

Carbapenemase-producing Enterobacteriaceae isolates resistant to last-line antibiotics in an Italian general hospital

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SUMMARY

The global dissemination of carbapenemase-producing Enterobacteriaceae (CPE) is of great concern for public health. These bacteria have the potential for rapid dissemination in healthcare settings and cause infections associated with high rates of morbidity and mortality. A total of 221 carbapenem non-susceptible Enterobacteriaceae isolates were collected from patients admitted to an Italian general hospital from January 2016 to March 2017. Among these isolates, 78.3% were carbapenemase producers: 96% were positive for the *bla*_{KPC} gene and the remainder for the *bla*_{VIM} gene (allelic variant VIM-1). CPE isolates were mainly *Klebsiella pneumoniae*, but we also detected carbapenemase enzymes in *Citrobacter freundii*, *Enterobacter cloacae* and *Escherichia coli*. Among CPE isolates, 79.2% exhibited co-resistance to two or more non-β-lactam agents and 38% of these isolates (all KPC-positive) were resistant to colistin. This percentage reached 55% among CPE isolated from the bloodstream. All patients with colistin-resistant CPE isolates recovered from blood samples showed an unfavorable outcome within 7 days from the first positive blood culture. Our data show the dissemination of a high percentage of CPE isolates co-resistant to last-line antibiotics. In addition, we report the first identification in our hospital of CPE isolates harboring the *bla*_{VIM} gene and *Escherichia coli* harboring the *bla*_{KPC} gene. These results underline the need to implement antibiotic stewardship and infection control programs, and emphasize the need for novel antimicrobial agents active against CPE.

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INTRODUCTION

Enterobacteriaceae are members of the human intestinal microbiota and are among the most common microorganisms causing both nosocomial and community-acquired infections, including those of the urinary and respiratory tracts, bloodstream, and intra-abdominal, skin and soft tissue infections (Wang *et al.*, 2015). Carbapenems are a class of β-lactam antibiotics with broad-spectrum antibacterial activity and are widely regarded as the drugs of choice for the treatment of severe infections caused by extended-spectrum β-lactamase-producing Gram-negative bacteria (Paterson and Bonomo, 2005; Nordmann *et al.*, 2011; Lee *et al.*, 2016). However, in recent years, carbapenem resistance has gradually increased among Enterobacte-

riaceae (Rhombert and Jones, 2009; Houghton *et al.*, 2010; Nordmann *et al.*, 2011; Rossolini, 2015) and it has reached high rates in many areas of the world, becoming a public health problem of global importance (Patel *et al.*, 2008; Tzouveleki *et al.*, 2012; Guh *et al.*, 2015). These bacteria have the potential for rapid dissemination in healthcare settings (Nordmann *et al.*, 2011; Tzouveleki *et al.*, 2012), often exhibit multidrug resistance phenotypes that leave very few therapeutic options (Hirsch and Tam, 2010; Falagas *et al.*, 2014) and cause infections associated with high rates of morbidity and mortality (Tzouveleki *et al.*, 2012; Falagas *et al.*, 2014). Bloodstream infection is the major clinical outcome caused by carbapenem-resistant Enterobacteriaceae (CRE), accounting for the majority of CRE infections (Daikos *et al.*, 2012; Hussein *et al.*, 2013). Several studies in the USA, Greece, Italy, Israel, Spain and Brazil have reported crude fatality rates from 24% to more than 72% in patients with CRE-bloodstream infections (Daikos *et al.*, 2009; Mouloudi *et al.*, 2010; Neuner *et al.*, 2011; Zarkotou *et al.*, 2011; Ben-David *et al.*, 2012; Tumbarello *et al.*, 2012; Hussein *et al.*, 2013; Navarro-San Francisco *et al.*, 2013; Daikos *et al.*, 2014; De Oliveira *et al.*, 2015).

Carbapenemase production is the key carbapenem resistance mechanism in CRE isolates (Nordmann *et al.*, 2011; Canton *et al.*, 2012). The most clinically relevant carbapenemases encountered in Enterobacteriaceae belong to Am-

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bler class A (mostly KPC-type) (Pitout *et al.*, 2015), Ambler class B (metallo- β -lactamases [MBLs] such as IMP-, VIM- and NDM-types) (Nordmann *et al.*, 2012; Dortet *et al.*, 2014), or Ambler Class D (OXA-48-like enzymes) (Albiger *et al.*, 2015). The first carbapenemase producer among the Enterobacteriaceae was identified in 1993 (Naas and Nordmann, 1994), and over the past 15 years the rate of carbapenemase-producing Enterobacteriaceae (CPE) has risen dramatically worldwide (Logan and Weinstein, 2017). Although carbapenemases have been identified in various species of Enterobacteriaceae and in non-fermentative bacteria (e.g., *Pseudomonas aeruginosa* and *Acinetobacter baumannii*), carbapenemase producers are principally identified among *Klebsiella pneumoniae* strains in which the most common carbapenemase detected was the KPC-type (Nordmann *et al.*, 2011; Canton *et al.*, 2012; Grundmann *et al.*, 2017).

