AKADÉMIAI KIADÓ

Acta Microbiologica et Immunologica Hungarica

68 (2021) 2, 65–72

DOI:
10.1556/030.2021.01364

ORIGINAL RESEARCH PAPER

Ceftazidime/avibactam and eravacycline susceptibility of carbapenem-resistant *Klebsiella pneumoniae* in two Greek tertiary teaching hospitals

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Received: December 9, 2020 • Accepted: January 7, 2021
Published online: January 29, 2021

ABSTRACT

The present study evaluated the carbapenem resistance mechanisms of *Klebsiella pneumoniae* strains isolated in two Greek tertiary teaching hospitals and their susceptibility to currently used and novel antimicrobial agents.

Forty-seven carbapenem resistant *K. pneumoniae* strains were collected in G. Papanikolaou and Ippokrateio hospital of Thessaloniki between 2016 and 2018. Strain identification and antimicrobial susceptibility was conducted by Vitek 2 system (Biomérieux France). Susceptibility against new antimicrobial agents was examined by disk diffusion method. Polymerase chain reaction (PCR) was used to detect bla_{KPC}, bla_{VIM}, bla_{NDM} and bla_{OXA-48} genes.

The meropenem–EDTA and meropenem–boronic acid synergy test performed on the 24 *K. pneumoniae* strains demonstrated that 8 (33.3%) yielded positive for metallo-beta-lactamases (MBL) and 16 (66.6%) for *K. pneumonia* carbapenemases (KPC) production. Colistin demonstrated the highest *in vitro* activity (87.7%) among the 47 *K. pneumoniae* strains followed by gentamicin (76.5%) and tigecycline (51%). Among new antibiotics ceftazidime/avibactam showed the highest sensitivity (76.6%) in all strains followed by eravacycline (66.6%). The bla_{KPC} gene was present in 30 strains (63.8%), the bla_{NDM} in 11 (23.4%) and the bla_{VIM} in 6 (12.8%). The bla_{OXA-48} gene was not detected.

Well established antimicrobial agents such as colistin, gentamicin and tigecycline and novel antibiotics like ceftazidime/avibactam and eravacycline can be reliable options for the treatment of invasive infections caused by carbapenem-resistant *K. pneumoniae*.

KEYWORDS

K. pneumoniae, carbapenem-resistance, carbapenemases, carbapenems

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INTRODUCTION

Carbapenems are widely considered the treatment of choice for severe infections caused by *Enterobacteriaceae* producing extended-spectrum beta-lactamases (ESBL). The increasing incidence of Gram-negative bacteria resistant to carbapenems is becoming a serious global health problem since the therapeutic options are significantly restricted [1]. Carbapenemase production is a major carbapenem-resistance mechanism of *Enterobacteriaceae* [2, 3]. Carbapenemases are a large group of various enzymes capable of hydrolysing several beta-lactam antibiotics including carbapenems [1]. *Klebsiella pneumoniae* carbapenemases (KPCs) are classified as Ambler class A β-lactamases (penicillinases) with an antibiotic spectrum activity similar to that of ESBLs and carbapenems. Ambler class B carbapenemases are metallo-beta-lactamases (MBLs) which hydrolyse all β-lactams except aztreonam. Among them, VIM, IMP and NDM group are the most commonly described. The Ambler class D carbapenemases or oxacillinases comprise several OXA-48 derivatives with low hydrolytic activity against carbapenems [3]. Carbapenemases have been widely spread [4] and although are predominantly carried by *K. pneumoniae* they have also been harboured by several other *Enterobacteriaceae* genera [2, 4]. In recent years, the predominant carbapenem-resistance mechanism among *K. pneumoniae* strains in many geographical regions is either KPC or MBL production [5]. Until 2004 KPC producing *K. pneumoniae* strains were restricted in the United States [1]. In the following years, KPC-producers have been spread worldwide [2] and European countries such as Greece [1–3] and Italy [3] are nowadays considered endemic areas for KPC-producing bacteria [1–3]. KPCs are encoded by the bla_{KPC} gene located within the Tn4401transposon which is highly transferable into different plasmids of Gram-negative bacteria, hence favouring its inter-species and geographic dissemination [2] outbreak onset [3] and multi-drug resistance [2, 3].

