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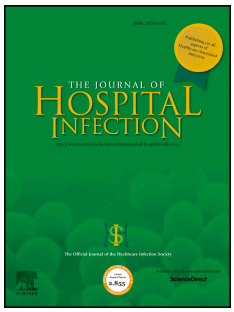

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Nosocomial transmission of carbapenem-resistant *Klebsiella pneumoniae* in an Italian university hospital: a molecular epidemiological study.

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Molecular epidemiology of KPC-*K. pneumoniae*

Abstract

Aim

To describe the phenotypic and genotypic profiles of *Klebsiella pneumonia* carbapenemase-producing *K. pneumonia* (KPC-Kp) strains isolated from patients with invasive infections at an Italian university hospital in order to assess the epidemiological trend.

Methods

An observational prospective study was carried out at the University Hospital of Sassari, Italy, to detect KPC-Kp strains in patients with invasive bacteraemia. Isolates were identified phenotypically; carbapenemase production was assessed using phenotypic and genotypic methods. Sequencing of *bla*KPC genes, pulsed-field gel electrophoresis, and multi locus sequence typing were performed.

Results

During the period 2015-2017 46 cases of invasive infection with *K. pneumoniae* were recorded. 67.4% of patients were male; mean age was 69.4 years. Most patients had at least one comorbidity (56.5%) and/or had been previously hospitalized (70.5%). 81.8% had current or recent medical device use and 85.4% had recent antibiotic exposure. Mortality rate was 52.3%. A multi-drug resistant pattern (including carbapenems, fluoroquinolones, third/fourth generation cephalosporins) was showed for all *K. pneumoniae* isolates. KPC-3 and -2 were produced by all strains. The most

frequent Sequence Types were 512 (91.3%) and 101 (8.7%), grouped into three clusters (A, A1, B).

Conclusions

A high incidence of KPC-Kp in patients with invasive infections was recorded at an Italian university hospital in comparison with that measured before 2015. It was confirmed the importance of KPC-3 carbapenemase variant, as previously reported by other Italian studies. High mortality and comorbidity rate seems associated with KPC-Kp infection.

Introduction

Carbapenems are antibiotics with a broad-spectrum anti-bacterial activity, including against extended-spectrum beta-lactamase (ESBL)-producing bacteria [1]. The spread of carbapenem-resistant *Enterobacteriaceae* (CRE) represents an important clinical and public health issue owing to few therapeutic options. In addition, many CRE strains frequently carry additional resistance genes to other non-β-lactam antibiotics, making these organisms resistant to the majority of the current antibiotic armamentarium. These bacteria are at best usually susceptible to only a few antimicrobials, such as polymyxins, tigecycline, fosfomycin, and nitrofurantoin [2].

Resistance to carbapenems may be associated with several mechanisms: modifications of the outer membrane permeability; up-regulation of efflux systems associated with hyperproduction of AmpC β-lactamases (cephalosporinases) or ESBLs; production of specific β-lactamases, such as carbapenemases [3]. Carbapenemases may be encoded by mobile genetic elements, which could be disseminated intra- and inter-species [3]

The Ambler classification separates β-lactamases (encoded by *bla* gene*)* into four classes A-D, based on their molecular structure [4]. Class A carbapenemase genes include *Klebsiella pneumoniae* carbapenemases (KPCs), the most frequent β-lactamases detected in carbapenem-resistant *K. pneumoniae* isolates [5].

Twelve variants of KPC have been reported (KPC-2 to KPC-13); however, the most frequently detected areKPC-2 and -3 [5,6].

Outbreaks of KPC-producing *K. pneumoniae* (KPC-Kp) have been reported worldwide and are responsible of numerous healthcare-associated infections [7],

particularly in hospital wards (e.g., long-term care, intensive care units –ICUs-) where vulnerable population groups are admitted [5,7]. Moreover, endemic distribution of KPC-Kp has been reported in US, China, Brazil, Argentina, Colombia, Taiwan, and Europe [8]. In Europe, the population-weighted mean percentage of KPC-Kp was 8.1% in 2015, ranging from 0.0% (Denmark, Estonia, Finland, Iceland, Latvia, Lithuania, Luxembourg, and Sweden) to 61.9% (Greece). Three countries (Greece, Italy, and Romania) reported carbapenem resistance rates that were substantially higher than in other European countries (61.9%, 33.5%, and 24.7 %, respectively) [9].