In Italy, since their discovery in late 2008 (Giani *et al.*, 2009), KPC-producing *K. pneumoniae* (prevalently KPC-2 and KPC-3) have undergone rapid and extensive dissemination, becoming endemic in 2010 (Giani *et al.*, 2013). Recently, Grundmann *et al.* (Grundmann *et al.*, 2017) reported that among confirmed carbapenemase-producing *K. pneumoniae* isolates, 95.9% of strains were KPC-positive, 3% were VIM-positive, and 1% were NDM- or OXA-48-like-positive. To monitor CPE and recommend control measures to limit their spread in healthcare settings, in 2013, the Ministry of Health issued a letter asking the Italian regions to report all cases of bloodstream infections due to carbapenemase-producing *K. pneumoniae* or *Escherichia coli* (*E. coli*) (Ministry of Health of Italy, 2013).

Bacteria-producing carbapenemases are commonly susceptible to only a few drugs. One worrisome development in recent years has been the rapid and countrywide dissemination of CPE resistant to antibiotics of last resort (Tangden and Giske, 2015; Van Duin and Doi, 2017) and, in particular, to colistin, the key component of treatment combinations for patients infected with these microorganisms (Munoz-Price *et al.*, 2013). The extent of the problem in Italy is difficult to assess, but it has recently been reported that among carbapenemase-producing *K. pneumoniae* isolates 40.1% of strains were resistant to colistin (Grundmann *et al.*, 2017).

The aim of the present study was to monitor the antimicrobial susceptibilities and the proportion of CPE among carbapenem non-susceptible Enterobacteriaceae (CNSE) recovered from patients admitted to Perugia General Hospital, Italy, from January 2016 to March 2017, with a special focus on the presence and proportion of colistin-resistant strains.

MATERIALS AND METHODS

Study design

From January 2016 to March 2017, non-replicate clinical isolates of Enterobacteriaceae recovered from patients hospitalized in Medical, Surgery, Intensive Care Units and Hematology/Oncology wards were collected by the Clinical Microbiology Laboratory of Perugia General Hospital, Italy. In this study, we included only isolates non-susceptible (resistant or intermediate) to at least one carbapenem antibiotic: ertapenem minimum inhibitory concentration [MIC] >0.5 mg/L, and/or imipenem MIC >2 mg/L, and/or meropenem MIC >2 mg/L (EUCAST, 2017). The only exceptions were for *Proteus* spp., *Providencia* spp. and

Morganella spp., which may have elevated imipenem MIC by mechanisms other than carbapenemase production (EUCAST, 2017), so the inclusion criteria for these species were based only on ertapenem and meropenem MIC results. According to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2014) and the European Antimicrobial Resistance Surveillance Network (ECDC, 2013) we included in this study only the first isolate from each patient, irrespective of the body site from which the specimen was obtained or the antimicrobial pattern. Isolates from surveillance or screening cultures were excluded. Mortality rate was assessed only in patients with documented bacteremia (Villegas *et al.*, 2016).

Identification and antimicrobial susceptibility tests

Bacterial identification was carried out by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany) as described elsewhere (Mencacci *et al.*, 2013). Antimicrobial susceptibility testing was performed using the Phoenix Automated Microbiology System (Becton Dickinson Diagnostic Systems, Sparks, USA) or the Vitek-2 System (BioMérieux, Marcy l'Etoile, France) (Giani *et al.*, 2013; Bonura *et al.*, 2015). Confirmatory MIC testing was performed by Etest (BioMérieux, Marcy l'Etoile, France) (Giani *et al.*, 2013; Bonura *et al.*, 2015; Lefebvre *et al.*, 2015; Mehta *et al.*, 2015; Monari *et al.*, 2016a; Monari *et al.*, 2016b) and data were interpreted using clinical breakpoints according to European Committee on Antimicrobial Susceptibility Testing criteria 2017 (EUCAST, 2017).