The phenotypic detection of KPC producers is important for restricting resistance and should be adjusted according to the prevalent resistant type in each region. In the laboratory practice, MBL production is ascertained by double-disk synergy tests in combination with EDTA and imipenem disks. The modified Hodge test (MHT) and the susceptibility to ertapenem are the most indicated methods to reveal the production of these enzymes [5]. The MHT is the only phenotypic screening method recommended by the Clinical and Laboratory Standard Institute (CLSI) [2, 6, 7] and is based on the inhibition zone formation around a carbapenemase disk when a KPC suspected organism is cultured on Mueller-Hinton agar [2]. The sensitivity of the test reaches almost the 100% [2] but diversities in specificity values [2, 7] and false positivity of the results raise a concern [6, 7]. Cultures of the isolates on chromogenic agar have also been recommended for KPC detection, demonstrating 100% sensitivity and specificity comparable to those of polymerase chain reaction (PCR) [2]. Moreover, the use of boronic acid disk test in combination with several antibiotic substrates has been evaluated as sensitive and highly specific for the phenotypic detection of KPC producing *K. pneumoniae* clinical isolates [5]. Undoubtedly, however, molecular techniques are the most indicated methods to confidently confirm KPC production [2], especially the OXA-type carbapenemases for which no specific phenotypic test has been established, yet [6].

The aim of the present study was to define the susceptibility profile of KPC producing *K. pneumoniae* strains collected in two Geek tertiary teaching hospitals against the currently used and novel antimicrobial agents as well as the molecular detection of the underlying resistance mechanisms.

MATERIAL AND METHODS

Forty-seven clinical samples (including urine, bronchial secretions, sputa, venous blood, venous catheter tips, tissue fragments, rectal swabs, trauma materials, soft tissue collections, drainage and ascitic fluids) were obtained from equal number of patients either hospitalised or attended the outpatient clinic of G. Papanikolaou General hospital and Ippokrateio hospital, both sited in Thessaloniki, Greece (Figs. 1 and 2). The 24 clinical samples were collected in G. Papanikolaou hospital between 1/11/2016 and 5/1/2018 and the 23 ones in Ippokrateio hospital between 26/1/2017 and 19/4/2017 and sent to the corresponding clinical laboratory. All samples were cultured according to the standard microbiological methods. Isolated *K. pneumoniae* strains were inoculated in nutrient broth containing 16% glycerol and stored at −70 °C until assessment.

Culture methods

The clinical samples were cultured according to their origin: urine samples were inoculated on 5% horse blood agar and MacConkey 2 agar and incubated for 24 h at 37 °C. Blood samples were distributed into dedicated bottles and placed in the automated blood culture monitoring system, BacT/Alert (BioMérieux, France). Positive blood cultures were subcultured on 5% horse blood agar, MacConkey 2, Chapman and Sabouraud agar. The other samples were plated on 5% horse blood agar and MacConkey 2 agar and incubated at 37 °C for 24 h as well as on chocolate agar incubated, accordingly. All specimens excluding sputa, were enriched with nutrient broth and incubated for another 24 h at 37 °C under aerobic conditions. *K. pneumoniae* isolates grown on cultures were stored at −70 °C into Brain Heart Infusion Broth (BHIB), containing 16% glycerol until being processed for the molecular detection of specific resistance genes.

Identification methods

K. pneumoniae isolates were identified to the species level with the automated system VITEK 2 Compact ((bioMérieux, Marc L' Étoile, France) using the GN ID test panel according to the manufacturer's recommendations. The GN

