Emergence of \( \text{bla}_{\text{KPC-3}}-\text{Tn4401a} \) associated with a pKPN3/4-like plasmid within ST384 and ST388 \textit{Klebsiella pneumoniae} clones in Spain

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Received 11 January 2010; returned 1 March 2010; revised 24 April 2010; accepted 27 April 2010

**Background:** KPC carbapenemase-producing \textit{Klebsiella pneumoniae} isolates have been increasingly detected in Europe. We report the first KPC-producing isolates characterized in Madrid, Spain.

**Methods:** Twelve \( \text{K. pneumoniae} \) isolates recovered from clinical and surveillance cultures from eight patients (September 2009 to February 2010) that were resistant to carbapenems and resulted in a positive modified Hodge test, were screened for carbapenemase genes (PCR, sequencing and hybridization). Clonal relatedness was established by PFGE and multilocus sequence typing. Plasmid characterization included incompatibility group assay and XhoI/HindIII restriction pattern comparison. The genetic environment was characterized by PCR based on the Tn\(4401\) sequence.

**Results:** All 12 isolates were resistant to all \( \beta \)-lactams, including imipenem and meropenem (MIC \( \geq 8 \) mg/L). Eleven of them, fully susceptible to aminoglycosides and fluoroquinolones, showed related PFGE patterns and belonged to sequence type (ST) 384 and harboured the \( \text{bla}_{\text{KPC-3}}, \text{bla}_{\text{OKP-5}}, \text{bla}_{\text{OXA-9}} \) and \( \text{bla}_{\text{TEM-1}} \) genes, whereas one isolate resistant to quinolones belonged to ST388, and also harboured the \( \text{bla}_{\text{CTX-M-10}} \) gene. The \( \text{bla}_{\text{KPC-3}}, \text{bla}_{\text{OXA-9}} \) and \( \text{bla}_{\text{TEM-1}} \) genes were located on an \( \sim 85 \) kb non-transferable plasmid that was a derivative of pKPN3/pKPN4 previously described in \textit{K. pneumoniae}. The \( \text{bla}_{\text{KPC-3}} \) gene was located on the Tn\(4401\) ‘isoform a’ variant, which is usually linked to \( \text{bla}_{\text{KPC-2}} \), but rarely to \( \text{bla}_{\text{KPC-3}} \).

**Conclusions:** The emergence of Tn\(4401\)-\(\text{bla}_{\text{KPC-3}}\) within a pKPN3/4-like plasmid and its novel association with ST384 and ST388 \textit{K. pneumoniae} clones in Spain is reported. Although \( \text{bla}_{\text{KPC-3}} \) has been scarcely reported in Europe, the location of this Tn\(4401\)a in a widespread \textit{K. pneumoniae} plasmid supports the possibility of broader future dissemination.

**Keywords:** clinical \textit{K. pneumoniae}, \( \beta \)-lactamases, transposons

**Introduction**

\textit{Klebsiella pneumoniae} carbapenemases (KPCs) are class A \( \beta \)-lactamases that confer resistance to penicillins, extended-spectrum cephalosporins, carbapenems and monobactams. They were first detected in 1996 in the USA and since then they have been increasingly reported worldwide, and are becoming endemic in hospitals in the North-East of the USA, Israel and Greece.\(^1\) In Europe, KPCs have been described since 2005 in France, Finland, Germany, Greece, Italy, Norway, Poland, Sweden and the UK.\(^2\)\(^-\)\(^9\) Their recent emergence in some of these countries seems to be linked to persons who have travelled to endemic areas.\(^2\)\(^-\)\(^3\)\(^5\)\(^8\)\(^9\) The rapid spread of these enzymes is of concern since infections caused by KPC producers are difficult to treat due to the scarce therapeutic options available and the high rate of associated morbidity.\(^1\)