Phenotypic detection of carbapenemase production

All isolates confirmed to be non-susceptible to at least one carbapenem antibiotic were tested for carbapenemase production using the commercial KPC + MBL Confirm ID Kit (Rosco Diagnostica A/S, Taastrup, Denmark) (Giske *et al.*, 2011; Jeong *et al.*, 2018) employing: MEM (10 μ g), MEM + phenyl-boronic acid (PBA; 600 μ g), MEM + dipicolinic acid (DPA; 1000 μ g), and MEM + cloxacillin (750 μ g) disks. Briefly, a 0.5 McFarland standards inoculum was prepared from CNSE isolates spread on cation-adjusted Mueller-Hinton II agar plates (Becton Dickinson Diagnostic Systems, Sparks, New Jersey, United States) and the disks were placed on each plate. Results were interpreted according to the manufacturer's instructions. In particular, production of KPC was considered when the growth inhibitory zone diameters seen around the MEM disk with PBA had increased by ≥ 5 mm compared to the growth inhibitory zone diameter seen around the disk containing MEM alone. Production of MBL was considered when the growth inhibitory zone diameter seen around the MEM disk with DPA had increased by ≥ 5 mm compared to the growth inhibitory zone diameter seen around the disk containing MEM alone.

Molecular detection of carbapenemase genes

Detection of *bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{NDM} genes in Modified Hodge Test (MHT)-positive isolates was performed to confirm the presence of carbapenemase in each isolate using a commercial multiplex PCR (hyplex SuperBug ID) according to the manufacturer's directions (Amplex Biosystems GmbH, Giessen, Germany) (Kaase *et al.*, 2012). All isolates recovered from blood samples or with the *bla*_{VIM} gene were sent to BMR Genomics (Padova, Italy) to amplify and sequence the *bla*_{KPC} or *bla*_{VIM} gene. For each

test isolate, one to two colonies were lightly picked from fresh overnight culture plates and suspended in 30 µL water. The suspension was transferred to a multiwell plate, heated at 100°C for 5 min, and sent to BMR Genomics to use as the template for subsequent DNA amplifications and sequencing. The full-length alleles of *bla*_{KPC} and *bla*_{VIM} were amplified and sequenced using primers: KPC_Forward: TGTCACGTATCGCCGTCTAG and KPC_Reverse: TTACTGCCCGTTGACGCCCAATCC (Kaczmarek *et al.*, 2006), and VIM1_Forward: ATGTAAAAGTTATTAGTAGTT and VIM1_Reverse: CTA CTGCGCGACTGAGCGATT (Del Franco *et al.*, 2015), respectively. The amplicons obtained were analyzed to identify the specific type of *bla*_{KPC} or *bla*_{VIM} gene by using the BLAST program available on the National Center for Biotechnology Information server (<http://blast.ncbi.nlm.nih.gov/>).

Data analysis

Data analysis of *in vitro* activity of antimicrobial agents was performed by using WHONET software, version 5.6, a program for the management of microbiology laboratory data that is available, free of charge, from the World Health Organization (O'Brien and Stelling, 2011). The chi-square test (χ^2) was used to analyze associations between categorical variables. A P-value ≤ 0.05 was considered statistically significant. Finally, to describe the association of resistant strains with patient outcome, survival distribution functions of both colistin resistant (Col-R) and colistin susceptible (Col-S) CPE isolated from blood samples were estimated using the Kaplan-Meier product-limit method (Geng *et al.*, 2018).

RESULTS

Characteristics of CNSE isolates

From January 2016 to March 2017, a total of 2936 non-replicate clinical isolates of Enterobacteriaceae were recovered from patients admitted to Perugia General Hospital. Among these isolates, 221 were non-susceptible (resistant or intermediate) to at least one of meropenem, ertapenem or imipenem (i.e., they were CNSE). The most common species was *K. pneumoniae* (n = 194, 87.8%), followed by *Enterobacter cloacae* (n = 13, 5.9%), *Citrobacter freundii* (n = 5, 2.3%), *E. coli* (n = 3, 1.4%), *Enterobacter aerogenes* (n = 2, 0.9%), *Proteus mirabilis* (n = 2, 0.9%), *Serratia marcescens* (n = 1, 0.4%), and *Morganella morganii* (n = 1, 0.4%). The majority of CNSE were from urine (n = 90, 40.7%) followed by the respiratory tract (n = 53, 24%), and blood samples (n = 31, 14%).

