Emergence of Pseudomonas aeruginosa with KPC-type carbapenemase in a teaching hospital: an 8-year study

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An outbreak of Klebsiella pneumoniae carbapenemase (KPC)-producing K. pneumoniae occurred at our institution. Multiresistant Pseudomonas aeruginosa could have acquired this transmissible resistance mechanism, going unnoticed because its phenotypic detection in this species is difficult. We compared P. aeruginosa isolates obtained before and after the KPC-producing K. pneumoniae outbreak. No blaKPC genes were detected in the isolates obtained before the outbreak, whereas 33/76 (43%) of the isolates obtained after the outbreak harboured the blaKPC gene. P. aeruginosa may thus become a reservoir of this transmissible resistance mechanism. It is very important to understand the epidemiology of these multiresistant isolates, in order to achieve early implementation of adequate control measures to contain and reduce their dissemination in the hospital environment.

INTRODUCTION

Pseudomonas aeruginosa is an important pathogen of hospital infections, characterized by high morbidity and mortality and increased hospital costs (Lister et al., 2009; Nicolau & Oliver, 2010). One of the main complications in the treatment of infections caused by P. aeruginosa is its high intrinsic resistance to different groups of antibiotics and its ability to develop and/or acquire new mechanisms of resistance, leading to an increase in the frequency of appearance of multiresistant strains and a significant reduction in therapeutic options (Lister et al., 2009; Nicolau & Oliver, 2010).

Carbapenems are generally used to treat severe infections caused by these micro-organisms. However, in recent years, an increase in resistance to these compounds has been observed worldwide. A study conducted in our hospital, in which the percentages of P. aeruginosa resistance to different β-lactam antibiotics were compared between two time periods (2000–2004 and 2005–2008), showed a statistically significant increase in the percentage of resistance to imipenem and meropenem from 21% and 19%, respectively, to 35% in both cases (García Ramirez et al., 2009).

The resistance of P. aeruginosa to carbapenems is due to the presence of several different mechanisms, which may be found either alone or in combination: alterations in outer membrane porin D (OprD), overexpression of the chromosomal cephalosporinase AmpC and efflux pumps (MexAB-OprM, etc.), and presence of class A, B, and D β-lactamases. Unlike other mechanisms, the β-lactamases can be horizontally transferred by plasmids carrying transposons and/or integrons with great clinical and epidemiological impact (Lister et al., 2009; Nicolau & Oliver, 2010).

One of these, the Klebsiella pneumoniae carbapenemase (KPC), was first detected in a strain of K. pneumoniae in the USA in 1996 (Yigit et al., 2001), and since then has spread, not only clonally but also to other bacterial species (Nordmann et al., 2009). The SENTRY Antimicrobial Surveillance Program reported that 3.3% of Enterobacteriaceae isolates collected during 2010 in five Latin American countries were KPC-2-producing, with a dramatic increase in carbapenem resistance (Castanheira et al., 2012). To date, 12 different types of blaKPC have been reported in various bacterial species (KPC-2 to KPC-13) with KPC-2 and KPC-3 the most widespread variants.

The genetic environment of some of these β-lactamases has been characterized recently. blaKPC is located in Tn4401, which in turn is located in plasmids, making these elements highly transferable and explaining their rapid dissemination in the hospital environment (Gomez et al., 2011; Pasteran et al., 2012; Rice et al., 2008; Naas et al., 2008; Curiao et al., 2010; Cuzon et al., 2011; Shen et al., 2009). KPC-producing K. pneumoniae was first isolated at our institution in June 2009 (index case). From October 2009...
to March 2010, there was a multiclonal outbreak of KPC-producing *K. pneumoniae*, with predominance of one clone, at our institution (Arduino et al., 2010). During this period, *bla*KPC was found in other species including *P. aeruginosa* with high levels of resistance to carbapenems. Since this outbreak, KPC-producing *Enterobacteriaceae* have been endemic in our hospital, raising the number of nosocomial infections. A surveillance programme of nosocomial infections caused by multiresistant strains thus has been implemented. Weekly surveillance cultures are carried out in all patients of intensive care units (ICUs); detection of colonization indicates that the patient must be isolated.