The first KPC-Kp infection in Italy was officially notified in 2008; since then, the proportion of KPC-Kp strains has increased significantly, associated with wide geographical spread within the country [8,10]. In 2015, the first case of bloodstream infection caused by KPC-Kp was described in Sassari, Italy, in a patient transferred out by a university hospital located in Northern Italy. Since then, several KPC-Kp cases have been recorded.

The aim of the present study is to describe phenotypically and genotypically strains of KPC-Kp isolated from invasive infections in Sassari, Italy, to better understand their epidemiological trend and which preventive measures could be implemented to address infection-related challenges.

Material and methods

Study design

An epidemiological, prospective study was designed following the implementation of a surveillance protocol for all invasive bacteremia caused by carbapenem-resistant or carbapenemase-producing *K. pneumoniae* and *Escherichia coli* at the University Hospital of Sassari, Italy, along with a systematic screening for the detection of colonized patients and contacts of newly infected patients in 2015 [12]. Study data were collected from medical records and microbiology laboratory databases. Definition of infection and infectious disease was based on microbiological and clinical data according to the agreement between the attending physician and the epidemiological team. They included the following variables: demographics (e.g., gender, age), comorbidities, history of community- or healthcare-associated infections, current and previous therapies prescribed during hospital stays, length of hospital and ICU stay, surgery, blood transfusion, invasive device use, previous hospitalizations, and recent antibiotic exposure.

Microbiological analysis

Identification of KPC-Kp strains and drug susceptibility test were performed using the Vitek-2 System (bioMérieux, Marcy l'Etoile, France). Drug susceptibility test and MIC values were assessed using breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [11]. Both phenotypic and genotypic methods were adopted to detect carbapenemase production.

All isolates resistant to imipenem and/or meropenem according to the EUCAST breakpoints and with minimum inhibitory concentrations for imipenem and/or meropenem ≥0.5 µg/ml [13] were further analyzed. Their resistance was assessed using a β-lactamase inhibitor disk placed on the surface of a Mueller Hinton Agar plates (Microbiol Diagnostici, Uta, Italy) ours. After overnight incubation at 37°C, a synergistic inhibition zone was interpreted as a positive result. The colorimetric RAPIDEC® CARBA NP test (bioMérieux, Marcy l'Etoile, France) was used to detect carbapenemase activity [14]. ESBL production was evaluated using the double-disk synergy test [15].

Carbapenem-resistant isolates were further investigated using molecular techniques through identification of the following genes: *bla*KPC, *bla*NDM, *bl*aVIM, *bla*OXA-48, and *bla*IMP (GeneXpert platform, Cepheid, Sunnyvale, CA). Sequencing of *bla*KPC genes through the BLAST program from the National Center for Biotechnology Information Web site was performed to detect KPC variants [16].

Pulsed-field gel electrophoresis (PFGE) was performed as previously described [17]. Isolates were considered clonally related if the Dice coefficient was >80%, while patterns with indistinguishable PFGE banding patterns (similarity coefficient>97%) were considered belonging to the same subtype [18]. Multi Locus Sequence Type was performed according to the protocol of the Pasteur Institute [19], amplifying and sequencing the internal fragments of seven *K. pneumoniae* housekeeping genes: gapA, infB, mdh, pgi, phoE, rpoB, and tonB. Sequence analysis was performed using Bioedit software and each single locus was compared with those included in the database of the Pasteur Institute to evaluate percentage of similarity and compatibility [19].

Statistical analysis

An electronic form was prepared to collect demographic, epidemiological, clinical, and microbiological variables. Absolute and relative (percentages) frequencies and means and standard deviations (SD) were used to summarize qualitative and normally-distributed quantitative variables, respectively. Data were analyzed using STATA version 14 (StataCorp, College Station, Texas).

