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*bla*KPC-2 and *bla*OXA-48 producing *Klebsiella pneumoniae* found in a Turkish hospital in the Balkans

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Carbapenem-resistant *Klebsiella pneumoniae* carrying *bla*KPC-2, *bla*Oxa48, and other metallo-β-lactamases (MBLs) are difficult to treat. This study was conducted to study the phenotypic and genotypic features of carbapenem resistance in isolates of *K. pneumoniae* isolated from a tertiary care hospital in the European region of Turkey. Isolates of *K. pneumonia* (n=100) resistant to at least one carbapenem (imipenem, meropenem, or ertapenem) were collected from 60 patients for 14 months. Carbapenem resistance was determined via the VITEK-2 system and the E-test confirmed this. The Modified Hodge Test (MHT) and Double Disc Synergy Test (DDST) were performed. Genes were analyzed by Sybr-Green real-time, multiplex and convensional polymerase chain reaction (PCR). Phylogenetic relatedness was analyzed by ERIC-PCR. The rate of resistance by E-test against ertapenem, imipenem, and meropenem were 98, 94, and 72%, respectively; 21% of isolates were somewhat susceptible to tygecycline. The MHT positivity was 98% and DDST was negative in all cases. There were 19 and 24% of isolates positive for *bla*KPC-2 and *bla*Oxa-48, respectively. ERIC-PCR showed that all *bla*KPC-2-positive isolates were branched into two main clusters with 80.5% similarity. The results indicate that *bla*KPC-2 mediated carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infection is spreading in Turkey and *bla*OXA-48 endemicity continues to be a serious problem. The molecular determination of carbapenemases will be useful for patients with concurrent carbapenem-resistant Enterobacteriaceae (CRE) infections. This could prevented outbreaks and complications.

Key words: *Klebsiella pneumoniae*, carbapenem resistance, *bla*KPC-2, *bla*Oxa-48.

INTRODUCTION

Carbapenems, among the beta-lactams, are the most effective agents against Gram-positive and Gram-negative bacteria presenting an expansive antimicrobial spectrum. Their unique molecular structure is due to the presence of a carbapenem together with the beta-lactam ring. This combination confers exceptional stability against most beta-lactamases (enzymes that inactivate beta-lactams) including ampicillin and carbenicillin (AmpC)

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and the extended spectrum beta-lactamases (ESBLs) (Meletis, 2016). The emergence and dissemination of carbapenem-resistant Gram-negative pathogens including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* as well as Enterobacteriaceae and Gram positive bacteria (e.g., *Staphylococcus* and *Streptococcus* species) is a significant contributor to patient morbidity and mortality (Patel et al., 2013). The emergence and spread of carbapenem-resistant Enterobacteriaceae is a major public health challenge. The most prevalent carbapenemases in *Klebsiella pneumoniae* are *K. pneumoniae* carbapenemases (KPC) in Amber class A which was first identified in 1996 in the United States (Yigit et al., 2001) and it is now endemic to several non-contiguous areas of the world especially China, Israel, Greece, and South America (Munoz-Price et al., 2013; Li et al., 2016). KPC-producing *K. pneumoniae* has only recently been reported in Turkey (Labarca, 2014). The other prevalent enzyme is Oxacillinase (OXA-like) carbapenemase in class D; specially *bla*Oxa-48 type with strong carbapenem-hydrolyzing activity was identified first in Turkey (Poirel et al., 2004). Over the years, numerous *bla*Oxa-48 type carbapenemase-producing Enterobacteriaceae have been reported in Belgium (Cuzon et al., 2008), Morocco (Benouda et al., 2010), Israel (Goren et al., 2011), Tunisia (Saidani et al., 2012), United Kingdom (Thomas et al., 2013), Lebanon (Hammoudi et al., 2014), India (Srinivasan et al., 2015), Argentina (Pasteran et al., 2012) and Czeck Republic (Skalova et al., 2017). The increased frequency of report shows that it has successfully spread and has even become endemic (Carrer et al., 2010; Nazik et al., 2014; Sahin et al., 2015). The main question still remains unclear; whether the carbapenemases spread within Enterobacteriaceae is chromosomal or whether horizontal transfer because the gene encoding this enzyme is located in the plasmid; for instance ST258 specific clone is associated carriage of *bla*KPC-2 and *bla*KPC-3 genes, is considered to be responsible for expansion of resistance (van Duina and Doi, 2017).

The aim of this study was to describe data collected for 14 months regarding the clinical *K. pneumoniae* isolates from intensive care unit (ICU) patients as well as their microbiologic and molecular features. The site was Trakya University Health Research and Application Center and State Hospital located in the Balkans.

MATERIALS AND METHODS

Sample collection and bacterial diagnosis

This study was performed at a 1100-bed tertiary care center. A total of 100 *K. pneumoniae* isolates were isolated from 60 patients. These patients were being treated at different wards between January 2013 and February 2014. The clinical samples consisted of blood, endotracheal aspirate, urine, tissue biopsy, central venous catheter, abscess fluids, or wound. An automated VITEK-2(BioMerieux, Bruz, France) system was used to identify the isolates.

