27	*A. baumannii*	OXA-51-like+ TEM	92	*P. aeruginosa*	OXA-48-like
28	*A. baumannii*	OXA-51-like+ TEM+ OXA-23-like	93	*P. aeruginosa*	OXA-48-like
29	*A. baumannii*	OXA-51-like+ OXA-23-like	94	*P. aeruginosa*	-
30	*A. baumannii*	OXA-51-like+ TEM+ OXA-23-like	95	*P. aeruginosa*	VIM1
31	*A. baumannii*	OXA-51-like+ OXA-23-like	96	*P. aeruginosa*	-
32	*A. baumannii*	OXA-51-like	97	*P. aeruginosa*	GES
33	*A. baumannii*	OXA-51-like+ OXA-23-like	98	*P. aeruginosa*	-
34	*A. baumannii*	OXA-51-like	99	*P. aeruginosa*	-
35	*A. baumannii*	OXA-51-like+ TEM	100	*P. aeruginosa*	CTX-M
36	*A. baumannii*	OXA-51-like+ TEM	101	*P. aeruginosa*	OXA-48
37	*A. baumannii*	OXA-51-like+ OXA-23-like	102	*P. aeruginosa*	-
38	*A. baumannii*	OXA-51-like+ OXA-23-like	103	*P. aeruginosa*	OXA-48
39	*A. baumannii*	OXA-51-like+ OXA-23-like	104	*P. aeruginosa*	-
40	*A. baumannii*	OXA-51-like+TEM+OXA-23-like	105	*P. aeruginosa*	OXA-23
41	*A. baumannii*	OXA-51-like+NDM-1+ OXA-23-like	106	*P. aeruginosa*	-
42	*A. baumannii*	OXA-51-like	107	*P. aeruginosa*	-
43	*A. baumannii*	OXA-51-like+TEM+OXA-23-like	108	*P. aeruginosa*	OXA-23
44	*A. baumannii*	OXA-51-like+TEM+ OXA-23-like	109	*P. aeruginosa*	-
45	*A. baumannii*	OXA-51-like+ OXA-23-like	110	*P. aeruginosa*	-
46	*A. baumannii*	OXA-51-like+ OXA-23-like	111	*P. aeruginosa*	GES
47	*A. baumannii*	OXA-51-like+- OXA-23-like	112	*P. aeruginosa*	OXA-23
48	*A. baumannii*	OXA-51-like+ TEM	113	*P. aeruginosa*	-
49	*A. baumannii*	OXA-51-like	114	*P. aeruginosa*	-
50	*A. baumannii*	OXA-51-like	115	*K. pneumoniae*	OXA-48+ CTX-M9
51	*A. baumannii*	OXA-51-like+ OXA-23-like+ OXA-24-like	116	*K. pneumoniae*	OXA-48+ CTX-M9
52	*A. baumannii*	OXA-51-like+ OXA-23-like	117	*K. pneumoniae*	-
53	*A. baumannii*	OXA-51-like+OXA-23-like	118	*K. pneumoniae*	OXA-48
54	*A. baumannii*	OXA-51-like	119	*K. pneumoniae*	IMP
55	*A. baumannii*	OXA-51-like+ OXA-23-like	120	*K. pneumoniae*	-
56	*A. baumannii*	OXA-51-like+ TEM	121	*K. pneumoniae*	OXA-24+-CTX-M9
57	*A. baumannii*	OXA-51-like+ TEM+ OXA-23-like	122	*K. pneumoniae*	OXA-24+CTX-M9
58	*A. baumannii*	OXA-51-like+TEM+OXA-23-like	123	*K. pneumoniae*	OXA-48
59	*A. baumannii*	OXA-51-like+TEM+OXA-23-like	124	*E.coli*	OXA-48
60	*A. baumannii*	OXA-51-like+CTX-M+OXA-23-like	125	*E.coli*	NDM-1
61	*A. baumannii*	OXA-51-like+ OXA-23-like	126	*E.coli*	OXA-48
62	*A. baumannii*	OXA-51-like+TEM+OXA-23-like	127	*Chryseobacterium indologenes*	-
63	*A. baumannii*	OXA-51-like+ TEM+ OXA-23-like	128	*Chryseobacterium indologenes*	-
64	*A. baumannii*	OXA-51-like+GES+OXA-23-like	129	*Enterobacter cloacae*	NDM-1+OXA-48+CTX-M1
65	*A. baumannii*	OXA-51-like+ TEM+ OXA-23-like+ OXA-48-like	130	*Proteus mirabilis*	-