Results

From November 2015 to May 2017 46 inpatients had invasive infections caused by *K. pneumoniae* resistant to carbapenems at the University Hospital of Sassari, Italy (Figure 1). There were 31 (67.4%) males and the mean (SD) age of the study population was 69.4 (13.0) years (Table 1). More than half (26 patients, 56.5%) had at least one comorbidity; the most prevalent were chronic obstructive pulmonary disease (COPD) (17.1%) and liver disease (11.4%) (Table 2).

The commonest wards where patients were admitted to were medicine (30.4%), ICU (26.1%), and long-term care (19.6%). Previous hospitalization was recorded in 31 (67.4%) patients. Of the 44 patients for whom full medical records were available 14 (31.8%) underwent surgical interventions and 36(81.8%) had at least one medical device (Table 1), most commonly central venous and urinary catheters.

More than two thirds (35/41, 85.4%) of patients for whom previous drug histories were available were previously treated with antibiotics, most commonly piperacillin/tazobactam (16 cases) (Table 3).

Forty two of the 44 patients whose full medical records were available were administered antibiotics during their stay, most frequently meropenem (20 cases) and colistin (17 cases).

The following specimens were collected for microbiological examination: blood cultures (33, 71.7%); respiratory secretions (8, 17.4%), and central venous catheter tips (5, 10.9%). Nineteen of 41 patients from whom rectal swabs were collected were positive for *K. pneumoniae*. The mean (SD) time between hospital admission and first detection of *K. pneumonia* was 33.1 (18.9) days.

Twenty three of 44 (52.3%) patients died. Demographic, clinical, and epidemiological variables were not statistically associated with mortality.

All isolates were resistant to carbapenems and other β-lactam antibiotics and most (91.0%) were ciprofloxacin resistant. Resistance to other antibiotic classes was more variable (Table 4). All isolates produced KPC: 28 (61.0%) and 18 (39.0%) were KPC-3 and -2 variants, respectively.

PFGE showed the poorest variability between isolates, with electrophoresis bands ranging from 18 to 22 and with a molecular weight ranging from 100 Kb to >1,000 Kb. Three distinct clusters (A, A_1, B), identified using Gel Compare II software, were sized 29, 10 and 7 isolates, with a total Dice's coefficient of 77.42% (Figure. 2). The most prevalent lineage was Sequence Type 512 (42, 91.3%), followed by Sequence Type101 (4,8.7%).

Discussion

Our study showed a high incidence of KPC-Kp infections during the study period (46 bacterial strains from November 2015-May 2017), in comparison with the time period preceding the implementation of an active and specific surveillance system in our university hospital. Based on patient medical history, we deemed that all cases were hospital-acquired. Two sequence types (*i.e.*, 512 and 101) were found.

WHO published a list of priority pathogens posing a greatest threat to human health, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and carbapenem-resistant *Enterobacteriaceae*, such as *K. pneumoniae*. In Italy, carbapenem-resistant *K. pneumoniae* has dramatically increased in both hospitals and long-term care settings after the detection of the first isolate in 2009 [20,21]. The current study shows the spread of epidemic clones of KPC-Kp within a hospital setting. KPC-3 was the predominant variant, confirming previous Italian estimates [22].

We found that the majority of patients had comorbidities, use of medical devices, recent antibiotic use and previous exposure to healthcare settings (usually hospitals); surgery and ICU admission were also common. These observations are consistent with previous epidemiological studies [23,24].

The high mortality rate (52.3%) could be at least partly explained by the severity of KPC-Kp infection. However, this hypothesis needs to be confirmed by an ad hoc analytical study, designed to collect all variables potentially associated with mortality. Studies have demonstrated that the Sequence Type 512, included in the most globally prevalent clonal group 258, has an estimated mortality rate higher than 50%. Both sequence types we found are endemic in Italy [25]. However, apart from the intrinsic virulence of these bacteria the serious underlying of infected patients is likely to itself be a risk factor for mortality.