Various KPC variants differing in one or two amino acids (KPC-2, -3, -4, -5, -6, -7, -8, -9 and -10) have been mainly identified in \textit{K. pneumoniae} and to a lesser extent in \textit{Pseudomonas aeruginosa} and \textit{Acinetobacter baumannii} clinical isolates, in which KPC-2 and KPC-3 are the most widespread variants.\(^1\)\(^,\)\(^10\) While KPC-2 is more frequently detected in the USA, KPC-3 has...
have been more commonly identified in Israel although recent studies have detected KPC-3 producers in South and North America and several European countries. Both blaKPC genes have been found as part of Tn4401, a Tn3-like element of 10 kb, which has been detected among different conjugative and non-conjugative plasmids. However, the enzyme encoded by blaKPC appears to confer a lower level of carbapenem resistance than that encoded by blaKPC-2. Clonal expansion of the K. pneumoniae ST258 clone and the spread of particular plasmids seem to have mainly fuelled the rapid dissemination of these genes worldwide.

Here, we describe the emergence of blaKPC-3 in Spain associated with a Tn4401 variant usually linked to blaKPC-2, but located in a similar plasmid context to that previously described for blaKPC-3. Such emergence was associated with an outbreak of two K. pneumoniae clones not previously associated with blaKPC genes. One is an apparently new highly transmissible clone and the other is a persistently recovered clone in our institution associated with a CTX-M extended-spectrum β-lactamase (ESBL). It can be hypothesized that the spread of these blaKPC-3-containing strains into different clones might increase the risk of broader dissemination of KPC-mediated carbapenem resistance.

Materials and methods

Bacterial strains, epidemiological background, clinical data and control measures

Twelve clinical isolates resistant to imipenem, meropenem and ertapenem (MIC ≥8 mg/L) and negative for the expression of metallo-β-lactamase (MBL) enzymes were recovered from clinical and colonization surveillance cultures from September 2009 to February 2010 from eight hospitalized patients in the tertiary Ramón y Cajal University Hospital, Madrid (Spain). Isolates were recovered from urine (n=3), faeces (n=3), pharynx (n=3), wound (n=2) and blood (n=1) (see Table 2). Bacterial identification was performed using the semi-automated WIDER system (Fco. Soria-Melguizo, Madrid, Spain). Relatedness among isolates was established by XbaI and SpeI PFGE as previously described and PFGE patterns were interpreted according to the criteria proposed by Tenover et al. with closely and possibly related patterns being considered as belonging to a single clone. Isolates were also characterized by multilocus sequence typing (MLST) according to the protocol described for K. pneumoniae (http://www.pasteur.fr/recherche/genopole/PFBB/mlst/Kpneumoniae.html).

Clinical records of patients with infection and/or colonization with these isolates were reviewed and the following data were included: age, sex, underlying disease, patient location (hospital ward), length of stay (LOS), LOS until the first positive culture, infection and colonization sites, use of antibiotics in the 2 weeks before culturing carbapenem-resistant K. pneumoniae isolates, and the antibiotic therapy directed to control the clinically significant isolate.

Infection control measures, including contact isolation, barrier precaution and rectal and pharyngeal surveillance cultures examined infected patients and those admitted to the same unit were immediately implemented after the detection of the first case. Samples from surveillance cultures were directly inoculated onto MacConkey agar plates supplemented with ceftazidime (4 mg/L) and using previous broth enrichment in brain heart infusion (BHI) supplemented with imipenem (1 mg/L). Colonies recovered on ceftazidime-containing MacConkey plates were screened for carbapenemase production.

Antibiotic susceptibility and screening for carbapenemase production

Antibiotic susceptibility testing was performed by standard microdilution and E-test. Clinical categorization was performed using the EUCAST clinical breakpoints (www.eucast.org). The epidemiological cut-off value (ECOFF) was also used in the absence of a EUCAST breakpoint. Phenotypic carbapenemase production was assessed by the modified Hodge test as recommended by the CLSI and disc diffusion-based methods using boronic acid and EDTA. In addition, the presence of ESBLs was also screened. Antibiotics were provided by the manufacturers or purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA).

Characterization of antibiotic resistance genes and blaKPC genetic environment

The presence of genes encoding class A (TEM, SHV, CTX-M and KPC), B (IMP and VIM) and D (OXA-9) β-lactamases was determined by PCR using specific primers. The presence of qnr genes was investigated by multiplex PCR as previously described. The genetic environment of blaKPC was sought by long-PCR mapping based on the sequence of Tn4401 (Table 1 and see Figure 2). All PCR products were sequenced and compared with available sequences in the GenBank database.