Phenotypic and molecular characterization of CPE isolates

CNSE were tested for carbapenemase production by phenotypic analysis using the commercial KPC + MBL Confirm ID Kit. The results showed that 173 (78.3%) isolates were CPE. The majority of CPE were from individuals aged 70 to 89 (n = 110, 64.3%). Demographic characteristics of the corresponding patients are listed in Table 1. Among these bacteria, the most common species was *K. pneumoniae* (n = 167, 96.5%), followed by *C. freundii* (n = 3, 1.7%), *E. cloacae* (n = 2, 1.2%), and *E. coli* (n = 1, 0.6%). Of note, this was the first detection of a carbapenemase-producing *E. coli* isolate in our hospital. The main sources of these bacteria were urine (n = 74, 42.7%), followed by the respiratory tract (n = 43, 24.9%), blood (n = 29, 16.8%), and

Table 1 - Demographic characteristics of patients (n = 171) admitted to Perugia General Hospital with carbapenemase-producing Enterobacteriaceae recovered from January 2016 to March 2017.

Gender	No. (%)
Male	90 (52.6)
Female	81 (47.4)
Age	Range
Mean	74 (19–98)
Median	78 (19–98)
Age group	No. (%)
0–39	8 (4.7)
40–49	4 (2.3)
50–59	10 (5.9)
60–69	25 (14.6)
70–79	51 (29.8)
80–89	59 (34.5)
≥ 90	14 (8.2)

wound exudates (n = 19, 11%). The remaining isolates (n = 8, 4.6%) were from other clinical specimens.

Molecular analysis showed that 166/173 (96%) of the isolates harbored the *bla*_{KPC} gene and 7/173 (4%) the *bla*_{VIM} gene. The *bla*_{KPC} gene was mainly detected in *K. pneumoniae* isolates (n = 164/166, 98.8%). The other two KPC-positive isolates were *E. cloacae* (n = 1/166, 0.6%) and *E. coli* (n = 1/166, 0.6%). The *bla*_{VIM} gene was harbored by *K. pneumoniae* (n = 3/7, 42.9%), *C. freundii* (n = 3/7, 42.9%), and *E. cloacae* (n = 1/7, 14.2%). Of note, all *C. freundii* (n = 3/3) harbored the *bla*_{VIM} gene. Given that the isolates harboring *bla*_{VIM} were from different bacterial species, we sequenced the alleles of this gene. All seven *bla*_{VIM}-containing isolates carried *bla*_{VIM-1}.

Antimicrobial susceptibilities of CPE isolates

Antimicrobial susceptibility tests against carbapenem antibiotics (Table 2) showed that 94.8% of the CPE isolates were resistant to ertapenem, 87.2% to imipenem, and 86.6% to meropenem. Most of the CPE (n = 140/173, 80.9%) were resistant to all three of the carbapenems tested. Antimicrobial susceptibility testing revealed that, among KPC-producing strains, 97% were resistant to ertapenem, 89.2% to imipenem and 89.2% to meropenem; among VIM-producing strains, 42.9% were resistant to ertapenem, 42.9% to imipenem and 28.6% to meropenem. In addition, 83.1% (n = 138/166) of KPC isolates were resistant to all three carbapenems tested, while 28.6% (n = 2/7) of VIM isolates showed pan-carbapenem resistance. Given that CPE are usually resistant to most antibiotics, we determined their antimicrobial susceptibility to third-generation cephalosporins and to last-line antibiotics recommended for treatment of infections caused by these pathogens. As shown in Table 2, all examined isolates were non-susceptible to all three cephalosporins tested; >50% of CPE were resistant to fosfomicin, amikacin and gentamicin and 38% of CPE (all belonging to *K. pneumoniae*) were resistant to colistin. The most active drug was tigecycline with only 12.3% of isolates being resistant. Antibiotic susceptibility testing showed that resistance to colistin was associated only with

Table 2 - Antimicrobial profile of all carbapenemase-producing Enterobacteriaceae (CPE) isolates and of CPE carrying bla_{KPC} or bla_{VIM}.

