Emergence of carbapenem-resistant 
*Klebsiella Pneumoniae* strains producing KPC-3
in Brescia Hospital, Italy

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**INTRODUCTION**

Nosocomial infections caused by multidrug-resistant microorganisms are increasing. Recently, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has rapidly emerged as a new multidrug-resistant nosocomial pathogen (Gupta *et al.*, 2011; Sisto *et al.*, 2012). Indeed, *K. pneumoniae* can survive for several hours on the hands of hospital personnel, which likely facilitates nosocomial spread (Casewell *et al.*, 1977), and silently colonize hospital personnel or patients by establishing residence in the gastrointestinal tract without causing any sign of infection (Selden *et al.*, 1971). Therefore, colonized individuals can be asymptomatic carriers for long periods of time and act as reservoirs for continuous transmissions, making it difficult to control the spread and to stop outbreaks (Poh *et al.*, 1993). Controlling the spread of this pathogen by screening patients, personnel and the environment should represent a high priority in infection control progression. Infections caused by CRKP strains, susceptible to colistin and tigecycline only (Livermore *et al.*, 2011), have limited treatment options and have been associated with high mortality rates (Patel *et al.*, 2008). The emergence of carbapenem-resistant strains has become critical for

**SUMMARY**

Carbapenem-resistant *K. pneumoniae* has recently been reported as a new multidrug-resistant nosocomial pathogen. This study reports the emergence of carbapenem-resistant *K. pneumoniae* strains in Brescia Civic Hospital, Italy.

Different samples, collected from April 2012 to February 2013, showed that 29 patients presented infections from multidrug-resistant *K. pneumoniae* and three of these patients were intestinal carriers. In total, 40 carbapenem-resistant *K. pneumoniae* strains were isolated from multiple specimens of these patients. In 39 out of 40 samples, we identified the *bla*<sub>KPC-3</sub> carbapenemase gene variant responsible for bacterial carbapenem resistance. The DiversiLab analysis showed four different genetic patterns within multidrug-resistant *K. pneumoniae* isolates, with pattern 1 and 2 including 95% of the bacterial strains. Carbapenem-resistant *K. pneumoniae* strains belonging to patterns 1 and 2 were also detected in the intestinal tract of the three asymptomatic carriers. Moreover, isolation of the same strains in other body sites of the same patients and in bronchial fluid of a non-colonized patient in the same ward indicates an initial dissemination of this pathogen.

Our results highlight the emergence of carbapenemase-producing *K. pneumoniae* strains in different hospital wards and the urgent need for infection control, antibiotic stewardship programmes and utilization of a surveillance and prevention system.

**KEY WORDS:** Klebsiella pneumoniae, Multidrug-resistant, Carbapenem-resistant, *bla*<sub>KPC</sub>, KPC-3, DiversiLab.
prevention and containment of *K. pneumoniae* infection, and has highlighted problems for the correct identification of resistant pathogens by diagnostic microbiology laboratories. Carbapenem resistance is primarily caused by plasmid-encoded KPCs (Nordmann *et al.*, 2009), a new variant of class A beta-lactamase enzymes capable of hydrolyzing all known beta-lactam antibiotics and displaying resistance to beta-lactamase inhibitors.

Ten KPC gene (*bla*KPC) variants described to date are characterized by nonsynonymous single nucleotide substitutions within four codons (nucleotides 147, 308, 716 and 814) (Chen *et al.*, 2011). KPC-2 and KPC-3 variants account for most epidemic outbreaks (Nordmann *et al.*, 2009) and are currently the most widespread in Europe and Northern Italy (Cantón *et al.*, 2012; Migliavacca *et al.*, 2013). Detection of carbapenemases (KPCs) by susceptibility testing is challenging, due to the heterogeneous expression of the beta-lactam resistance by multiple determinants (Marshall *et al.*, 2009), and results vary with different methods (Bulik *et al.*, 2010). Moreover, detection of isolates harbouring carbapenemases can be inconsistent using automated systems, often requiring subsequent confirmatory tests (Tenover *et al.*, 2006; Bulik *et al.*, 2010). Given these limitations, molecular detection of *bla*KPC genes by polymerase chain reaction (PCR) has been proposed as the gold standard for detection of KPC-bearing microorganisms (Nordmann *et al.*, 2009).