Since the resistance of *P. aeruginosa* to carbapenems is due to several mechanisms, it is difficult to determine phenotypically which is responsible for the resistance. Inhibition with boronic acid (300 µg), used for the phenotypic detection of *bla*KPC in *K. pneumoniae*, also inhibits AmpC, the chromosomal cephalosporinase present in *P. aeruginosa*, so it is not possible to discriminate between these two mechanisms in this species when using this method as a sole screening test. The combination of several tests including inhibition with boronic acid (600 µg) and cloxacillin (a specific inhibitor of AmpC) together with the modified Hodge test for phenotypic detection of *bla*KPC in *P. aeruginosa* has recently been proposed (Pasteran et al., 2011a, b).

The rapid dissemination of KPC-type carbapenemases, and the difficulty in their phenotypic detection in *P. aeruginosa*, means that this mechanism of resistance is frequently unnoticed in clinical laboratory routines.

It is extremely important to understand whether resistance to carbapenems in *P. aeruginosa* is due to chromosomal mechanisms or to enzymes that can be located in highly transferable elements, since the latter mechanism requires behavioural modifications to prevent hospital outbreaks.

**METHODS**

**Bacterial strains and growth conditions.** A total of 113 unique isolates of *P. aeruginosa* from inpatients of the Centro de Educación Médica e Investigaciones Clínicas ‘Dr. Norberto Quirno’ (Buenos Aires, Argentina) were studied. Thirty-seven were obtained between January 2005 and June 2009, prior to the first case of the outbreak of KPC-producing *Enterobacteriaceae*, and 76 were obtained between July 2009 and April 2012, after the outbreak. All isolates were selected based on resistance to imipenem and meropenem (disc zone ≤ 15 cm).

**Susceptibility assay.** The disk diffusion method on Mueller-Hinton agar (Oxoid) was used according to the recommendations of the CLSI (2011). We evaluated the following antibiotics (Oxoid): aztreonam, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, gentamicin, amikacin and colistin. Susceptibility breakpoints were determined and interpreted as recommended by the CLSI (2011); all plates were incubated at 37 °C for 18 h. The MICs of imipenem, meropenem, ceftazidime and cefepime were determined by standard agar dilution (CLSI, 2011).

**Detection of *bla*KPC genes.** *bla*KPC genes were amplified by PCR, using the specific oligonucleotides *KPC-F* and *KPC-R* (Table 1), in *P. aeruginosa* and *K. pneumoniae* (index case). PCR assays were performed using 5.0 µl DNA, 0.75 µl each primer (10 µmol µl⁻¹), 5.0 µl 5 × GoTaq buffer (Promega), 0.4 µl dNTP solution (10 mM each dNTP; Genbiotech), 0.15 µl GoTaq polymerase (5 U µl⁻¹; Promega), 13 µl PCR-grade water, and the following conditions: 5 min at 95 °C; 36 cycles of 1 min at 95 °C; 30 s at 53 °C; 1 min at 72 °C; and a final step of 10 min at 72 °C. The amplification products were sequenced and analysed with tools available at www.ncbi.nlm.nih.gov/.

**Analysis of the genetic environment of the *bla*KPC gene.** The genetic environment of the *bla*KPC gene in *P. aeruginosa* was analysed by PCR mapping as described previously, using primers designed taking into account known structures containing this gene (Table 1). The sequences of the amplified products were analysed with tools available at www.ncbi.nlm.nih.gov/.

**Repetitive element palindromic PCR (Rep-PCR).** Rep-PCR assays were performed using 5.0 µl DNA, 9 µl of each primer (10 µmol µl⁻¹), 10 µl 5 × GoTaq buffer (Promega), 0.5 µl dNTP solution (Genbiotech; 10 mM each dNTP), 0.25 µl GoTaq polymerase (Promega; 5 units µl⁻¹), 11.25 µl PCR-grade water, 5 µl DMSO and the following conditions: 5 min at 95 °C; 45 cycles of 1 min at 95 °C; 1 min at 42 °C; 8 min at 65 °C; and a final step of 10 min at 65 °C. Rep-PCR patterns were interpreted according to the criteria proposed by Tenover et al. (1995).

**Multilocus sequence typing (MLST).** Amplification and sequencing of the seven housekeeping genes (*gacA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppA* and *trpE*) was performed following previously published protocols (Curran et al., 2004). The sequences were compared with those available at the *P. aeruginosa* MLST database (http://pubmlst.org/paeruginosa/) to determine allele numbers and sequence types.