Antimicrobial agent	TOT (n = 173)							bla _{KPC} (n = 166)							bla _{VIM} (n = 7)							*P-value
	%R	%I	%S	MIC ₅₀	MIC ₉₀	Range	%R	%I	%S	MIC ₅₀	MIC ₉₀	Range	%R	%I	%S	MIC ₅₀	MIC ₉₀	Range				
Ertapenem	94.8	4.1	1.2	16	16	0.25-64	97	3	0	16	16	1-64	42.9	28.6	28.6	1	16	0.25-16	<0.0001			
Imipenem	87.2	8.7	4.1	32	32	1-64	89.2	7.8	3	32	32	1-64	42.9	28.6	28.6	8	64	1-64	<0.0001			
Meropenem	86.6	9.3	4.1	32	32	0.25-64	89.2	8.4	2.4	32	32	0.25-64	28.6	28.6	42.9	4	64	0.5-64	<0.0001			
Ceftazidime	100	0	0	128	128	32-256	100	0	0	128	128	23-256	100	0	0	64	256	64-256	-			
Cefotaxime	100	0	0	128	128	4-128	100	0	0	128	128	4-128	100	0	0	32	128	8-128	-			
Cefepime	94.7	5.3	0	64	128	2-128	95.2	4.8	0	64	128	2-128	71.4	28.6	0	16	64	2-64	-			
Fosfomycin	65.9	-	34.1	64	>256	6-2048	65.7	-	34.3	64	256	1-2048	0	-	100	16	64	2-64	-			
Amikacin	62.9	11.2	25.9	32	128	1-128	65.2	12.2	22.6	64	128	1-128	0	0	100	2	6	1-6	<0.0001			
Gentamicin	59.4	7.6	32.9	16	48	0.38-128	59.6	7.8	32.5	16	64	0.38-128	14.3	0	85.7	1	32	0.75-32	<0.0001			
Colistin	38	-	62	2	32	0.125-32	39.4	-	60.6	2	32	0.125-32	0	-	100	0.5	0.5	0.125-0.5	<0.0001			
Tigecycline	12.3	32.2	55.6	1	3	0.25-16	12.1	32.7	55.2	1	3	0.25-16	14.3	14.3	71.4	0.5	8	0.38-8	0.0280			

R = resistant; I = intermediate; S = susceptible. *Resistant isolates harboring bla_{KPC} vs. resistant isolates harboring bla_{VIM}. MICs interpreted using clinical breakpoints according to EUCAST (version 7.1), 2017. Range, MIC₅₀ and MIC₉₀: minimum inhibitory concentration in mg/L.

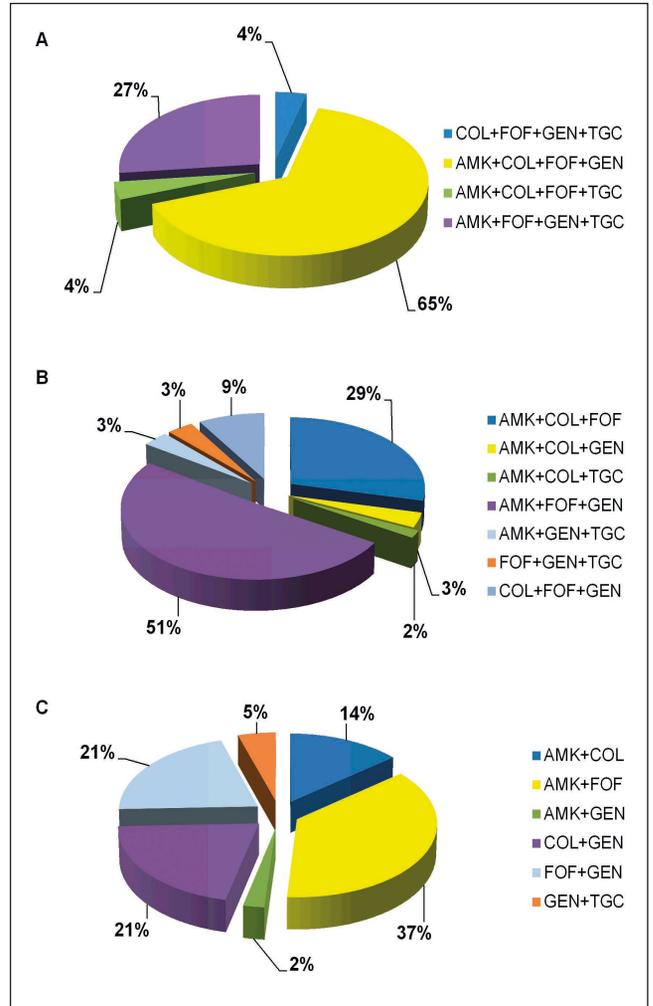


Figure 1 - Percentage of carbapenemase-producing Enterobacteriaceae (CPE) isolates resistant to four (n = 26) (A), three (n = 59) (B), or two (n = 43) (C) non-β-lactam agents.

isolates that were KPC-positive. In addition, no VIM-producing strains exhibited resistance to fosfomicin and amikacin (Table 2). Analysis of co-resistance to non-β-lactam agents revealed that: 1.7% (n = 3/173) of the isolates were resistant to fosfomicin, amikacin, gentamicin, colistin and tigecycline; 15.2% (n = 26/173) were resistant to four antibiotics; 34.1% (n = 59/173) to three drugs; and 24.8% (n = 43/173) to two antibiotics. Co-resistance to two or more non-β-lactam agents was exhibited by 79.2% of isolates (n = 137/173). In addition, 14.4% (n = 25/173) were resistant to only one drug (four isolates to amikacin, three to colistin, five to fosfomicin, 11 to gentamicin, two to tigecycline) and 9.8% (n = 17/173) were not resistant to any non-β-lactam agent tested. Antibiotic co-resistance percentages of CPE are shown in Figure 1 A, B, C.