The isolates were stored in the CryoBilles (AES Laboratoire, France) medium tube at the -80°C until molecular analysis.

Antimicrobial drug susceptibility testing and phenotypic screening of carbapenemase production

Antimicrobial susceptibility testing was conducted by automated VITEK-2 susceptibility card AST-GN325 (BioMerieux, Bruz, France) containing imipenem and meropenem tests according to the manufacturer's instructions. The MIC calling range for meropenem and imipenem on the AST-GN28 card was ≤0.25 to ≥16 µg/ml in doubling dilutions. The MIC results were categorized as susceptible, intermediate, or resistant based on 2014 CLSI breakpoints (susceptible, ≤1 µg/ml; intermediate, 2 µg/ml; resistant, ≥4 µg/ml) (Wayne, 2014).

All isolates were tested for MIC values of imipenem, meropenem, ertapenem, and tigecycline via the E-test strip (BioMerieux, Durham, NC, USA). The results were evaluated for CLSI breakpoints for imipenem, meropenem, and ertapenem and US Food and Drug Administration breakpoints were used to evaluate to determine susceptibility of tigecycline: susceptible, ≤2 µg/ml; intermediate, 4 µg/ml; resistant, ≥8 µg/ml (US FDA, 2009).

The Double Disc Synergy Test (DDST) and Modified Hodge Test (MHT) were used to phenotypically screen metallo-β-lactamases (Ramana et al., 2013). An imipenem (10 µg) disc and indicator isolate *E. coli* ATCC 25922 were used for MHT.

Detection of carbapenemase genes

Three different types of PCR techniques were applied via published primers (Table SD, supplementary data). SYBR-Green real-time PCR coupled with Melt-Curve analysis for *bla*KPC and *bla*NDM.

The other β-lactamases *bla*VIM-1, 2, *bla*SIM, *bla*GIM, and *bla*SPM were screened by multiplex PCR. Conventional single PCR for *bla*Oxa-48 and for ERIC-PCR (details in supplemented data) were conducted before phylogenetic program analyses.

The PCR products were sequenced at Med Santek (İstanbul, Turkey) using the same primers used in PCR reactions with ABI 3100 Capillary Electrophoresis sequence detection system (Applied Biosystem, CA).

RESULTS

During the study period, 100 carbapenem-resistant *K. pneumoniae* (CRKP) isolates (one is susceptible to all antibiotics; clinical negative control) were isolated from different clinical materials from 60 patients. The maximum of four isolates of identical species isolated from different clinical materials or at the different dates of isolation were included. The isolates were obtained from blood (36%), urine (22%), endotracheal aspirate (21%), intravenous catheter (IVC) (15%), abscess aspiration (3%), sputum (2%), and wound tissue (1%). Most (76%) patients were in the intensive care unit and the other clinics were the internal medicine clinics (oncology, hematology, physical therapy and rehabilitation) (12%), surgical clinics (7%), and the emergency unit (5%). Antibiotic susceptibility test results indicated that all isolates were resistant to ampicillin, amoxicillin-clavulanic acid, and piperacillin-tazobactam. Between 94 and 68%

Table 1. MIC values of the strains according to the VITEK-2 system.

Antibiotic	MIC values (µg/ml)		
Meropenem	≥8 (96%)	2 (3%)	≤0.5 (1%)
Ertapenem	≥8 (99%)	-	≤0.5 (1%)
Imipenem	≥8 (97%)	2 (2%)	≤0.5 (1%)
Ampicillin	≥32 (100%)	-	-
Ampicillin/Clavulanic acid	≥32 (99%)	-	≤2 (1%)
Piperacillin tazobactam	≥128 (99%)	-	≤4 (1%)
Cefuroxime-Cefuroxime/Axetil	≥16-64 (98%)	-	≤1-4 (2%)
Cefoxitin	32-64 (96%)	-	≤4 (4%)
Ceftazidime	≥16-64 (68%)	8 (13%)	≤4 (19%)
Ceftriaxone	32-64 (94%)	2 (1%)	≤1 (5%)
Cefepime	≥16-64 (68%)	4-8 (5%)	≤2 (27%)
Trimethoprim-sulfamethoxazole	≥320 (60%)	-	≤20 (40%)
Ciprofloxacin	≥4 (49%)	-	≤0.25 (51%)
Amikacin	≥64 (28%)	16 (6%)	≤2-4 (67%)
Gentamicin	≥16 (38%)	-	≤1-4 (62%)
Colistin	≥16 (3%)	-	≤0.5-2 (97%)

Table 2. Susceptibility of the strains for carbapenems and tigecycline according to E-test.