**Table 1. Description of the oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Designation</th>
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<th>Use</th>
<th>Reference</th>
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<tbody>
<tr>
<td>KPC-R</td>
<td>CTTTGTGCTACCTCTTTGTTAG</td>
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<td>Yigit et al. (2001)</td>
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<td>PCR mapping</td>
<td>This study</td>
</tr>
<tr>
<td>ISKpn6-F</td>
<td>CTACAACGGGTTACAGCAC</td>
<td>This study</td>
<td></td>
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<tr>
<td>4281</td>
<td>GGACGGCGCAATGACTA</td>
<td>This study</td>
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<tr>
<td>Kpn7-F</td>
<td>CAGGACCTTACAGCTTAC</td>
<td>Rep-PCR</td>
<td>Snelling et al. (1996)</td>
</tr>
<tr>
<td>Kpn7-R</td>
<td>CGTACACCGATGGAGCG</td>
<td></td>
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<tr>
<td>REP-1</td>
<td>IIICGGCICGATCIGGGC</td>
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<td>2</td>
<td>ICGCCTTATCIGGGCTAC</td>
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Surveillance cultures. After the outbreak, weekly surveillance for KPC-harbouring strains was carried out in all patients at the ICUs of our institution. Presence was confirmed by means of culture of perianal swabs in a KPC chromogenic medium (CHROMagar KPC; Mediatec). Colonies suspected of being KPC-producing Enterobacteriaceae were investigated with phenotypic methods according to CLSI (2011) recommendations and confirmed by PCR.

Outbreak detection. The CUSUM method for the detection of outbreaks in health events was used; this is based on monitoring of cumulative differences between observed and expected data in a time window compared to a threshold. If the cumulative sum of these values, the ‘CUSUM’, exceeds a warning value, an outbreak is confirmed (Unkel et al., 2012): \( C_i = \max \{0, C_{i-1} - X_i - k\} \) where \( C_0 = 0, C_i \) is the CUSUM value, \( k \) is half of the shift in the process mean that we wish to detect, expressed as a multiple of the SD \( \sigma \) [\( k = (\delta/2)\sigma = (\mu_1 - \mu_0)/2 \) where \( \delta \) is the shift size of the population mean, \( \sigma \) is the SD and \( \mu \) is the mean], in our case \( k = 2.42 \) for K. pneumoniae and 1.5 for P. aeruginosa, and \( X_i \) is the outcome measure for the standardized procedure where \( X_i = X(N/T) + n \) with \( X \) as the number of KPC-producing isolates per month, \( N \) the total number of isolates, \( T \) the total number of months evaluated and \( n \) the number of isolates per month.

\( h \) (specified threshold) values are generally selected to achieve a good balance between type I and type II error probabilities; we have accepted a false outbreak every 24 months. In these conditions, our \( h \) is 3 for both K. pneumoniae and P. aeruginosa.

Because we were interested only in detecting increases in mean KPC-producing frequency, the CUSUM was not allowed to fall below zero. If a negative value was obtained, the CUSUM was reset to zero.

Changes in detection rate of KPC-producing isolates. Changes in the detection rate of KPC-producing isolates (D) was calculated as: \( D = R_p/R_p \) where \( R \) is KPC-producing isolates/isolate total per period, \( p \) is evaluated period and \( pp \) is previous evaluated period.

RESULTS AND DISCUSSION

Infections with KPC-producing Enterobacteriaceae have great clinical impact. Reports show that the presence of KPC is an indicator of poor prognosis, with a higher number of therapeutic failures and increased hospital costs, because the treatment options for such patients are few or null (Neuner et al., 2011).

In the present study, no \( \text{bla}_{\text{KPC}} \) genes were found in any P. aeruginosa isolate obtained before the index case; 33 out of 76 (43 %) isolates obtained after the outbreak were KPC producers. In the second period, 195 out of 457 P. aeruginosa isolates were resistant to carbapenems (disc zone \( \leq 15 \) cm), from which 84 (18 %) isolates were KPC producers. These results show that, in contrast to initial expectations, KPC-type carbapenemase is a significant mechanism of resistance to carbapenems in P. aeruginosa isolates in our hospital.

P. aeruginosa may contain different mechanisms conferring high levels of resistance to carbapenems, hindering phenotypic detection of the presence of KPC-type carbapenemases. In this way, these bacteria can become a reservoir of transmissible mechanisms of resistance in the hospital environment. The CUSUM graph for K. pneumoniae (Fig. 1) showed three points where the CUSUM value exceeded the threshold (\( h = 3 \)) indicating that the process was out of control (an outbreak). The CUSUM graph for P. aeruginosa (Fig. 2) indicated an outbreak at one point, after which the CUSUM values did not return to zero, indicating that the process was never controlled. These events show clearly that there is a reservoir of this resistance mechanism in our hospital. Similar results were obtained from the graph plotting the difference in the detection rate of KPC-producing isolates (Fig. 3), which

**Fig. 1.** CUSUM graph for K. pneumoniae; arrows mark the points where the process is out of control (outbreaks).
showed that the proportion of KPC-producing *P. aeruginosa* increased during the period in which the proportion of KPC-producing *K. pneumoniae* decreased after the first outbreak.