Epidemiological typing is important in order to quickly detect ongoing outbreaks and define the nosocomial epidemiology of multidrug-resistant pathogens. Molecular typing approaches, such as pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing, have been used (Poh *et al.*, 1993; Podschun *et al.*, 1998). PFGE is generally considered the “gold standard” method for typing many bacterial species, but this approach has the drawbacks of being time-consuming and poorly reproducible (Podschun *et al.*, 1998; Chuang *et al.*, 2010; Brolund *et al.*, 2010; Ligozzi *et al.*, 2010).

On the other hand, the recently introduced DiversiLab (DL) system (BioMerieux) potentially overcomes these limitations (Podschun *et al.*, 1998; Chuang *et al.*, 2010; Brolund *et al.*, 2010; Ligozzi *et al.*, 2010).

Here, we report on the presence of CRKP strains at the Civic Hospital of Brescia, Italy. We analyzed the antibiotic susceptibility of the *K. pneumoniae* strains and investigated the presence of plasmids bearing the KPC genes. Finally, we typed the *K. pneumoniae* strains by DL system to identify the major clusters circulating in the Brescia hospital wards and to detect possible clonal relationships among isolates.

**MATERIALS AND METHODS**

**Bacterial identification and antimicrobial susceptibility**

From April 2012 to February 2013, CRKP strains isolated from different samples (urine samples, tracheal aspirates, rectal swabs, injury swabs, blood cultures, abdominal drainage liquid and vaginal swabs) were collected at the Microbiology Laboratory of the Civic Hospital of Brescia, Italy. Early screening cultures for *K. pneumoniae* were performed on MacConkey agar (BioMerieux, Florence, Italy) and on selective media for carbapenem-resistant Gram negative bacteria (CHROMagar KPC medium; BioMerieux), at 37°C for 24/48 h in aerobic atmosphere. Identification and determination of antibiotic susceptibility of all *K. pneumoniae* isolates were performed using the VITEK 2 system (BioMerieux, Florence, Italy). *E. Coli* ATCC 25922 was used as quality control strain for antibiotic susceptibility testing. *K. pneumoniae* isolates with a pattern of multiresistance (MIC for meropenem >0.5 mg/L) were also cultured on MacConkey agar containing 1 µg/mL of meropenem at 37°C for 24h in aerobic atmosphere (Wiener *et al.*, 2010) to confirm their carbapenem-resistance. Carbapenem resistance was evaluated according to the breakpoint diameters established by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (D<=16 mm).

**Molecular detection of *bla*KPC gene**

Detection of *bla*KPC gene and its variants in CRKP isolates was performed by PCR. Plasmid DNA was extracted from bacterial colonies using the PureYield Plasmid Miniprep System.
KPC-3 producing K. pneumoniae in Brescia, Italy

(KPromega, USA) and used as template in a polymerase chain reaction (PCR). Molecular probes were designed to be complementary to the target gene and to detect all known bla<sub>KPC</sub> genes, since no polymorphisms were present at the primer binding sites. The Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov) was used to perform similarity searches to rule out potential cross-reactivity between probes and nonspecific targets. Primer sequences were 5'-TATCGTTGATGTCACTGTATCG-3' (forward) and 5'-ATTTTCA-GAGCCTTACTGCCC-3' (reverse) (Tm 53°C, fragment size 901bp) (Integrated DNA Technologies, San Jose, CA, USA). PCR conditions were as follows: a denaturation step at 95°C for 5 min, followed by 40 cycles 95°C/45 s, Tm°C/45 s, 72°C/60 s and finally an elongation step 72°C/7 min. Then, plasmid DNA sequencing of a single KPC<sub>K. pneumoniae</sub> isolate was performed.