Antibiotic	MIC values (µg/ml)		
Ertapenem	≥12-no zone (98%)	-	0.012 (2%)
İmipenem	≥4-no zone (94%)	3 (4%)	0.24 (2%)
Meropenem	≥4-no zone (72%)	1.5-3 (19%)	≤1 (9%)
Tigecycline	-	3-4 (21%)	0.019-≤2 (79%)

were resistant to cefuroxime, cefuroxime/axetil, cefoxitin, and ceftazidime. Variable resistances were observed to ceftriaxone (94%), cefepime (68%), trimethoprim-sulfamethoxazole (60%), and ciprofloxacin (49%). Resistance rates to ertapenem, imipenem, and meropenem were 99, 97, and 96%, respectively. The MIC values for all antibiotics (based on VITEK-2) are shown in Table 1. The resistance rates for ertapenem, imipenem, and meropenem were determined by the E-test to be 98, 94, and 72%, respectively. None of the isolates were resistant to tigecycline; 21% had intermediate resistant. The E-test results are shown in Table 2. The MHT result was positive for 98% of the isolates, but the DDST was negative for all.

Molecular analysis revealed that 19 and 24 isolates were positive for *bla*KPC-2 and *bla*OXA-48 genes, respectively. The *bla*KPC-2 positive isolates were isolated from 17 patients in the ICU. Four of the isolates were isolated from both the endotracheal aspirates and blood samples of two patients. The characteristics of the patients and the carbapenem MIC values are shown in Table 3. SYBR-green RT-PCR was used for only end-point detection of *bla*KPC-2. The amplicon was distinguished by its specific Tm value. The melting curve analysis of the *bla*KPC-2 gene indicated that the products peaked at 89°C under determined conditions (Figure 1).

ERIC-PCR results showed that *bla*KPC-2 was positive in 17 out of 19 (two isolates were not successfully clustered). These isolates were grouped into two main clusters (A and B) that shared ~81% similarity. Cluster A was dominant with 10 isolates (~92% similarity), and cluster B had 7 isolates with ~86% similarity. The phylogenetic tree indicated a group of isolates called A_1/A_2 that is the control isolate for *K. pneumoniae* ATCC®-BAA 1705™. Cluster A_1 was isolated from Internal Medicine Intensive Care Units (IMICU) in the middle of 2013. İsolates B_1/B_2 were from the Surgical Intensive Care Unit (SICU) in early 2014. Six isolates (60% of cluster A isolates) (A_{1a}) displayed a single profile with 100% similarity indicative of dissemination. The isolates with five and six numbers were in A_{1a}, and 14 and 15 were in B_2 groups. These were in two different patients. All patient isolates shared the same patterns (Figure 2). It was found that 24 of the *K. pneumoniae* isolates carried *bla*OXA-48 that was isolated from 14 patients; six had two or more identical isolates isolated

Table 3. Demographic, clinical and laboratory characteristics of the patients infected with CRKP positive for *bla*KPC-2.

Strain No.	Patient	Age (Years)/Gender	Hospitalization (days)	Underlying disease	Hospitalization ward	Date of isolation	Isolation material	Treatment	Patient outcome	Carbapenem MICs by E-test			
										ERT	IMP	MRP	TGC
1	1	83/F	18	Pneumonia	IMICU	03.03.2013	Urine	Colistin	Improved	≥32	≥32	≥32	2
2	2	56/M	13	Colon carcinoma	Oncology	11.03.2013	CVC	Colistin	Improved	≥32	≥32	≥32	2
3	3	18/M	7	Subdural hematoma	IMICU	30.03.2013	Urine	Colistin+Meropenem	Improved	≥32	3	1	1.5
4	4	65/M	3	Septic shock	IMICU	30.03.2013	Blood	Ertapenem	Deceased	No zone	No zone	No zone	2
5	5	63/F	14	Pneumonia	SICU	09.04.2013	ETA	Meropenem	Deceased	≥32	4	2	2
6	5	-	-	-	-	09.04.2013	Blood	-	-	≥32	4	2	2
7	6	59/F	21	Pneumonia	IMICU	04.04.2013	Blood	None	Deceased	No zone	No zone	No zone	2
8	7	69/M	22	Congestive Heart Failure	IMICU	05.04.2013	ETA	Colistin	Improved	No zone	No zone	No zone	2
9	8	64/M	21	Cerebrovascular disease	IMICU	14.04.2013	Blood	Ampicillin/Sulbactam	Deceased	No zone	No zone	No zone	1.5
10	9	59/M	20	Cholinergic carcinoma	IMICU	26.01.2014	ETA	Colistin+Meropenem	Improved	≥32	4	8	4
11	10	85/M	13	Parotid tumor	SICU	03.02.2014	ETA	Colistin+Meropenem	Deceased	≥32	≥32	24	4
12	11	22/F	36	Trauma	SICU	01.02.2014	Blood	Colistin+Meropenem	Improved	≥32	≥32	12	2
13	12	62/M	87	GIS hemorrhage	SICU	17.02.2014	Blood	Colistin+Meropenem	Deceased	≥32	≥32	8	4
14	12	-	-	-	-	17.02.2014	ETA	-	-	≥32	≥32	≥32	4
15	13	88/F	31	Cerebrovascular disease	SICU	02.02.2014	ETA	Colistin+Meropenem	Deceased	≥32	4	3	2
16	14	68/M	9	Intracerebral hemorrhage	SICU	01.02.2014	Blood	Meropenem	Deceased	≥32	≥32	6	4
17	15	74/F	10	Acute myeloid leukemia	Hematology	26.05.2014	Urine	Colistin	Improved	≥32	≥32	≥32	4
18	16	61/M	87	Paraplegia	SICU	03.02.2014	ETA	Colistin+Meropenem	Deceased	≥32	≥32	16	1.5
19	17	62/M	17	Pancreatic carcinoma	IMICU	11.02.2014	Wound	Colistin+Meropenem	Deceased	≥32	16	12	4