Transferability of the blaKPC gene and plasmid characterization

Transfer of blaKPC was assayed by a filter-mating protocol at a 1:10 donor/recipient ratio for 24 h at 37°C. Clinical isolates were used as plasmid donors and an Escherichia coli K-12 strain BM21 (rifampicin and nalidixic acid resistant and plasmid free) was used as recipient. Transconjugants were selected on Luria–Bertani (LB) plates supplemented with ceftazidime (2 mg/L), imipenem (2 mg/L) or meropenem (1 mg/L) and rifampicin (100 mg/L; Sigma, Poole, UK) after incubation at 37°C for 24 h. Location of blaKPC was determined by hybridization of S1-digested DNA or plasmid DNA from clinical isolates with specific probes for the β-lactamases genes.

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Designation</th>
<th>Sequence (5’ - 3’ )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>IR Tn4401F</td>
<td>AGG GGT TCT AAT CCG GAA</td>
<td>this study</td>
</tr>
<tr>
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<td>IR Tn4401R</td>
<td>TCC GGC ATT CCG CAT TGT</td>
<td>this study</td>
</tr>
<tr>
<td>3</td>
<td>tnpAF</td>
<td>CAC CAT CAC CAC GAA CC</td>
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</tr>
<tr>
<td>4</td>
<td>KPC-R</td>
<td>GAC GAG CAT ACA GTG AAT T</td>
<td>this study</td>
</tr>
<tr>
<td>5</td>
<td>KPC-F2</td>
<td>GGC GTC AAC GGG CAG TAA</td>
<td>this study</td>
</tr>
<tr>
<td>6</td>
<td>istBR</td>
<td>GCG ACC GGT GTC TCT GTC T</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>tnpAR</td>
<td>TCG AAT TGG CAT CAC AGG</td>
<td>this study</td>
</tr>
<tr>
<td>8</td>
<td>P1.1</td>
<td>GTG CGA CGA TGG CCC ACA</td>
<td>this study</td>
</tr>
<tr>
<td>9</td>
<td>P1.1.1</td>
<td>TGC CGG ATG TCG ATG AGT</td>
<td>this study</td>
</tr>
<tr>
<td>10</td>
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<td>this study</td>
</tr>
<tr>
<td>11</td>
<td>KPC-F</td>
<td>ATG TCA CTG TAT GCG CTC</td>
<td>36</td>
</tr>
<tr>
<td>12</td>
<td>KPC-R</td>
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<td>36</td>
</tr>
<tr>
<td>13</td>
<td>repB</td>
<td>ATG TCA GTG TCA GCA GAT AA</td>
<td>this study</td>
</tr>
<tr>
<td>14</td>
<td>repA</td>
<td>ATG GGC CAG TCG GTC TGA</td>
<td>this study</td>
</tr>
<tr>
<td>15</td>
<td>OXA-9F</td>
<td>ATG AAG GAT ACC TTG ATG A</td>
<td>this study</td>
</tr>
<tr>
<td>16</td>
<td>OXA-9R</td>
<td>CAT TTG TTA CCC ATC AAC</td>
<td>this study</td>
</tr>
</tbody>
</table>

Primers were designed based on known sequences: GenBank accession no. CP000649; and GenBank accession no. DQ174113.
Plasmids containing \( \text{bla}_{\text{KPC}} \) were characterized according to their incompatibility group, which was determined based on sequences related to replication and conjugation modules obtained by PCR-based typing methods designed by Carattoli et al.\(^{25} \) and Alvarado et al.,\(^{26} \) respectively. Correspondence of PCR products with plasmids harboured by the cell was determined by hybridization of S1-digested DNA or plasmid DNA with specific probes. Comparison of XhoI- and HindIII-digested plasmid DNA was performed to assess similarity between plasmids.