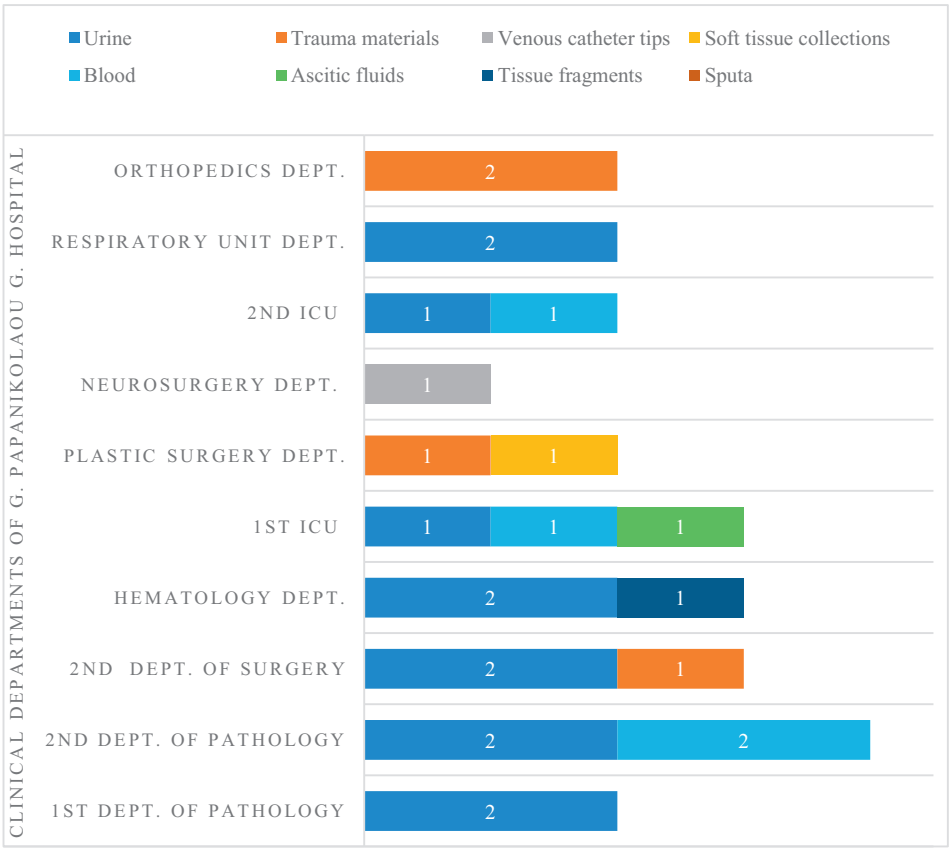

Fig. 1. Distribution per clinical department of the 24 clinical samples obtained from G. Papanikolaou hospital

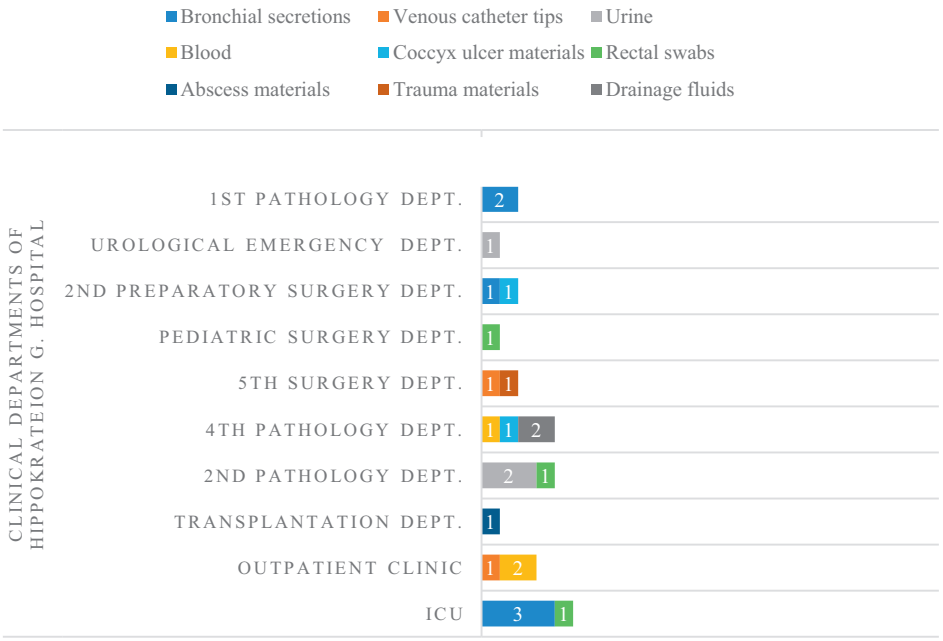

Fig. 2. Distribution per clinical department of the 23 clinical samples obtained from Ippokrateio General hospital

card is based on documented biochemical methods and implementation of innovative substrates which measure the use of carbon source, enzymatic activity and resistance. The final result is available within 10 hours or less [8]. For both identification and susceptibility, the isolates were suspended into 3 ml normal saline and the turbidity of the inoculum was adjusted to 0.5–0.63 (±0.1) of the MacFarland (McF) scale using Densichek (bioMérieux).