CRKP: Carbapenem resistant *Klebsiella pneumoniae*; F: female; M: male; IMICU: Internal Medicine Intensive Care Unit; SICU: Surgical Intensive Care Unit; CVC: central venous catheter; ETA: endotracheal aspirate; ERT: ertapenem; IMP: imipenem; MRP: meropenem; TGC: tygecycline.

from different clinical samples. The characteristics of the patients and carbapenem MIC values are shown in Table 4. The carbapenem MIC values were similar in *bla*KPC-2- and *bla*OXA-48-positive isolates other than tigecycline. Despite none of the isolates being resistant to tigecycline, seven isolates had higher MIC values for *bla*KPC-2 positive isolates and one had higher MIC values for *bla*OXA-48-positive isolates.

All isolates were negative for the rest of the MBLs: *bla*GES, *bla*NDM-1, *bla*VIM-1, *bla*VIM-2, *bla*GIM, *bla*SIM, and *bla*SPM. But the remaining 56 out of 99 isolates (57%) lost ompK 36 and 44 out of 56 (79%) isolates lost both *omp*K35 and 36. OmpK35 and 36 porin loss was also detected in 14 and four *bla*OXA-48 and *bla*KPC-2 positive isolates, respectively.

Before DNA sequence analyses, selected *bla*KPC-2 and *bla*OXA-48-positive samples were subjected to conventional PCR. The product sizes were confirmed on agarose gel electrophoresis (Figure 3). The DNA sequences were performed in forward directions for *bla*KPC-2 and both directions for *bla*OXA-48. The alignment of all sequenced DNA showed high identity with *bla*KPC-2 and *bla*Oxa-48; sequences are available online through the GenBank databases with the accession numbers: MG171145, MG171146, MG171147, MG171148, MG171149, MG171150, MG171151, MG171152, MG171153, and MG171154.

DISCUSSION

Carbapenem-resistant *K. pneumoniae* is a major problem in nosocomial infections with high mortality rates especially in immunocompromised patients in the intensive care unit (Patel et al., 2008; Ulu et al., 2015). CRE were listed as one of the most urgent antibiotic resistance threats by the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) (Band et al., 2018). According to the CDC tracking program; KPC and OXA-48 type CRE have