Proportion of CPE isolates by hospital ward and clinical samples

The proportion of CPE strains among clinical CNSE isolates was evaluated by hospital ward and biological source (Table 3). The highest proportion of CPE was from Hematology/Oncology wards (88.9%), and, concerning the clinical specimens, from blood samples (93.5%). In particular, all CNSE recovered from blood of patients hospitalized in

Intensive Care Units and in Hematology/Oncology wards were carbapenemase producers.

Distribution of the 173 CPE isolates by hospital ward and biological source was also determined; 55.5% (n = 96) of these isolates were from Medical Units, 22.5% (n = 39) from Intensive Care Units, 17.4% (n = 30) from Surgical Units and 4.6% (n = 8) from Hematology/Oncology wards. As for the body source, 42.8% (n = 74) were from urine, 24.9% (n = 43) from the respiratory tract, 16.8% (n = 29) from blood, 11.0% (n = 19) from wound exudates and 4.6% (n = 8) from other sites.

Analysis of CPE recovered from the bloodstream

Given that 93.5% of CNSE isolated from blood samples were carbapenemase producers and that the detection of CPE in the bloodstream is usually indicative of a serious infection, these isolates were further analyzed. Among 29 such isolates, 28 were *K. pneumoniae* and one was *C. freundii*. Sequence analysis of the carbapenemase genes revealed that all the *K. pneumoniae* isolates harbored *bla_{KPC-3}*, while the *C. freundii* isolate harbored *bla_{VIM-1}*. Of these isolates, 89.6% (n = 26/29) were resistant to all three carbapenems tested and, among the remainder,

6.9% (n = 2/29) were resistant only to ertapenem. The *C. freundii* isolate was resistant only to imipenem. Antibiotic susceptibility testing using non-β-lactam agents showed that 55.2% (n = 16/29) of these CPE were Col-R. Analysis of co-resistances showed that these isolates, besides being resistant to colistin, were also resistant as follows: two to four other drugs (fosfomycin, gentamicin, amikacin and tigecycline); two to three other drugs (one isolate to fosfomycin, gentamicin and amikacin, one isolate to fosfomycin, amikacin and tigecycline); six to two other drugs (four to amikacin and gentamicin, two to amikacin and fosfomycin); five to one other antibiotic (three to gentamicin and two to amikacin). Details of the drug resistance profiles are presented in Table 4.

The overall 7- and 28-day mortality rates after bacteremia onset were 20.7% (n = 6/29) and 31% (n = 9/29) respectively. The majority (77.7%, n = 7/9) of the deaths occurred within 7 days of the first positive blood sample and, of note, all patients from whom Col-R strains were isolated, died within 6 days of bacteremia onset. The difference in mortality rate between Col-R and Col-S isolates (P=0.016) is shown by Kaplan-Meier survival estimates at 7 days (Figure 2) (Villegas et al., 2016).

Table 3 - Distribution of carbapenem non-susceptible Enterobacteriaceae (CNSE) isolates and proportion of carbapenemase-producing Enterobacteriaceae (CPE) isolates by Perugia General Hospital ward and clinical sample source.

Wards	Isolates											
	Urine		Respiratory tract		Wound exudates		Blood		Others		Total	
	CNSE	CPE (%)	CNSE	CPE (%)	CNSE	CPE (%)	CNSE	CPE (%)	CNSE	CPE (%)	CNSE	CPE (%)
Medical	66	54 (81.8)	19	14 (73.7)	23	16 (69.6)	11	10 (90.9)	5	2 (40.0)	124	96 (77.4)
Surgical	15	15 (100)	4	2 (50.0)	4	2 (50.0)	8	7 (87.5)	8	4 (50.0)	39	30 (76.9)
Intensive Care Unit	8	4 (50.0)	30	27 (90.0)	1	0	7	7 (100)	3	1 (33.3)	49	39 (79.6)
Hematology/Oncology	1	1 (100)	0	0	1	1 (100)	5	5 (100)	2	1 (50.0)	9	8 (88.9)
Total	90	74 (82.2)	53	43 (81.1)	29	19 (65.5)	31	29 (93.5)	18	8 (44.4)	221	173 (78.3)

Table 4 - Antimicrobial susceptibility of carbapenemase-producing Enterobacteriaceae (CPE) isolates recovered from clinical blood samples.