Antimicrobial susceptibility testing

The susceptibility to antibiotic agents was performed by the VITEK 2 system using the AST card for Gram-negative bacilli. The AST card methodology is based on the minimum inhibitory concentration (MIC) technique and is basically a miniaturised and abbreviated version of the doubling dilution technique for MIC determined by the microdilution method. Each card contains a control well filled only with nutrient medium and multiple wells filled with selected antibiotics of increasing concentrations in the medium. The system continuously monitors the organism growth in each well over time and at the completion of the incubation period the MIC values for each antibiotic are automatically reported [8]. The results for antibiotics available in the European Committee of Antimicrobial Susceptibility Testing breakpoints were interpreted according to EUCAST. The strains, whose susceptibility was examined with antibiotics unavailable to EUCAST, were defined as sensitive, intermediate or resistant according to the CLSI interpretive criteria.

Meropenem–EDTA and meropenem–boronic acid synergy test

All 24 *K. pneumoniae* strains from G. Papanikolaou hospital resistant to carbapenems were tested for MBL and KPC production by synergy test using disks containing meropenem-EDTA and meropenem-boronic acid respectively. The phenotypic differentiation of KPC-type was performed as follows: 20 µL of phenylboronic acid solution (containing 400 µg of boronic acid) and 10 µL 0.1 M EDTA were used as reagents and dispensed onto commercially available meropenem containing antibiotic disks. A microbial suspension of 0.5 McF turbidity was inoculated on Mueller-Hinton plate onto which four meropenem disks-a meropenem-phenylboronic acid disk, a meropenem-EDTA disk, a meropenem-phenylboronic acid plus EDTA disk and a meropenem disk alone-were placed at suitable distance. After incubation at 37 °C for 18 h inhibition zones >5 mm of diameter around disks were evaluated. The test was considered positive for KPC or MBL production when the inhibition zone around the disk of meropenem-boronic acid or meropenem-EDTA respectively was greater than the disk containing meropenem alone. Similarly, concurrent production of KPC and MBL was suspected when the inhibition zone was >5 mm only around the disk containing both reagents [5, 9].

Susceptibility to new antimicrobial agents

The strains were tested for susceptibility to the following antibiotics: minocycline, tetracycline, ceftazidime/clavulanic acid, doripenem, ertapenem/cloxacillin, meropenem/dipicolinicacid, eravacycline and ceftazidime/avibactam. The susceptibility testing was carried out by the disk diffusion method on Mueller-Hinton agar according to the EUCAST and CLSI guidelines and interpretive standards (Table 1) [5, 9].

Table 1. Susceptibility breakpoints of the new antibiotics tested by disk diffusion method

Antimicrobial agent	Inhibition zone (mm)		
	S	I	R
*Minocycline (MN) 30 µg	≥16	13–15	≤12
*Tetracycline (TE) 30 µg	≥15	12–14	≤11
**Ceftazidime/Clavulanic acid (CAL) 40 (30/10) µg	≥26	23–25	≤22
*Doripenem (DOR) 10 µg	≥23	20–22	≤19
**Ertapenem/Cloxacillin (ET/CL)	≥22	19–21	≤18
**Meropenem/Dipicolinic acid (MR/DP)	≥23	20–22	≤19
**Eravacycline (ERV) 20 µg	≥15	12–14	≤11
**Ceftazidime/Avibactam (CZA) 50 (30/20) µg	≥13	-	≤13

S: susceptible, I: intermediate, R: resistant.
*Susceptibility breakpoints as established by CLSI (M100 ED30, 30th edition, 2020).
**Susceptibility breakpoints not established by CLSI and EUCAST (Clinical Breakpoint Tables v. 10.0, valid from 2020-01-01). Data also retrieved from J.A. Sutcliffe et al., 2013 [26] and Helio S. Sader et al., 2018 [28].