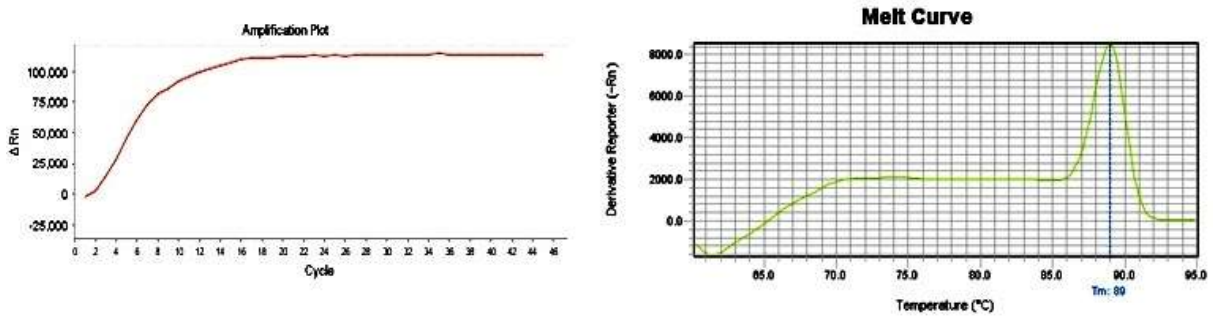
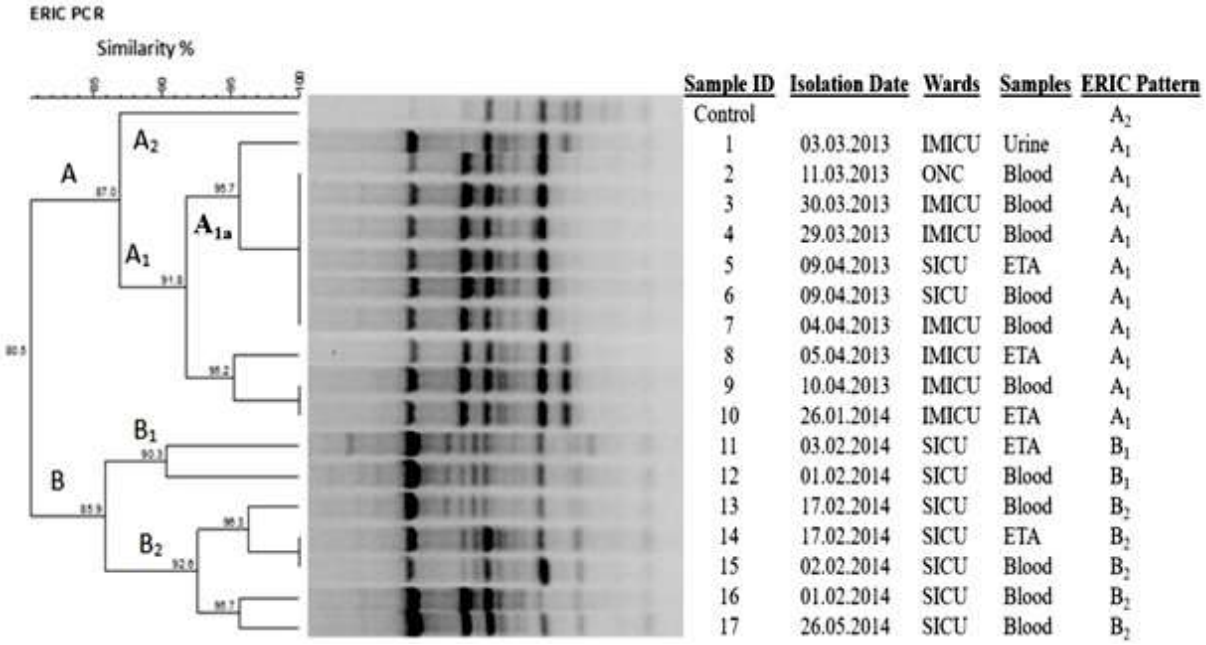


Figure 1. Amplification plot vs. cycle and Melt Curve plot; negative derivative of the normalized fluorescence generated by reporter during PCR amplification vs. temperature of *K. pneumoniae bla*KPC-2 positive control isolate *BAA* 1705.



Sample ID	Isolation Date	Wards	Samples	ERIC Pattern
Control				A_2
1	03.03.2013	IMICU	Urine	A_1
2	11.03.2013	ONC	Blood	A_1
3	30.03.2013	IMICU	Blood	A_1
4	29.03.2013	IMICU	Blood	A_1
5	09.04.2013	SICU	ETA	A_1
6	09.04.2013	SICU	Blood	A_1
7	04.04.2013	IMICU	Blood	A_1
8	05.04.2013	IMICU	ETA	A_1
9	10.04.2013	IMICU	Blood	A_1
10	26.01.2014	IMICU	ETA	A_1
11	03.02.2014	SICU	ETA	B_1
12	01.02.2014	SICU	Blood	B_1
13	17.02.2014	SICU	Blood	B_2
14	17.02.2014	SICU	ETA	B_2
15	02.02.2014	SICU	Blood	B_2
16	01.02.2014	SICU	Blood	B_2
17	26.05.2014	SICU	Blood	B_2

Figure 2. ERIC PCR: Clinical features and molecular relatedness of the 17 *bla*KPC-2-producing *K. pneumoniae* isolates. The dendrogram was developed using the Quantity One 1-D software (Biorad-Germany) with the Unweighted Pair Group Method employing arithmetic averages (UPGMA). İsolates ID: CTRL: BAA 1705 (*bla*KPC positive *K. pneumoniae*); 1-17: patient numbers; Wards: HEME: hematology; ONC: oncology; IMICU: Internal Medicine Intensive Care Unit; and SICU: Surgical Intensive Care Unit.

spread rapidly, with cases reported in all 50 and 26 states in USA, respectively (CDC, 2017). The European Survey on Carbapenemase-Producing Enterobacteriaceae (EUSCAPE) reported that Greece, Italy, Malta, and Turkey have endemic carbapenemase-producing Enterobacteriaceae (CPE). Greece has VIM and KPC, Italy has KPC, and Malta and Turkey have OXA-48 (Dortet et al., 2017). The first *bla*KPC-2-producing *K. pneumoniae* in Turkey was reported in 2014 in Istanbul. It was subsequently found in 4.052 *Escherichia coli* isolates including two *bla*KPC-2 isolates and five *bla*OXA-48 positive isolates (Kuskucu et al., 2016).