Antimicrobial agent	CPE (n = 29)					
	%R	%I	%S	MIC ₅₀	MIC ₉₀	Range
Ertapenem	96.6	0	3.4	8	16	0.5–64
Imipenem	75.9	20.7	3.4	16	32	2–64
Meropenem	75.9	13.8	10.3	16	64	1–64
Ceftazidime	100	0	0	128	128	64–256
Cefotaxime	100	0	0	128	128	8–128
Cefepime	96.6	3.4	0	64	128	2–128
Fosfomycin	51.7	-	48.3	48	256	16–512
Amikacin	51.7	20.7	27.6	24	128	2–128
Gentamicin	58.6	10.3	31	16	32	0.5–48
Colistin	55.2	-	44.8	4	16	0.5–32
Tigecycline	10.3	34.5	55.2	1	4	0.5–16

R = resistant; I = intermediate; S = susceptible. Range, MIC₅₀ and MIC₉₀; minimum inhibitory concentration in mg/L. MICs interpreted using clinical breakpoints according to EUCAST (version 7.1), 2017.

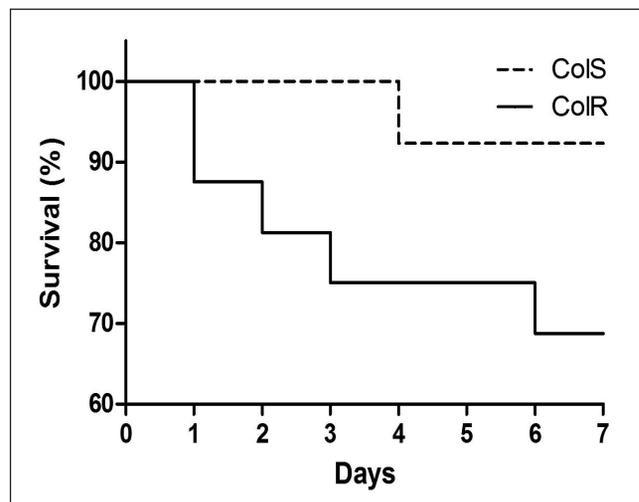


Figure 2 - Kaplan-Meier survival estimates at 7 days after bacteremia onset of patients with positive blood samples due to CPE isolates according to colistin resistance (Col-R) or susceptibility (Col-S) of the isolates. P=0.016 (log-rank test).

DISCUSSION

The worldwide dissemination of CPE isolates represents a serious public health threat because these bacteria are usually resistant to most antibiotics. Infections due to these organisms are often associated with high morbidity and mortality (Zarkotou *et al.*, 2011).

In this study, a total of 221 non-replicate clinical CNSE isolates were identified from patients admitted to Perugia General Hospital and among these bacteria the majority (78.3%) were CPE. Although *K. pneumoniae* was the main representative of the CPE isolates, the production of carbapenemase was also detected in *C. freundii*, *E. cloacae* and *E. coli*. KPC was the type of carbapenemase most frequently identified, and the remaining CPE produced VIM-type carbapenemases. Among the CPE, 38% were resistant to colistin and, of note, no VIM-positive strains exhibited resistance to colistin. The highest proportion of CPE among CNSE isolated from clinical samples was from blood samples. In addition, our results show that CPE were isolated not only in high-risk wards (ECDC, 2017) but in all sectors of Perugia General Hospital, confirming the high dissemination capacity of these bacteria.

In Europe, the most important CPE are *K. pneumoniae* and *E. coli*, albeit carbapenemases occur much less commonly in the latter than in *K. pneumoniae*. The most frequently detected carbapenemases are, among *K. pneumoniae* isolates, KPC enzymes, and, among *E. coli* isolates, OXA-48-like enzymes (Grundmann *et al.*, 2017). It has recently been reported that in Italy KPC-producing *K. pneumoniae* account for almost 96% of carbapenemase-producing *K. pneumoniae* isolates (Grundmann *et al.*, 2017), and that, on the contrary, KPC-producing *E. coli* isolates were very few (0.3% of the total number of invasive isolates tested in 2016) (ECDC, 2017). In line with previous studies (Grundmann *et al.*, 2010; Giani *et al.*, 2013; Monari *et al.*, 2016b; Grundmann *et al.*, 2017), here we report that most CPE isolates (166/173, 96%) were KPC producers and that *K. pneumoniae* was the species most frequently identified among them (164/173, 98.8%). In addition, we identified for the first time in our setting a KPC-positive *E. coli* isolate. This result is of concern given that this species is one of the most frequent causes of nosocomial and community-acquired bacterial infections (Tangden and Giske, 2015) and is able to spread into the community more easily than *K. pneumoniae*. Concerning the distribution of VIM enzymes, although this type of carbapenemase was first recognized in Italy in 1997 (Lauretti *et al.*, 1999), its exact distribution in our country is still difficult to establish. It has recently been reported (Grundmann *et al.*, 2017) that among carbapenemase-producing *K. pneumoniae* 3% of isolates produce a VIM-type carbapenemase. In this study, we report the detection for the first time in our hospital of the *bla*_{VIM} gene among Enterobacteriaceae. Although it was identified in only a few strains (7/173, 4%), this gene was detected in isolates belonging to different species (*K. pneumoniae*, *C. freundii* and *E. cloacae*). Sequence analysis revealed that all seven strains carried *bla*_{VIM-1}. VIM genes are often situated within class 1 integrons harbored on broad-host range plasmids (Matsumura *et al.*, 2017) that can easily be transferred among different species (Mathers *et al.*, 2015). Thus, our results suggest probable plasmid-dependent interspecies transmission. Further-