**RESULTS**

**Screening for K. pneumoniae isolates and determination of their antibiotic susceptibility**

*K. pneumoniae* was isolated in different samples (22.5% in urine samples, 42.5% tracheal aspirates, 7.5% rectal swabs, 15% injury swabs, 7.5% blood cultures, 2.5% abdominal drainage liquid and 2.5% vaginal swabs) from patients admitted to medical wards (55%), surgical wards (15%) and intensive care units (30%) from April 2012 to February 2013. In total, 40 multidrug-resistant *K. pneumoniae* were detected in multiple clinical samples from 29 patients (76% males, 24% females), with a mean age of 66 years (range 24 to 83). All isolates proved resistant to tested beta-lactam antibiotics, whereas 2.5% of the examined isolates were sensitive to amikacin (MIC<=8 mg/L), 22.5% to fosfomycin (MIC<=16 mg/L) and 32.5% to gentamicin (MIC<=2 mg/L) (Table I). Instead, all strains were sensitive to both colistin (MIC<=16 mg/L) and tigecycline (MIC<=1 mg/L) (Tab. 1). Moreover, nine of the 29 patients presented multiple *K. pneumoniae* infections in different body sites. In the same period of observation, 122 hospitalized patients at the intensive care unit 2 (ICU2) of Brescia Civic Hospital were screened by rectal swabs for the detection of intestinal colonization of multidrug-resistant *K. pneumoniae*. Among 122 patients in ICU2, three (2.5%) were found to be colonized by CRKP and two of the colonized patients also had positive CRKP cultures from other body sites, suggesting a self-infection. Among the patients who were found to be non carriers (97.5%), one patient proved positive for CRKP in bronchial fluid, thus excluding self-infection in the absence of any intestinal colonization and suggesting a possible nosocomial infection.

**Typing of K. pneumoniae strains by the DL system**

The 40 isolates of CRKP were then analyzed by the DL system, revealing four different genetic clusters. Figure 1 shows the results of rep-PCR by comparing virtual gel images. The dendogram analysis showed two main patterns, 1 and 2, characterized by an average of 90% similarity. Pattern 2, the major rep-PCR clus-
ter, included 30 isolates (75%), whereas pattern 1, the second major cluster, included eight isolates (20%). Patterns 3 and 4 integrated a single isolate each. The three CRKP strains isolated from intestinal carriers belonged to pattern 1 (n=1) and 2 (n=2) and the same bacterial isolate was observed in different body site of colonized patients. Figure 2 shows the distribution of rep-PCR patterns in the different hospital wards. Strains belonging to pattern 2 were dispersed among the most hospital units (9 out of 9; 100%). On the other hand, strains belonging to pattern 1 were present only in four out of nine wards examined (44.4%), namely in Infectious Diseases, General Surgery and ICU1 and ICU2 wards. Both patterns 1 and 2 were circulating in the ICUs, Infectious Diseases and General Surgery wards. The two bacterial strains belonging to patterns 3 and 4 were isolated in the Medicine ward and ICU2, respectively.

Detection of bla <sup>KPC</sup> gene variants responsible for <i>K. pneumoniae</i> multidrug-resistance

Plasmid DNA extracted from all 40 isolates of CRKP was analyzed by PCR for the presence of the bla <sup>KPC</sup> gene and its variants. The bla <sup>KPC</sup> gene was present in 39 out of 40 samples (97%). Gene sequencing of KPC <i>K. pneumoniae</i> iso-
lates for each DL cluster identified in patterns 1, 2 and 3 disclosed the \textit{bla}_{KPC-3} (C\textsubscript{\textsuperscript{T}} 814) gene as the agent responsible for bacterial multidrug resistance. On the other hand, this gene was not detected in the bacterial isolate representative of pattern 4.