Increasing availability of advanced molecular tools as point of care diagnostics could help detect plasmids and genes involved in the hyper-virulence of KP strains. A recent study carried out in China on an outbreak caused by hyper-virulent strains showed that plasmid curing experiments and whole genome sequencing can better characterize the molecular profile of the bacteria, allowing the prescription of tailored therapies and more appropriate infection control interventions [26].

Our genotypic analysis based on PFGE and Multi Locus Sequence Type, with detection of only 2 different Sequence Types (512 and 101) during a long-term surveillance (*i.e.*, 20 months), underscores poor standards of hospital infection control [27]. Whilst our study did not ascertain the entire molecular pattern of isolates, including the detection of hyper-virulence plasmids, we were able to show the relatedness of isolates, which will be important in planning infection control interventions.

Clinical and public health concerns have been raised on the emergence and spread of colistin-resistant KPC-Kp strains [28]. This antibiotic resistance is usually associated with a MDR pattern and, then, with few therapeutic options [29]. For the first time, we found three colistin-resistant cases in our geographical area. National and local strategies and policies need to reinforce the need for antimicrobial stewardship interventions to combat this emerging public health threat.

A recent study showed the role played by 3 major risk factors associated with invasive infections caused by carbapenem-resistant *K. pneumoniae*: colonization pressure, previous carbapenem exposure, and comorbidities [24]. Two of these can be potentially modifiable through screening of patients for the presence of

carbapenemase-producing bacteria and good infection control, and reduction of carbapenem prescription through antimicrobial stewardship.

Our study shows several limitations. A part from the above-mentioned availability of advanced molecular techniques, we could not retrieve some clinical and epidemiological information (missing data for a few patients); furthermore, we did not collect important host-related variables to carry out a comprehensive analysis of the mortality- and morbidity-associated risk factors (e.g., types of medical devices). Design of analytical studies aimed at evaluating the association between mortality and epidemiologic, demographic, and clinical covariates would be key to plan preventative interventions.

Based on the Italian experience, a surveillance system for WHO priority agents is needed in order to better assess the dynamic relationship between human beings and MDR bacteria. Recently, Italian regional governments have issued a set of recommendations for healthcare professionals and policy-makers aimed to reinforce surveillance programs for biological agents based on monitoring activities of infections caused by alert organisms, networking of laboratories, conventional and molecular epidemiological investigations, and antimicrobial stewardship programs. Implementation and scale-up of molecular typing services and point of care diagnostics should emerge from these recommendations.

In conclusion, our study underlines the growing problem of KPC-Kp in Italian hospitals, and highlights that better hospital infection prevention and control will be needed to combat the threat posed by these bacteria.

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Declaration of interest

The Authors declared no competing interests.

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Table 1: Demographic, epidemiological, and clinical characteristics of a cohort of patients with *Klebsiella pneumoniae* infection in Sassari, Italy.

Variable		**No. (%) cases**
Male (n=46)		31 (67.4)
Mean (SD) age, years (n=46)		69.3 (13.0)
Hospital stay (n=46)	*Medicine*	14 (30.4)
	ICU	12 (26.1)
	Long-term care	9 (19.6)
	Hematology	6 (13.0)
	Infectious diseases	5 (10.9)
Previous hospital admissions (n=44)		31 (70.5)
*Medical device (n=44)**		36 (81.8)
Surgical intervention (n=44)		14 (31.8)
Comorbidity (n=46)		26 (56.5)
Previous ICU admission (n=44)		15 (34.1)
Deaths (n=44)		23 (52.3)
Positive rectal swab for KPC (n=41)		19 (46.3)

* Central venous catheter 14; Peripheral venous catheter 2; Urinary catheter 8; Arterial line 2; Percutaneous endoscopic gastrostomy 1; Portacath 1; Tracheal tube 1

362 **Table 2: .Comorbidities in the cohort of 46 patients with *Klebsiella pneumoniae***