To the best of our knowledge, this is the third and one of the largest study to identify Turkish *bla*KPC-2 and the first one to use real-time PCR for screening of *bla*KPC in Turkey. The *bla*OXA-48 was first identified in Turkey and then subsequently emerged in the Middle East, India, Europe, North Africa, and Saudi Arabia (Aktas et al., 2008; Azap et al., 2013; Carrer et al., 2008, 2010; Daikos and Markogiannakis, 2011; Gulmez et al., 2008; Karabay et al., 2016; Al-Zahrani et al., 2018). The *bla*OXA-48 is frequently carried in *K. pneumoniae* but it was also identified in other Enterobacteriaceae members like *E. coli, Proteus mirabilis*, *Enterobacter* species (Fursova et al., 2015; Yu et al., 2017). Here, a nearly similar positive number of *bla*KPC-2- and *bla*OXA-48 was detected

Table 4. Demographic, clinical and laboratory characteristics of the patients infected with CRKP positive for *bla*OXA-48.

Strain No.	Patient	Age (Years)/Gender	Hospitalization (days)	Underlying Disease	Hospitalization Ward	Date of isolation	Isolation material	Treatment	Patient Outcome	Carbapenem MICs by E-test			
										ERT	IMP	MRP	TGC
20	18	69/M	45	COAH	SICU	23.03.2013	ETA	Clostin+Meropenem	Deceased	≥32	3	1.5	2
21	19	66/M	36	Intraabdominal abscess	SICU	26.03.2013	ETA	None	Deceased	≥32	12	2	2
22	19	-	-	-	-	26.03.2013	Blood	None	-	≥32	≥32	≥32	2
23	20	45/M	45	Necrotizing fasciitis	SICU	22.02.2013	Wound	Colistin+Tigecycline	Improved	≥32	2	1	2
24	21	83/M	24	GIS hemorrhage	SICU	17.02.2013	Urine	Colistin	Improved	≥32	8	2	2
25	22	55/M	109	Paraplegia	PTR	24.02.2013	Urine	Ciprofloxacin	Improved	16	3	1	1.5
26	23	18/M	7	Subdural hematoma	IMICU	30.03.2013	Urine	Colistin+Meropenem	Improved	≥32	≥32	≥32	2
27	24	57/F	35	Guillain barre syndrome	SICU	02.06.2013	ETA	Colistin+Meropenem	Improved	≥32	12	2	2
28	25	88/M	18	COAH	SICU	16.05.2013	ETA	Colistin+Meropenem	Deceased	≥32	4	1.5	0.19
29	25	-	-	-	-	16.05.2013	Blood	Colistin+Meropenem	-	≥32	4	1	0.25
30	26	80/F	37	CVD	IMICU	24.04.2013	Urine	None	Deceased	≥32	≥32	≥32	0.25
31	26	-	-	-		24.04.2013	ETA	None		≥32	≥32	≥32	0.19
32	26	-	-	-	-	01.05.2013	Blood	Colistin+Meropenem		≥32	≥32	≥32	0.19
33	26	-	-	-	-	08.05.2013	CVC	Colistin+Meropenem	-	≥32	4	1	0.19
34	27	76/M	18	COAH	SICU	13.05.2013	ETA	Colistin+Meropenem	Deceased	≥32	≥32	≥32	0.38
35	27	-	-	-	-	02.06.2013	Blood	Colistin+Meropenem	-	≥32	8	1.5	0.25
36	28	77/F	24	CVD	SICU	26.12.2013	ETA	Colistin+Tigecycline+Meropenem	Deceased	≥32	≥32	3	2
37	29	18/F	11	Trauma	Neurosurgery	01.04.2013	ETA	Colistin+Meropenem	Improved	No zone	No zone	No zone	2
38	29	-	-	-	-	04.04.2013	CVC	-	-	No zone	No zone	No zone	2
39	30	84/M	22	Colon cancer	SICU	26.11.2013	ETA	Colistin+Meropenem	Improved	≥32	≥32	1.5	1
40	31	70/F	15	Gastric cancer	IMICU	19.01.2014	Urine	Colistin	Deceased	No zone	No zone	No zone	2
42	31	-	-	-	-	19.01.2014	ETA	-	-	≥32	≥32	≥32	2
43	31	-	-	-	-	24.01.2014	ETA	-	-	≥32	≥32	≥32	2
44	31	-	-	-	-	03.02.2014	Abscess aspirate	-	-	≥32	24	3	4

CRKP: Carbapenem resistant *Klebsiella pneumoniae*; F: female; M: male; COAH: chronic obstructive pulmonary disease; GIS: gastrointestinal system; CVD: cardiovascular disease; PTR: physical therapy and rehabilitation; IMICU: Internal Medicine Intensive Care Unit; SICU: Surgical Intensive Care Unit; CVC: Central venous catheter; ETA: endotracheal aspirate; ERT: ertapenem; IMP: imipenem; MRP: meropenem; TGC: tygecycline.