All of our isolates of KPC-producing *P. aeruginosa* showed a particular susceptibility pattern, high levels of resistance to imipenem, meropenem, cefepime (MICs $>128$ mg/mL) and ceftazidime (MIC range 32–64 mg/mL), remaining sensitive to gentamicin, amikacin and colistin. By contrast, the *P. aeruginosa* lacking KPC showed a different phenotypic pattern with less resistance to β-lactam antibiotics. We studied the clonal relationships among the KPC-producing *P. aeruginosa* in order to determine whether this resistance mechanism was acquired by a single clone which then spread or whether different clones were present with the same resistance mechanism. All isolates of KPC-producing *P. aeruginosa* evaluated showed the same Rep-PCR pattern (Fig. 4) and presented an identical allelic pattern.
profile in MLST (acrA 17, aroE 5, guaA 26, mutL 3, nuoD 4, ppsA 4 and trpE 26) corresponding to the sequence type 654 (ST654), which has been reported previously in Argentina (Pasteran et al., 2012). We found only one clone (ST654) to be present among the studied isolates, which successfully acquired the bla\textsubscript{KPC} gene and spread in the hospital, indicating that it plays an important role in the dissemination of this resistance mechanism.

The most serious problem with infections by these KPC-harbouring multiresistant bacteria is the rapid dissemination of the resistance mechanism not only within a species, but also to other species (Cuzon et al., 2011; Shen et al., 2009). The horizontal transfer of plasmids is infrequent in \textit{P. aeruginosa}. However, Tn4401 is flanked by different duplication sites and can be inserted into plasmids of a wide range of hosts and so be transferred to a wide range of species, including \textit{P. aeruginosa} (Naas et al., 2008). Our analysis of the sequence of the amplicons showed that both the \textit{P. aeruginosa} and \textit{K. pneumoniae} isolates obtained during the outbreak had the same KPC variant, identified as KPC-2. We compared a sequence of approximately 3519 bp obtained by PCR mapping of \textit{P. aeruginosa} with available Tn4401 sequences in the GenBank database. It was identical to the variant Tn4401b (pBC633, pBC2303, and pCOL1) and, unlike Tn4401a (pNYC), there is an insertion of 100 pb between bla\textsubscript{KPC}-2 and istB. On the other hand, in our isolates there was no truncated bla\textsubscript{TEM} upstream of the bla\textsubscript{KPC}-2 gene and IS\textsubscript{kp}n6 is complete, unlike the novel structure characterized in PA-2 from Colombia (Cuzon et al., 2011). Tn4401b initially characterized from two \textit{K. pneumoniae} isolates and one \textit{P. aeruginosa} isolate from Colombia seems to be the most frequently encountered in the USA and in Argentina (Nordmann et al., 2009; Pasteran et al., 2012; Rice et al., 2008).

A worrying issue arising from evaluation of our results was that most of the infectious episodes by KPC-producing \textit{P. aeruginosa} had not been previously detected. We reviewed the history of infection and colonization by KPC-producing \textit{Enterobacteriaceae} for the patients from whom we obtained KPC-producing \textit{P. aeruginosa} isolates after the outbreak. Interestingly, 11 (41%) out of the 27 isolates of KPC-producing \textit{P. aeruginosa} were obtained from patients with a history of colonization and/or infectious episodes by KPC-producing \textit{Enterobacteriaceae} (we did not analyse six isolates of KPC-producing \textit{P. aeruginosa} because we only began to perform surveillance cultures with a regular schedule after the outbreak). A warning indicator to suspect the presence of KPC in a multiresistant \textit{P. aeruginosa} could be a history of colonization and/or infection with KPC-producing \textit{Enterobacteriaceae}.

It is very important not only to detect this mechanism of resistance but also to perform investigations to determine the current epidemiological situation in each hospital. Only then will it be possible to implement appropriate epidemiological control measures to prevent further spreading of this type of resistance in hospitals and to prevent future outbreaks.

REFERENCES