Table 12 Carbapenemase genes determined by sequence analysis

	OXA-48	GES	VIM	NDM
A. baumannii	3* OXA-48	5*GES-11	-	2*NDM-1
P. aeruginosa	4* OXA-48	1*GES-11+1*GES-12	1*VIM-1	-
K. pneumoniae	4* OXA-48	-	-	-
E.coli	2* OXA-48	-	-	1*NDM-1
Chryseobacterium indologenes	-	-	-	-
Enterobacter cloacae	1* OXA-48			1*NDM-1

Moreover, the strains that form the A1, A2, C1, D, F, H3, L1, N, O1, O2, P1, P2 and R clusters among these clusters were 100% similar. The largest cluster was formed by the L cluster with four sub-members (L1- 100%, L1-L2 96.8%, L2-L3 96%, L3-L-4 94%), and the second-largest cluster was the four sub-membered C cluster (C1-100%, C1-C2 95.8%, C2-C3 93.3%, C3-C4 87.9%). When we examined the distribution of β-lactamase genes in *A. baumannii* isolates, which we evaluated as closely related, OXA-51-OXA-24 genes in A cluster, OXA-51-OXA-23-OXA-24 genes in B, C, D clusters, OXA-51-TEM gene in cluster E, OXA-51-TEM-OXA23 genes in clusters F, G, H, I, J, OXA-51- OXA23 genes in clusters K, N, O, P, OXA-51-OXA-48- OXA-23 genes in cluster L, also OXA-51-GES-OXA-23 genes in cluster M. OXA-51 gene were detected in 88 *A. baumannii* isolates. OXA-23 gene was the second most common in closely related isolates. Later, TEM and OXA-24 genes were detected. In addition to this, *A. baumannii* isolates were persistent at Balcali Hospital and these isolates were settled in different clinics of the hospital (Figure 2).

When the phylogenetic relationships of *P. aeruginosa* isolates (n: 26) were evaluated, it was observed that they were distributed in six clusters (A-B-C-D-E-F) in close relation. It was analyzed that the C1 cluster was 100% similar and three sub-members (C1 100%, C1-C2 91.4%) constituted the largest cluster C. In addition, we determined that the members in the C cluster had the OXA-48 gene, the D cluster members had the GES gene, and the members in the E and F clusters had the OXA-23 β-lactamase gene (Figure 3).

As a result of the phylogenetic analysis of *K. pneumoniae* isolates (n: 9), we found that they were distributed into two closely related clusters (A-B). We determined that A1 and A2 clusters, which were sub-members of the four-member A cluster that made up the largest cluster, were 100% similar. We determined that the A1 cluster was closely related to the A2 cluster at a rate of 94.1% and the B1-B2 members that formed the B cluster were closely related to each other at the rate of 92.1%. We determined that the OXA-48-CTXM-9 genes in the A1 cluster, the OXA-24-CTXM-9 genes in the A2 cluster and the OXA-48 gene in the B cluster (Figure 4).

Figure 1 - Gel image showing the band profile of the 281 bp OXA-48 gene, 399 bp GES gene, 129 bp NDM-1 gene.

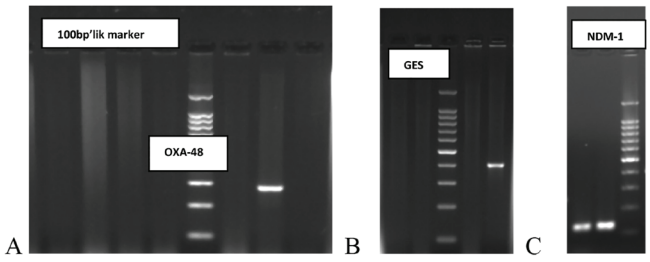


Figure 2 - Phylogenetic relationships of *A. baumannii* isolates.