**DISCUSSION**

Carbapenem resistance is primarily caused by KPCs (Nordmann \textit{et al.}, 2009) and the genes encoding carbapenemases are located on mobile genetic elements, which may readily transfer among \textit{Enterobacteriaceae}, principally \textit{E. Coli}, \textit{Enterobacter spp}, and \textit{K. pneumoniae} (Woodford N \textit{et al.}, 2011; Livermore DM, 2012). Carbapenem resistance among \textit{K. pneumoniae} strains is an emerging public health concern of clinical significance and worldwide importance (Gupta \textit{et al.}, 2011) and \textit{K. pneumoniae} strains carrying acquired carbapenemases are increasingly reported (Queenan AM \textit{et al.}, 2007). From April 2012 to February 2013, 40 CRKP strains were identified from different multiple clinical samples from 29 patients admitted in different wards of the Civic Hospital in Brescia, Italy. These bacterial isolates proved sensitive to colistin and tigecycline only (Tab. 1). However, these two antibiotics possess significant therapeutic limitations: colistin causes nephrotoxicity and shows uncertain efficacy in pulmonary infections, whereas tigecycline is not useful in urinary infections owing to low urinary levels (Livermore \textit{et al.}, 2005; Li \textit{et al.}, 2006). Moreover, even if 32.5\% of the examined CRKP isolates were susceptible to gentamicin and 22.5\% to fosfomycin, these antibiotics do not represent an optimal treatment choice. In fact, fosfomycin may be highly appropriate for urinary infections, but its use is questionable for severe infections at other body sites (Falagas \textit{et al.}, 2010), whereas gentamicin inhibits less than 50\% of the isolates (Sundin \textit{et al.}, 2001). However, detection of carbapenem resistance by antibiotic susceptibility testing is challenging, due to the heterogeneous expression of \(\beta\)-lactam resistance by multiple determinants (Anderson, 2007; Chen \textit{et al.}, 2011). Indeed, various studies have reported that carbapenem-resistant bacteria may be incorrectly identified, thus resulting in inappropriate selection of therapy (Anderson, 2007; Chen \textit{et al.}, 2011). In order to overcome these shortcomings, the

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>% Resistant</th>
<th>% Susceptibility</th>
<th>% Intermediately Active</th>
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<tbody>
<tr>
<td>Amikacin</td>
<td>97.5%</td>
<td>2.5%</td>
<td>0%</td>
</tr>
<tr>
<td>Amoxicillin + Clavulanic Acid</td>
<td>100%</td>
<td>0%</td>
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<tr>
<td>Cefepime</td>
<td>97.5%</td>
<td>0%</td>
<td>2.5%</td>
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<tr>
<td>Ceftazidime</td>
<td>100%</td>
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<td>Ciprofloxacin</td>
<td>100%</td>
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<tr>
<td>Colistin</td>
<td>0%</td>
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<tr>
<td>Fosfomycin</td>
<td>77.5%</td>
<td>22.5%</td>
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<tr>
<td>Gentamicin</td>
<td>5%</td>
<td>32.5%</td>
<td>62.5%</td>
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<tr>
<td>Imipenem</td>
<td>97.5%</td>
<td>0%</td>
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<td>Meropenem</td>
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<td>Piperacillin + Tazobactam</td>
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<td>Trimethoprim + Sulfamethoxazole</td>
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<td>Tigecycline</td>
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EUCAST has recently lowered the susceptibility breakpoints for meropenem and imipenem to ≤2 μg/ml (Giske et al., 2012). Nevertheless, susceptibility testing results still vary among the different methods (Marschall et al., 2009). Given these limitations, molecular detection of blaKPC genes by PCR has been proposed as the gold standard for detection of KPC-bearing microorganisms (Nordmann et al., 2009). Moreover, plasmid profiling is particularly useful to track the spread of plasmids encoding antimicrobial resistance genes among strains (Bingen et al., 1993). In the light of a potential rapid horizontal transmission of these genes between Enterobacteriaceae, the prompt recognition of KPC-producing strains is critical to control their spread in long-term care hospital settings. This study undertook a specifically designed PCR to detect all known blaKPC genes on plasmid DNA extracted from K. pneumoniae isolates. Moreover, some evidence suggests that mutations within blaKPC may have functional significance (Alba et al., 2005; Wolter et al., 2009). For instance, KPC-3 (C→T 814) exhibits a higher rate of ceftazidime hydrolysis than KPC-2, but has lower affinity for cefoxitin (Alba et al., 2005). Consequently, the identification of a specific blaKPC gene variant might be relevant in the selection of an appropriate therapy. At present, plasmid gene sequencing is the definitive method for the identification of blaKPC variants. In our study, gene sequencing showed that KPC-3 was the only KPC variant represented in carbapenem-resistant K. pneumoniae strains circulating at Brescia Civic Hospital and that a single CRKP isolate was blaKPC negative. Although we did not determine the mechanism of resistance of this isolate, it is likely that alternative mechanisms of resistance are involved, such as a reduced outer-membrane permeability associated with porin loss (Rodriguez-Martinez et al., 2009).