### Molecular techniques

Genomic DNA was extracted using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany). Plasmid DNA was obtained by using a modified protocol of the alkaline lysis method from an overnight LB culture supplemented with ceftazidime (2 mg/L) plus meropenem (1 mg/L).\(^{14} \) PCR assays to amplify fragments <2 kb were carried out using the conditions previously described.\(^{27} \) Long-PCR assays (>2 kb) were performed using 2.5 mM MgCl\(_2\), 0.2 mM of each deoxynucleoside triphosphate, 0.1 μM each primer, 2.5 U of Takara LA Taq polymerase (Takara Bio Inc., Shiga, Japan) and the following conditions: 1 min at 94°C; 30 cycles of 20 s at 96°C, 1 min at 52-60°C and 1.5–4 min at 72°C; and a final step of 10 min at 72°C. Primers used are listed in Table 1. The probes used in the hybridization assays were generated by PCR using positive controls as template DNA.\(^{27} \) Southern blot DNA transfer and hybridization were performed by standard procedures.\(^{28} \) Labelling and detection were carried out using Gene Images Alkphos Direct Labelling system kit, following the manufacturer’s instructions (Amersham Life Sciences, Uppsala, Sweden).

### Sequence analysis

GenBank searches were performed with the NCBI BLASTN alignment tool. The sequence of \( \text{Tn}4401\)a containing \( \text{bla}_{\text{KPC}-3} \) shown in Figure 2 is available at the GenBank database as GU386376.

### Results and discussion

#### Bacterial strains, epidemiological background and clinical data

All isolates were resistant to all β-lactams tested by standard microdilution including imipenem, meropenem and aztreonam (MICs ≥ 8 mg/L), but displayed lower carbapenem MICs when susceptibility was determined using Etest (MIC range 0.5–2 mg/L). Nevertheless, colonies growing within the zone of inhibition were found in all isolates, denoting the hetero-resistance in the expression of carbapenem resistance that complicates the detection of carbapenemase-producing isolates.\(^{1,29,30} \) The modified Hodge test and the boronic acid disc diffusion assay were positive. In contrast, the disc diffusion assays to detect the phenotypic pattern of either MBL (using EDTA and either imipenem or ceftazidime) or ESBL were negative. Unlike the situation previously described for carbapenemase-producing isolates,\(^{5,7,11,12,31} \) 11 of these isolates were susceptible to amikacin and tobramycin and to both nalidixic acid and ciprofloxacin, as well as to trimethoprim/sulfamethoxazole. Moreover, they were resistant to tetracycline and minocycline and showed an intermediate level of susceptibility to tigecycline (MIC = 2 mg/L). All these isolates were considered clonally related and they were assigned to the new sequence type ST384 (Figure 1), which might represent an emerging highly transmissible

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<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (gender)</th>
<th>Underlying disease</th>
<th>Hospital ward</th>
<th>LOS (days)</th>
<th>LOS until first positive culture</th>
<th>Previous antibiotic use (&lt;2 weeks)</th>
<th>Infection site (date of isolation)</th>
<th>Colonization site</th>
<th>Treatment (dose)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66 (M)</td>
<td>pulmonary carcinoma with cerebral metastasis</td>
<td>oncology</td>
<td>68</td>
<td>61</td>
<td>vancomycin, meropenem</td>
<td>urine (16/09/2009)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>pharyngeal</td>
<td>ciprofloxacin (750 mg/12 h, oral)</td>
<td>recovered</td>
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<tr>
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<td>73 (F)</td>
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<td>internal medicine</td>
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<td>10</td>
<td>amoxicillin/clavulanate, metronidazole</td>
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<td>rectal (28/09/2009)&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>pharyngeal and rectal (26/10/2009)&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>recovered</td>
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<td>not documented</td>
<td>blood and urine (25/01/2010)&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>not detected</td>
<td>pharyngeal (03/02/2010)</td>
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</table>

M, male; F, female.

<sup>a</sup>Isolates belonging to ST384 (allelic profile: gapA, 18; infB, 23; mdh, 56; pgi, 63; phoE; 80; rpoB, 43; and tonB, 51).

<sup>b</sup>Isolate belonging to ST388 (allelic profile: gapA, 16; infB, 24; mdh, 59; pgi, 27; phoE; 29; rpoB, 22; and tonB, 105).