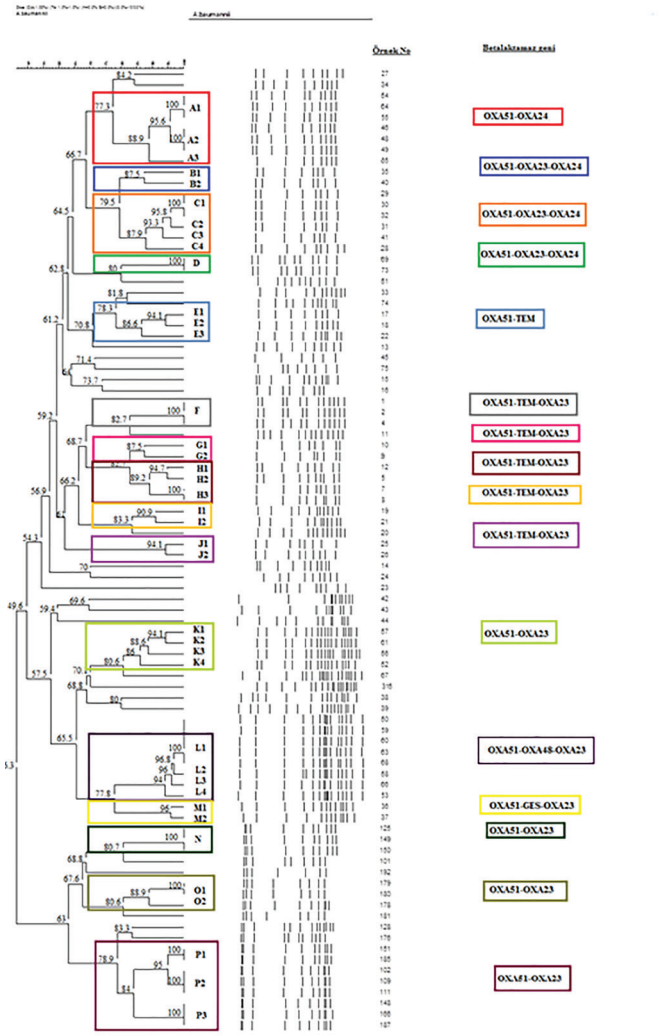


Figure 3 - Phylogenetic relationships of *P. aeruginosa* isolates

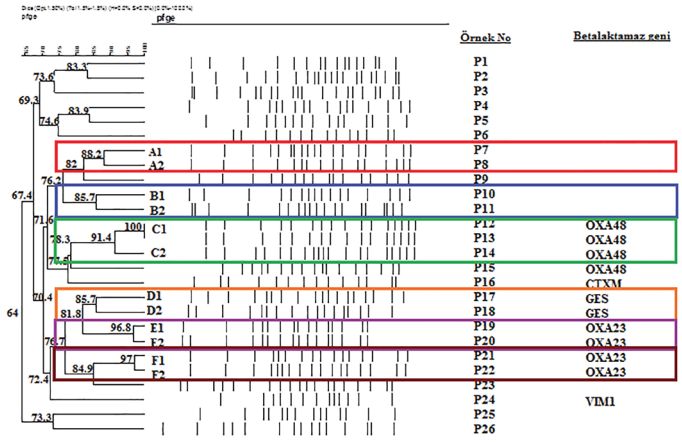
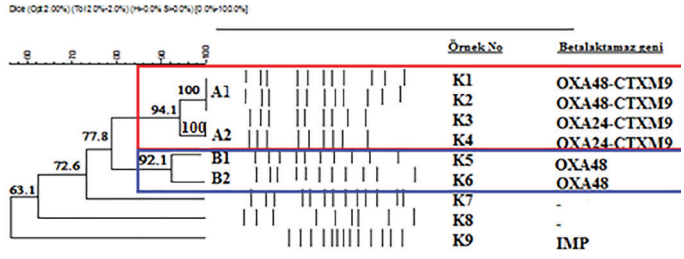


Figure 4 - Phylogenetic relationships of *K. pneumoniae*



Discussion

Bacteria showing resistance to antibiotics have become a major problem in the world and our country. Incorrect and unconscious use of antibiotics has led to an increase in multi-resistant Gr (-) bacilli. Resistance genes are studied with considerable interest by researchers worldwide.