Molecular detection of resistance genes

For the microbial DNA extraction 2–3 colonies of each strain were diluted into 100 µL distilled water. After incubation at 95 °C for 10 minutes into water bath and centrifugation for 2 minutes at 14,500 rpm the supernatant containing the microbial DNA (template DNA) was transferred into sterile tubes and either used for amplification or stored at −20 °C for future assessment. For each *K. pneumoniae* strain four different PCRs were conducted to detect the carbapenemase genes genes bla_{KPC}, bla_{VIM}, bla_{NDM} and bla_{OXA-48} using four different primer couples (Table 2) as previously described by Poirel et al. and Ellington et al [10, 11]. In brief, 2 µL of the template DNA and 48 µL of reagent mixture (50 µL final volume) were used for each amplification. The reagent mixture contained deoxynucleotide triphosphate mix (dNTPs) (Invitrogen 55083, 55082, 55084, 55085), $MgCl_2$, Taq DNA polymerase (AmpliTaq Gold Biosystems 5 Units/µL 4486226), the primer couples (Invitrogen) and the reaction buffer at a content. The DNA amplification was carried out using the 2,720 Thermal Cycler (Applied Biosystems) according to the following protocol: one PCR cycle for the initial DNA denaturation at 94 °C for 10 min and 36 PCR cycles including a) denaturation of the DNA at 94 °C for 30 s, b) annealing at 52 °C for 40 s, allowing primers to bind at target sites on the template and c) primer extension at 72 °C for 50 s. Subsequently, the final product was extended at 72 °C for 6 min. The PCR products were electrophorised on 2% (w/v) agarose gel for 30 min using a horizontal electrophoresis device of 100V constant voltage. The electrophoresis buffer used was 1x TAE solution (40 mmol/L Tris-HCl [pH 8.3], 2 mmol/L acetate, 1 mmol/L EDTA). The electrophoresis gel was stained with ethidium bromide 0.5 µg/mL and read under UV light [9–11].

Table 2. Primer couples used for the detection of carbapenemase genes

Carbapenemase genes	DNA primer couples	PCR product	Reference
bla_{KPC}	F: 5′-TGTCACTGTATCGCCGTC-3′ R: 5′-CTCAGTGCTCTACAGAAAACC-3′	798 bp	Poirel et al.
bla_{VIM}	F: 5′-GTTTGGTCGCATATCGCAAC-3′ R: 5′-AATGCGCAGCACCAGGATAG-3′	390 bp	Ellington et al.
bla_{NDM}	F: 5′-GCAGCTTGTCGGCCATGCGGGC-3′ R: 5′-GGTCGCGAAGCTGAGCACCGCAT-3′	621 bp	Poirel et al.
bla_{OXA-48}	F: 5′-GCGTGGTTAAGGATGAACAC-3′ R: 5′-CATCAAGTTCAACCCAACCG-3′	438 bp	Poirel et al.

RESULTS

Meropenem–EDTA and meropenem – boronic acid synergy test

The meropenem–EDTA and meropenem–boronic acid synergy test for the 24 *K. pneumoniae* isolates demonstrated that 8 strains were positive for MBL and 16 for KPC production.

Antimicrobial susceptibility

Susceptibility testing results of all *K. pneumoniae* strains conducted with the automated system Vitek 2 are illustrated in Table 3. All of the isolates demonstrated 100% resistance to piperacillin/tazobactam, cefoxitin, ceftazidime, ceftriaxone, cefepime, aztreonam, imipenem and meropenem. Also, most of them are proven resistant to ciprofloxacin and levofloxacin. In total, the most active antimicrobial agent was shown to be colistin (87.2% sensitivity), followed by gentamicin (76.5% sensitivity). Moreover, 24 of 47 isolates were susceptible to tigecycline.

Particularly, the isolates from Ippokrateio hospital were resistant to carbapenems, all demonstrating MIC values ≥4 μg/mL for imipenem and meropenem. The highest *in vitro* activity was shown for gentamicin (82.6% sensitivity) followed by colistin (73.9%) and tigecycline (69.5%). Similarly, all 24 isolates from G. Papanikolaou hospital were resistant to carbapenems while the highest *in vitro* activity was displayed for colistin (100% sensitivity), followed by sulfamethoxazole/trimethoprim and gentamicin (75 and 70.8% respectively).