363 **infection.**

Comorbidity	**No. (%) cases**
COPD	6 (17.1)
Hepatitis/cirrhosis	4 (11.4)
Leukaemia/Lymphoma	3 (8.6)
Cardiomyopathy	3 (8.6)
Diabetes	3 (8.6)
Hypertension	3 (8.6)
Anaemia	2 (5.7)
Cholecystitis	2 (5.7)
Gammopathy	1 (2.9)
Neutropenia	1 (2.9)
Bladder cancer	1 (2.9)
Psoriasis	1 (2.9)
Oesophageal cancer	1 (2.9)
Multiple sclerosis	1 (2.9)
Tetraplegia	1 (2.9)
Pulmonary embolism	1 (2.9)
Chronic renal failure	1 (2.9)

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366 **Table 3: Previous and current exposure to antibiotics in patients with *Klebsiella***

367 ***pneumoniae* infection.**

	No. (%) patients with antibiotic exposure:	
Antibiotics	**Previously (n=41)**	**Current (n=44)**
Amoxicillin/clavulanic acid	7 (17.1)	-
Piperacillin/tazobactam	16 (39.0)	10 (22.7)
Meropenem	7 (17.1)	20 (45.5)
Imipenem	2 (4.9)	2 (4.5)
Vancomycin	4 (9.8)	1 (2.3)
Teicoplanin	3 (7.3)	-
Colistin	4 (9.8)	17 (38.6)
Claritromycin	3 (7.3)	-
Cefixime	-	1 (2.3)
Cefotaxime	3 (7.3)	-
Ceftriaxone	1 (2.4)	-
Cotrimoxazole	1 (2.4)	1 (2.3)
Linezolid	4 (9.8)	-
Tigecyclin	1 (2.4)	9 (20.5)
Gentamycin	1 (2.4)	5 (11.4)
Cyprofloxacin	1 (2.4)	2 (4.5)
Levofloxacin	3 (7.3)	7 (15.9)
Fosfomycin	-	2 (4.5)
Rifampicin	-	2 (4.5)
Metronidazole	-	1 (2.3)

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370 **Table 4: Antibiotic susceptibility testing of 46 isolates of *Klebsiella pneumoniae***

371 **isolates from the University Hospital of Sassari, Italy.**

Antibiotic	**No. (%) of isolates that were:**	
	Intermediately resistant	**Resistant**
Amikacin	-	27 (58.7)
Amoxicillin/clavulanic acid	-	46 (100)
Cefepime	-	44 (95.6)
Cefotaxime	-	46 (100)
Ceftazidime	-	46 (100)
Ciprofloxacin	-	42 (91.3)
Colistin	-	6 (13.0)
Ertapenem	-	46 (100)
Fosfomycin	-	18 (39.1)
Gentamicin	6 (13.0)	13 (28.3)
Imipenem	-	42 (91.3)
Meropenem	-	46 (100)
Piperacillin/tazobactam	-	46 (100)
Tigecycline	8 (17.4)	23 (50)
Trimetoprim/sulfametoxazole	-	38 (82.6)

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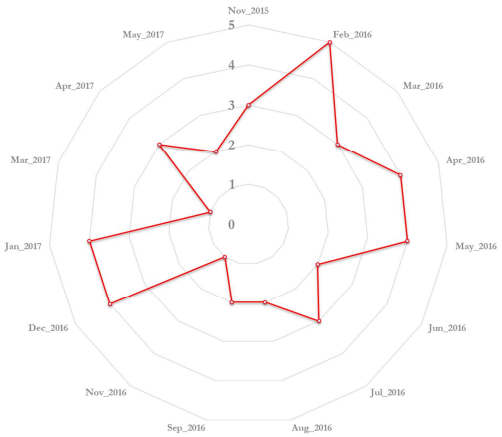
375 **Figure 1: Temporal distribution of *Klebsiella pneumoniae*-KPC strains detection in the**

376 **University Hospital of Sassari, Italy.**



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379 **Figure 2: Dendrogram and electrophoretic bands of *Klebsiella pneumoniae*-KPC**

380 **isolates.**

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