In our study, as a result of phenotypic antibiotic susceptibility tests, we found that the isolates were 99.23% (129/130) resistant to meropenem, 98.46% (128/130) to imipenem, 88.46% (115/130) to ciprofloxacin, 86.92% (113/130) to cefepime and ampicillin- sulbactam, 70% (91/130) to tetracycline group antibiotics and 5.38% (7/130) to colistin. It is thought that there is a high degree of resistance against almost all antibiotics among non-fermenter bacillus and Enterobacteriaceae strains that have clinical importance in our region, and colistin can be used as the last option in untreatable infections.

After determining the antibiotic susceptibility of the isolates, carbapenemase genes were investigated genotypically. In *A. baumannii* isolates, OXA-51-like (100%), OXA-23-like (61.36%), TEM (22.73%), OXA-24-like (17.04%), CTX-M (6.82%), GES (5.69%), OXA-48-like (3.40%) and NDM-1 (2.28%) type β-lactamase genes were identified, respectively. In the study conducted by Ergin et al., they detected the genes OXA-23-like (31%), OXA-58-like (23%) and OXA-51-like [17]. When compared with our study, we found that OXA-51-like and OXA-23-like genes became more prevalent over the years. Another finding in our study was the detection of GES-11 type A group carbapenemase in five isolates (5.69%). The first isolate producing GES enzyme in our country was found by Bogaerts et al. in a patient transferred to Belgium in 2010 [18]. Later in 2013, it was reported that the GES enzyme was synthesized by *A. baumannii* isolates in two different studies conducted by the groups of Cicek and Zeka [19,20]. The high resistance rates in *A. baumannii* isolates in our study can be interpreted due to either carbapenemase genes or combined β-lactamases. However, future studies on porin protein permeability and efflux systems are important in terms of elucidating these mechanisms.

In our study, OXA-23-like, OXA-48, GES-11, GES-12, CTX-M and VIM-1 β-lactamase genes were detected in P. aeruginosa isolates, respectively. In their review of the literature, Breidenstein et al. attributed the reason why *P. aeruginosa* is much more resistant to antimicrobials than other Gr (-) bacilli due to its reduced outer membrane permeability, improved efflux systems, different porin numbers and structures from other bacteria, and various resistance mechanisms, such as these [21]. Fernandez et al. reported that it could adapt better than other bacteria against various environmental stress factors, such as antibiotic pressure, nutrient deficiency, and insufficient breeding environment [22]. Such changes are controlled by genes, such as *crc, lon, psrA, ampD, gyrA, nalA, nfxB, mexZ, phoQ* in the genome [21]. In line with this information, the fact that β-lactamase genes were detected in *P. aeruginosa* isolates was because carbapenem resistance occurred with efflux systems or membrane permeability modifications.

We identified OXA-48 (44.44%), OXA-24 (22.22%), CTX-M (44.44%) and CTX-M9 genes in *K. pneumoniae* isolates included in this study, respectively. Guran et al., in a study they conducted in 2011, determined the frequency of CTX-M genes in community-acquired *K. pneumoniae* isolates as 88.8% [23]. However, in a similar study conducted in 2014, they found the total CTX-M frequency among Carbapenem-resistant *K. pneumoniae* isolates as 52% (13/25) [11]. In this study, the frequency of CTX-M was 44.44%. The most common β-lactamase genes in *K. pneumoniae* isolates in our study were OXA-48 and CTX-M genes, with a rate of 44.44%. Alp et al. reported in their epidemiological study that the prevalence of the OXA-48 gene in *K. pneumoniae* isolates was 91.5% in 2013 [24]. Nazik et al. reported that the prevalence of the OXA-48 gene in K. pneumoniae isolates was common in their study between 2011-2012 [25, 26]. In our study, we detected NDM-1 in one of the 3 *E.coli* isolates and OXA-48-like genes in 2. OXA-48 enzyme in *K. pneumoniae* isolates was identified for the first time in 2001 in Turkey. In later years, it was detected in various Enterobacteriaceae species and *E. coli* [27, 28]. Turkey is a country that is now considered to be endemic to the OXA-48 enzyme. It is stated that the strains carrying this enzyme are now circulating in society [29].