carrying isolates (17 and 14%, respectively; repeat samples are not included). This high rate of *bla*KPC-2 positivity is alarming because it indicates to risk an endemic carbapenemase as *bla*OXA-48. Carbapenemases raise the MIC values of carbapenems, but the resistance level range is variable. In this study, *bla*KPC positivity elevated to markedly MIC values for tigecycline compared to *bla*OXA-48 (8>MICs>2, 37% vs. 4%); however, tigecycline was still the most active agent. van Duin et al. (2015) and Chiu et al. (2017) reported that the tigecycline resistance rate was 18% in the USA and 100% in Taiwan and Greece (Bathoorn et al., 2016). The previous reports indicate that meropenem can be used for screening of CPE (Fattouh et al., 2016). However, in this study, the meropenem resistance rate was not significantly increased in *bla*KPC-2-positive isolates (90% vs. 70%; intermediate resistance was not included). The mortality rates for *bla*KPC-2 and *bla*OXA-48 positive isolates were 59% and 50, respectively. All patients had serious multiple underlying medical comorbidities.

The clonal relationship of the *bla*KPC-2-positive isolates suggests that the epidemic has expanded beyond the hospital and resistance is spreading to other cities as well as to Greece and Bulgaria where medical tourism is popular. In addition, analyses of multi-locus sequence typing (MLST) of *bla*KPC-2 is worth further study because it can identify resistant and virulent clones like ST258 (in America and Europe) and ST11 (in Europe and Asia) (Andrade et al., 2014; Tzouvelekis et al.,

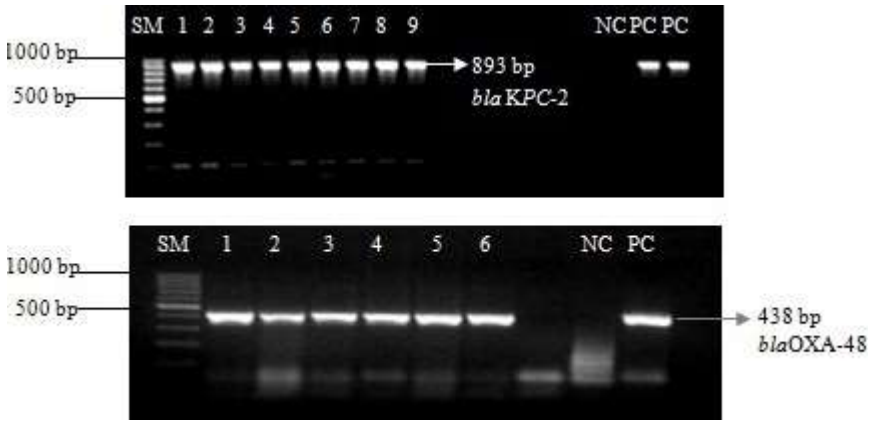


Figure 3. Detection of *bla*KPC-2 and *bla*OXA-48 by conventional PCR. The amplicons were separated by agarose gel electrophoresis and visualized with etidium bromide staining. First lane on both gels shows the molecular size marker (100 to 1000 bp) and lanes 1-9 (on first gel), 1-6 (on second gel) shows the presense of carbapenemases. NC: Negative control, PC: positive control.

2012; van Duin et al., 2017).

In contrast, other Turkish studies have found co-expression of MBLs (VIM, IMP and NDM) or AmpC type enzymes (CMY-1,2, CTX-M and SHV) with *bla*OXA-48 and *bla*KPC2 (Baran and Aksu, 2016; Karabay et al., 2016; Cizmeci et al., 2017). However, OmpK35 and 36 porin loss was found in fourteen *bla*OXA-48 and four *bla*KPC-2 positive isolates. Furthermore, 56 of 99 isolates (57%) showed moderate to high intensity carbapenem resistance, but no production of carbapenemases. These lost *omp*K 36; 44 out of 56 isolates (79%) lost both *omp*K35 and 36. This study shows that colistin (97% susceptibility) and tigecycline (79% susceptibility) might still be treatment options for CRKP infections in our center. Nevertheless, all isolates had aminoglycosides resistance; 32 to 65% of the isolates were *bla*OXA-48 and *bla*KPC-2-positive. The co-existence of plasmid-mediated *bla*KPC-2 carbapenemase in *K. pneumoniae* with the 16S rRNA-methylase *rmt*B-encoding gene offers aminoglycoside resistance. It allows methylate binding site of drugs used clinically (Sheng et al., 2012). 16S rRNA-methylase *rmt*B genes were screened by single PCR in carbapenemase-positive isolates, but none of the isolates were positive.

Conclusion

SYBR-Green RT-PCR is economical and useful tool for rapid screening of beta-lactamases rather than conventional PCR when coupled with post amplification melt-curve analysis. This offers reliable and discriminative results for carbapenemases. Furthermore, this study indicates that *bla*KPC-2-mediated CRKP infection is spreading in Turkey. Furthermore, *bla*OXA-48 is endemic and continues to be a serious problem. This situation is alarming and should be investigated via molecular characterization to find efficient antibiotics that decrease CPKP mortality. Molecular determination of carbapenemases was proposed to be useful for patients with concurrent CRE infections. This could prevent outbreaks and complications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table 1. Primers used in this study.