Epidemiological typing tools are essential to rapidly track the source and trace the spread of hospital-associated pathogens. PFGE analysis currently represents a widely used method due to its excellent discriminatory power. However, PFGE typing is time-consuming, requires rigorous standardization and experienced personnel to achieve reproducible results over time and place. Furthermore, there is no consensus nomenclature for PFGE patterns and no common international database available for comparison. Our study typed the CRKP strains by DL, a semi-automated rep-PCR system (Healy et al., 2005), in agreement with other typing methods (Ligozzi et al., 2008; Brolund et al., 2010; Fluit, 2010). DL possesses the ability to distinguish isolates belonging to different phylogenetic clonal groups with a slightly more discriminatory capacity than PFGE (Bonacorsi et al., 2009). Indeed, due to its high discriminatory power, DL is useful in detecting clonal relationships during a bacterial outbreak (Wiener, 2010). Analysis of the 40 CRKP isolates by the DL system revealed four different patterns. In particular, the dendogram analysis showed two main patterns, patterns 1 and 2. CRKP strains belonging to patterns 1 and 2 were also detected in the intestinal tract of three hospitalized patients at the ICU2 during the study period. It is worth noting that two out of three patients colonized with CRKP strains showed the same bacterial isolate in different body sites. Moreover, a CRPK strain was isolated in a patient admitted to the ICU2 ward in the absence of any intestinal colonization. This finding suggests the possible contribution of colonized patients to clonal dissemination and nosocomial transmission of these bacteria.

Effective control of K. pneumoniae dissemination requires a detailed understanding of how transmission occurs. Both clonal and horizontal transmission may account for the hyper-transmissibility of the epidemic strains. Therefore, the possibility to identify the blaKPC variants by plasmid gene sequencing and epidemiological typing tools should guide future infection control efforts. Accurate detection of the emerging clone is crucial both for the selection of appropriate therapeutic regimens and to control the spread of KPC-type enzymes. Genetic and typing data could allow not only the identification of unexpected modes of transmission but also link patients directly to environmental or infrastructure isolates, allowing a better refinement in cleaning and decontamination procedures by providing insight as to how and when contamination occurred. Beyond application to outbreak containment, typing by DL might provide a powerful tool to define the nosocomial epidemiology of important health care-associ-
ated pathogens with unprecedented precision. The presence of CRKP strains in Brescia Civic Hospital wards should alert medical authorities to include guidelines on strategies for carrier screening to avoid or control possible outbreaks of these microorganisms in the hospital environment.

REFERENCES