Carbapenemase enzyme types differ between regions or countries depending on the preferred frequency of antibiotic use. The movements of the strains producing carbapenemase enzyme should be limited with the help of molecular epidemiological methods in the community and hospital also the development of new resistance should be minimized. In molecular epidemiological studies conducted in many countries, especially in developing countries. It was observed that there was a large increase in the prevalence of strains producing carbapenemase enzyme in Gr (-) bacilli isolated from both hospital and community-acquired infections.

In the study conducted by Yang et al. in Korea, it was determined that all the carbapenem-resistant *A. baumannii* strains were clonally related to PFGE method [30]. In a study conducted in Greece, Pournaras et al. analyzed 17 carbapenem-resistant *A. baumannii* isolates using the PFGE method and identified six different clones [31]. In the 2009 SENRTY surveillance study covering ten countries (China, India, Indonesia, Thailand, Korea, Taiwan, Singapore, Australia, Hong Kong, and the Philippines), the results of the PFGE analysis of the Carbapenem-resistant *A. baumannii* isolates carrying the OXA-23 gene were determined and it was emphasized that it had an epidemic potential for these countries [32]. In a study conducted in the USA, it was stated that Carbapenem-resistant *A. baumannii* isolates carrying the OXA-23 gene were a significant threat for hospital infections [33]. Vahapoğlu et al. stated in their study that the genes in carbapenem-resistant *A. baumannii* isolates were plasmid-derived and showed multiple clonalities with PFGE [34]. In a study conducted in South Korea, it was determined that there was 85% and more clonal association in 35 *P. aeruginosa* isolates with the IMP-6 gene using the PFGE method. It was observed that a single isolate spread throughout the country [35]. Between 2002 and 2006, Pitout et al. investigated MBL production and the presence of clonal association in 528 carpaneme-resistant *P. aeruginosa* isolates, 518 of which were isolated from four different hospitals and 10 from environmental samples. They detected VIM-2 in 178 isolates and IMP-7 in seven isolates. 178 VIM-positive isolates showed a binary closely related pattern. One hundred fifty-four of them formed a group, while 21 formed a different group. They found that 3-VIM-positive isolates were unrelated to these patterns. They reported that the IMP positive isolates were in a different group from these groups [36].

In our study, which was conducted to monitor the prevalence and movements of the strains producing hospital and community-acquired carbapenemase enzyme, it was observed that *A. baumannii* isolates (n: 88) were distributed in 17 clusters (A1, A2, C1, D, F, H3, L1, N, O1, O2, P1, P2, R) closely related, the strains forming these clusters were 100% similar. The most

distributed β-lactamase genes were OXA-51, OXA-23, TEM, OXA-24. It was detected that *P. aeruginosa* isolates (n: 26) were distributed in six clusters (A-B-C-D-E-F) closely related, C1 cluster was 100% similar, members in cluster C had OXA-48, E and F cluster members had OXA-23 β-lactamase genes. *K. pneumoniae* isolates (n: 9) were distributed in two closely related clusters (A-B). It was determined that the sub-member A1-A2 cluster of the four-member A cluster forming the largest cluster was 100% similar and the closely related *K. pneumoniae* isolates had OXA48-CTXM9 β-lactamase genes. In our study, similar to other studies, it was observed that some clones that we evaluated in close relation with the PFGE method persist in our hospital and these clones were located in different clinics. In our study, it was determined that there was a high degree of resistance against almost all antibiotics among non-fermenter bacillus and Enterobacteriaceae strains, which were clinically important in our region as in the whole world. An increase in the prevalence of strains with carbapenemase activity was found. In addition, it was observed that strains synthesizing NDM-1 type carbapenemase, which are very difficult to treat, became widespread in our country.

As a result, considering the data of epidemiological surveillance studies in our region, it is necessary to take urgent measures to develop new and more effective control measures, review and revise existing control measures, and develop and implement more rational, disciplined strategies. More regional epidemiological studies are needed in our country to restrict the motility of strains carrying antibiotic resistance genes.

Disclosures: There is no conflict of interest for all authors.

Acknowledgments: We would like to thank Prof. Dr. Akgun Yaman for his contributions to our study, and we would like to thank Cukurova University for its support.

Funding: This study was supported by Cukurova University Research Projects as project number TF2014DI.

Ethics approval: This study was carried out with the approval of Cukurova University, Faculty of Medicine, Non-Interventional Clinical Research Ethics Committee (Date: 06.12.2013 and Decision No: 19).

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