Primer	Forward	Reverse	Size bp	Reference
NDM-1	GGTTTGGCGATCTGGTTTT	CGGAATGGCTCATCACGATC	621	Poirel et al. (2011)
KPC-2	ATGTCACTGTATCGCCGTCT	TTTTCAGAGCCTTACTGCCC	893	Schechner et al. (2009)
OXA-48	GCGTGGTTAAGGATGAACAC	CGGTTGGGTTGAACTTGATG	438	Poirel et al. (2011)
GES-2	GTTTTGCAATGTGCTCAACG	TGCCATAGCAATAGGCGTAG	371	Queenan and Bush (2007)
GES-7	ATCTTGAGAAGCTAGAGCGCG	GTTTCCGATCAGCCACCTCT	548	Tim (2014)
VIM	GATGGTGTTTGGTCGCATA	CGAATGCGCAGCACCAG	390	Ellington et al. (2006)
GIM-1	TCGACACACCTTGGTCTGAA	AACTTCCAACTTTGCCATGC	477	Ellington et al. (2006)
SPM-1	AAAATCTGGGTACGCAAAGC	ACATTATCCGCTGGAACAGG	271	Ellington et al. (2006)
SIM-1	TACAAGGGATTCGGCATCG	TAATGGCCTGTTCCCATGTG	571	Ellington et al. (2006)
OMPK35	CAGACACCAAACTCTCATCAATGG	AGAATTGGTAAACGATACCCAG	1125	Kaczmarek et al. (2006)
OMPK36	CAGCACAATGAATATAGCCGA	GCTGTTGTCGTCCAGCAGGTT	1054	Ellington et al. (2006)
RMTB	GAGCTGGATACCCTGTACGA	GGAAAGGTAAAATCCCAAT	240	Teo et al. (2012)
ERIC-2	AAGTAAGTGACTGGGGTGAGG	-	-	Poirel (2011)

SUPPLEMENTARY DATA

Detection of carbapenemase genes

Three different types of PCR techniques have been designed via published primers (Table 1). This approach is fast, accurate, and repeatable. SYBR-Green real-time PCR coupled with Melt-Curve analysis was used for the plasmid-mediated carbapenemases *bla*KPC and *bla*GES as well as the chromosome/plasmid-mediated blaNDM. Bacterial DNA was extracted by boiling the bacterial suspension prepared by pure and fresh *K. pneumoniae* cultures in 100 µl of distilled water (Jin-Long, 2008). The DNA concentration was measured with a Nanodrop-200 (AllSheng, Mainland, China). The real time PCR amplification for *bla*KPC, *bla*NDM-1, and *bla*GES genes was performed in 20 µl of the Master mix reaction containing 10 µl of KiloGreen2X qPCR master mix (Applied Biological Materials, Vancouver, BC, Canada), 10 µM of each primer and 4 µl of genomic DNA. The PCR run used a 96-well StepOne Plus real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The real-time PCR run conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s; 60°C for 60 s; and 72°C for 20 s. The melt curve step started from 52°C and gradually increased by 0.18 C/s to 94°C with fluorescence data acquisition every 1 s.

Since SYBR Green dye binds every non-specific double strand DNA and the false positive results were evaluated via melt-curve analysis. The other β-lactamases *bla*VIM-1, 2, *bla*SIM, *bla*GIM, and *bla*SPM were screened by multiplex PCR. The master mixture contained 5 µl of master mix (Fermentase, Thermo Fisher Scientific Inc., Dreieich, Germany), 3 µl of 25 mM MgCl2, 200 µM of dNTPs, 4 µl of mix primer (10 µM of each primer pairs), 4 µl of DNA, and 1 U of Taq polymerase in a final volume of 50 µl. The PCR programmed the initial denaturation at 95°C for 5 min followed by 40 cycles of DNA denaturation at 95 for 30 s. Primer annealing at 58°C for 45 s, primer extension at 72°C for 1 min and a final extension step at 72°C for 10 min after last cycle. For ERIC-PCR, containing 6 µl of 25 mM MgCl2, 400 µM of dNTPs, 4 µl of 10 µM of ERIC primers and 4 µl of DNA (50 ng of DNA) and 2 U of Taq polymerase in a final volume of 100 µl (Jin-Long et al., 2008) with small modifications). A programmed Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) thermal cycler was used (95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, 40°C for 1 min and extension at 72°C for 5 min with a single extension at 72°C for 16 min).

Conventional single PCR for blaOxa-48 was conducted as described in multiplex PCR only with minor modifications to the primers (10 µM of Oxa-48 primer each). All PCR products (real-time, multiplex, and single PCR) were run on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide. The agarose gels were subsequently imaged under UV light using Quantity One instrument (Bio-Rad, California, USA) and recorded. The PCR products were sequenced at Med Santek (İstanbul, Turkey) using the same primers used in PCR reactions. This worked used an ABI 3100 Capillary Electrophoresis sequence detection system (Applied Biosystem, CA).