more, the presence of *K. pneumoniae* isolates harboring *bla*_{VIM} and *bla*_{KPC} suggests that these strains are not genetically correlated. Indeed, KPC-producing *K. pneumoniae* isolates are usually associated with sequence type (ST) 258 (Conte *et al.*, 2016; Peirano *et al.*, 2017; Samuelsen *et al.*, 2017), whereas VIM-producing *K. pneumoniae* are associated with sequence types unrelated to clonal group 258 (Sanchez-Romero *et al.*, 2012; Suzanne *et al.*, 2014; Ocampo *et al.*, 2016; Esposito *et al.*, 2017; Matsumura *et al.*, 2017).

Analysis of antimicrobial susceptibility showed that nearly all the CPE were resistant to at least one of the three carbapenems we tested. This result is worrying given that, to date, few other therapeutic options (tigecycline, aminoglycosides and polymyxins) (Kelesidis *et al.*, 2008; Tzouveleki *et al.*, 2012) remain for the treatment of patients infected with CPE. Recent data from the European Surveillance of Antimicrobial Consumption Network show that the consumption of polymyxins, mainly colistin, in Europe increased considerably between 2009 and 2015 (ECDC, 2014; ECDC, 2016). In parallel to this increase in colistin consumption, colistin resistance is increasing in CRE (ECDC, 2014; Monaco *et al.*, 2014; Grundmann *et al.*, 2017). Recently, in a surveillance study, Grundmann *et al.* (Grundmann *et al.*, 2017) reported that among carbapenemase-producing *K. pneumoniae* isolates, colistin resistance reached 40% and, in addition, 66.4% were resistant to fosfomycin. The rise of Col-R CPE isolates is very worrying given that colistin is the core component of treatment combinations (Munoz-Price *et al.*, 2013). Our data show that in Perugia General Hospital 38% of CPE (all *K. pneumoniae* KPC-positive) were resistant to colistin, an increase compared to our previous study (August 2014-January 2015) in which resistance to colistin was detected in 33.3% of carbapenem-resistant *K. pneumoniae* (Monari *et al.*, 2016b). Of note, the present study found colistin resistance only in strains producing KPC enzymes, but not in those producing VIM enzymes. This finding supports a previous study reporting that colistin sensitivity was higher among MBL-positive Enterobacteriaceae isolates than among those positive for KPC (Bradford *et al.*, 2015). Furthermore, our data show that all VIM-positive CPE were susceptible to fosfomycin and to amikacin and were generally less resistant than KPC isolates, suggesting that they could have less pathogenicity than KPC-positive isolates (Gaibani *et al.*, 2013).

Antimicrobial susceptibilities of CPE recovered from blood samples showed that 55% of these isolates were Col-R. In addition, the majority of deaths, within 7 days of bacteremia onset, occurred in patients infected with *K. pneumoniae* Col-R isolates. These data are alarming. As suggested by Giacobbe *et al.* (Giacobbe *et al.*, 2015), a high prevalence of colistin resistance among KPC-*K. pneumoniae* isolates has an unfavorable impact on the mortality of patients with bloodstream infections caused by these pathogens (Tumbarello *et al.*, 2012; Capone *et al.*, 2013; Tumbarello *et al.*, 2015).

In conclusion, despite the limitations deriving from a relatively short study period in a single hospital, our data confirm the strong need for antimicrobial stewardship programs to reduce the antibiotic selective pressure that favors the emergence and consequent spread of threatening bacteria and the need for surveillance systems to prevent and control nosocomial infections.

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