Table 3. Antibiotic susceptibility of the 47 isolates

Antimicrobial agent	Number of isolates		
	S	I	R
Ampicillin/Sulbactam			47
Piperacillin/Tazobactam			47
Cefoxitin			47
Ceftazidime			47
Ceftriaxone			47
Cefepime			47
Aztreonam			47
Imipenem			47
Meropenem			47
Amikacin	25		22
Gentamicin	36	1	10
Ciprofloxacin	2	1	44
Levofloxacin	2		45
Tigecycline	24	9	14
Fosfomycin	17		30
Colistin	41		6
Trimethoprim/Sulfamethoxazole	29		18
Amoxicillin/Clavulanic acid*			24
Ticarcillin*			24
Cefotaxime*			24
Ertapenem*			24

S: susceptible, I: intermediate, R: resistant.
*Only half of the isolates were examined for susceptibility to this particular antibiotic agent.

Susceptibility to novel antibiotics

The susceptibility profile of all KPC positive strains to novel antibiotics was examined by disk diffusion method and results are illustrated in Table 4. The combination of ceftazidime/avibactam had the highest activity (76.6% sensitivity) followed by eravacycline (66%). The 55.3% of strains demonstrated susceptibility to minocycline, followed by doripenem (27.7%) and tetracycline (25.5%). Susceptibility to meropenem/dipicolinic acid and ceftazidime/clavulanic acid was reported in 8.5% of strains whereas only 2.1% were sensitive to ertapenem/cloxacillin. It is noteworthy that all *K. pneumoniae* strains carrying the bla_{KPC} gene were susceptible to ceftazidime/avibactam.

Table 4. Susceptibility of all isolates to new antibiotics by disk diffusion method

Antimicrobial agent	Number of isolates (%)		
	S	I	R
Minocycline	26 (55.3)	2 (4.3)	19 (40.4)
Tertacycline	12 (25.5)	3 (6.4)	32 (68.1)
Ceftazidime/Clavulanic acid	4 (8.5)	3 (6.4)	40 (85.1)
Doripenem	13 (27.7)	6 (12.7)	28 (59.6)
Ertapenem/Cloxacillin	1 (2.1)	2 (4.3)	44 (93.6)
Meropenem/Dipicolinic acid	4 (8.5)	3 (6.4)	40 (85.1)
Eravacycine	31 (66.6)	6 (12.7)	10 (21.3)
Ceftazidime/Avibactam	36 (76.6)	–	11 (23.4)

S: susceptible, I: intermediate, R: resistant.

Molecular screening for carbapenemase production

According to PCR results the bla_{KPC} gene was present in 30 isolates (63.8%), the bla_{NDM} in 11 (23.4%) while 6 (12.8%) were carriers of the bla_{VIM} gene. In none of the examined strains the bla_{OXA-48} gene was detected.

DISCUSSION

The present study was designed to investigate the resistance mechanisms to carbapenems of 47 carbapenem resistant *K. pneumoniae* strains reported from two Greek tertiary teaching hospitals as well as their susceptibility to both well-established and novel antimicrobial agents. All isolates demonstrated MIC values within the resistance range for imipenem, ertapenem and meropenem. Twenty-four isolates were tested for carbapenemase production with meropenem-EDTA and meropenem-boronic acid synergy test, which demonstrated KPC and MBL production in 16 and 8 strains, respectively. All 47 strains were processed for the detection of the carbapenemases genes bla_{KPC}, bla_{NDM}, bla_{VIM} and bla_{OXA-48} by PCR. The bla_{KPC} was the most prevalent variant (30%) followed by bla_{NDM} (23.4%) and bla_{VIM} (12.8%). None of the examined isolates was carrier of bla_{OXA-48} gene nor harboured KPC and MBL-producing genes concomitantly. KPC production is a common resistance mechanism of *Enterobacteriaceae* against carbapenems and is highly prevalent in Mediterranean countries [12]. In Greece, according to a 10-year single-center study, an alarming increase in the number of KPC-producing *K. pneumoniae* cases was recorded [13]. As reported by EARS-Net, Greece displayed the highest prevalence of carbapenem-resistant *K. pneumoniae* isolates in 2014 in Europe but with a decreasing trend [14]. Similarly, in the European Survey on Carbapenemase-Producing *Enterobacteriaceae* (EuSCAPE) project KPC-producing *K. pneumoniae* strains have been detected in large proportions in several countries [15, 16]. A surveillance study among 119 Greek hospitals revealed that KPC production was the most prevalent resistance mechanism among *K. pneumoniae* strains followed by VIM production [17]. Currently, NDM is the second ranking carbapenemase in Greece [15] whereas the frequency of VIM is gradually decreased in 2016 [16]. In our study, none of the examined *K. pneumoniae* strains were positive for OXA-48 nor for a double carbapenemase production, possibly due to the relatively small number of our collection.

Colistin, gentamicin and tigecycline are the last line treatment against carbapemen-resistant *K. pneumoniae* infections. Non-susceptibility rates against these agents have been steadily increasing [13] and are mainly attributed to their previous administration [18–21] as well as to the lack of preventive measures in health-care units [21, 22]. In our study, resistance to colistin, gentamicin and tigecycline displayed the 26.1, 17.4 and 30.5%, respectively of the 23 carbapenem-resistant *K. pneumoniae* strains. By contrast, none of the rest 24 strains was resistant to colistin but only 33.3% were sensitive to tigecycline. Among the examined strains 29.2% of them displayed resistance to gentamicin. An important finding of the present study was the relatively high susceptibility prevalence to trimethoprim/sulfamethoxazole demonstrated by the 12 out of 17 *K. pneumoniae* strains of our collection recovered from urine samples. Considering that trimethoprim/sulfamethoxazole is mainly administered for urinary tract infections, this observation should raise concerns regarding the unwarranted use of new, powerful but costly antibiotics instead of earlier, less expensive and until today, effective drugs.

The 47 *K. pneumoniae* strains of our study were also examined for their susceptibility to other antibiotics such as ceftazidime/avibactam and eravacycline. Clinical data suggest that ceftazidime/avibactam is active against most multidrug resistant *K. pneumoniae* isolates and could be a valuable treatment option either alone or in combination with other drugs, such as aztreonam, in patients with KPC-producing *K. pneumoniae* infections [22]. Hackel et al. in an *in vitro* assessment of ceftazidime/avibactam activity against multi-drug resistant *K. pneumoniae* strains confirmed that 96.6 % of them were susceptible. Moreover, the combination was the most effective together with tigecycline and colistin [23]. In a study conducted by Castanheira et al. the activity of ceftazidime/avibactam in a large collection of clinical isolates was retained against the 99.3% of the examined *Enterobacteriaceae* harbouring bla_{KPC} gene and the 99.2% of bla_{KPC} *K. pneumoniae* carriers [24]. In line with this are the results of our study in which all 30 *K. pneumoniae* strains carrying bla_{KPC} were susceptible to ceftazidime/avibactam. The in *vitro* activity of eravacycline against a large Gram-negative pathogen collection was preserved against multidrug resistant isolates, including those producing OXA and KPC carbapenemases [25]. Accordingly, Sutcliffe et al. demonstrated that eravacycline was ≥ 2-fold active *in vitro* compared to tigecycline for many *Enterobacteriaceae* including the 46% of the examined *K. pneumoniae* isolates [26]. In our study, non-susceptibility to ceftazidime/avibactam and eravacycline was displayed by the 23.4 and 34% of the examined stains, respectively. Resistance to ceftazidime/avibactam has been sporadically reported in the United States and Europe and has been associated to KPC gene or CTX-M-14 mutations [27, 28].

Our study has a limitation regarding the susceptibility testing results of colistin for which both EUCAST and CLSI strongly recommend broth microdilution instead of the semi-automated devises [29]. Despite the problems reported with colistin on these systems, these are extensively used at clinical laboratories in our country and elsewhere, therefore we presented the VITEK colistin susceptibility results as reported in the actual clinical cases included in our study.

In conclusion, the *in vitro* susceptibility of the 47 *K. pneumoniae* strains demonstrated that colistin, gentamicin and tigecycline may be beneficial alternatives for the treatment of invasive infections caused by KPC-producing pathogens. New antibiotic agents such as ceftazidime/avibactam and eravacycline possess key advantages in the treatment of those infections but careful monitoring of their susceptibility profile coupled with the identification of the

carbapenemase produced are important for defining the optimal therapeutic regimen. Additionally, given the high morbidity and mortality of KPC-producing *K. pneumoniae* infections and the limited therapeutic choices, preventive strategies, such as strict adherence to basic hygiene practices in healthcare facilities should be urgently implemented for restricting the spread of those infections [30].

Funding: None.

Competing interests: None declared.

Ethical approval: Not applicable.

Consent for publication: All authors have consented for publication.

ACKNOWLEDGEMENTS

None.

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