<sup>c</sup>This patient was previously hospitalized in October and December 2009.
isolate was associated with therapy-refractory bacteraeemia whereas in one case the carbapenemase-producing patients who were successfully treated with ciprofloxacin, penems. Urinary tract infection was documented in two previously treated with broad-range antibiotics including carbapenemase-producing K. pneumoniae had recent periods of hospitalization. In six patients, the first positive culture for carbapenemase-producing K. pneumoniae isolate was obtained close to the time of hospital discharge (range 10–61 days) (Table 2). Six out of the eight patients were previously treated with carbapenemase-producing K. pneumoniae isolate was associated with a therapy-refractory bacteremia episode (Table 2). In all cases, infection control practices were implemented after the recovery of these isolates. Three patients were found to be colonized with carbapenemase-producing K. pneumoniae in surveillance cultures. Overall, rectal and pharyngeal colonization was detected in two patients each, another single patient being colonized in both locations. Despite the implementation of epidemiological measures currently recommended for control of infections with carbapenem-resistant Enterobacteriaceae we apparently failed to control the outbreak of these isolates.

**β-Lactamase genes and other resistance genes**

Carbapenemase activity was due to the presence of KPC-3 in all of the isolates studied, which also contained the bla*OXA-5*, **blabKP*, and **blaTEM* genes. The **blabKP*, **blaOXA* and **blaTEM* genes were located on the same plasmid as described in other studies and both showed identical sequences to those located in the recently sequenced plasmid pKpQIL from Israel (GenBank accession no. GU595196). Despite negative phenotypic detection of ESBLs, the isolate grouped within ST388 was positive for **blaCTX-M-10**. We did not detect ESBL-*blaSHV, qnrA*, qnrB or qnrS genes, which have also been linked to KPC-3 in other countries, in any of the isolates.

**blaKPC** is located on Tn4401 within a pKPN3/4-like plasmid

**blaKPC-3** was part of a Tn4401 platform identical to those identified in plasmids carrying **blaKPC-2** such as pNYC (80 kb, GenBank accession no. EU176011), pNGR (80 kb), pKN633 (12 kb, GenBank accession no. EU176012), pCOL (45 kb, GenBank accession no. EU176013), pBC2303 (75, 35 kb, GenBank accession no. EU176014) or **blaKPC-3** as pS12 (75 kb, GenBank accession no. FI223605). It corresponded to the variant of Tn4401 designated as ‘isoform a’, which contains a 100 bp deletion between **blaKPC** and **istB**. This transposon type is usually associated with **blaKPC-2** although it has also been detected in some KPC-3-producing isolates from Israel and Italy (Figure 2). As previously stated, we could not demonstrate an epidemiological link of our patients with known countries with KPC-3 isolates. The similarity in genetic background of our **blaKPC-3** to that found for other **blaKPC** genes might also suggest a local emergence of the KPC-3 variant in our country, as this enzyme only differs from KPC-2 in one amino acid position (H272Y).

Nevertheless, this variant has not yet been reported in Spain.

All isolates carried a non-transferable plasmid of 85–90 kb (harbouring **blaKPC-3**, **blaOXA-9** and **blaTEM-1**) and a large plasmid ranging from 180 kb (all but one isolate) to 320 kb (one isolate) as reported for other KPC-producing strains. The KPC plasmids in ST384 isolates showed identical restriction fragment length polymorphism (RFLP) patterns, but a different pattern was found for the ST388 isolate. Both KPC plasmids showed a positive amplification with primers specific for the Salmonella FII virulence plasmids. However, nucleotide sequences corresponding to the replicase and the relaxase obtained by the PCR schemes and Gastroenterology) or admitted to the emergency room between July 2009 and January 2010. The Oncology and Internal Medicine wards are located in the same area of our hospital, but not the other wards. Table 2 summarizes the clinical features of these patients. Unlike in previous reports, none of these patients had a history of a recent journey to countries where KPC producers are widely spread, indicating circulation of KPC-producing isolates within the clinical environment in our country. All but one patient were elderly (>65 years) and all had multiple underlying illnesses and a prolonged hospital stay (range 10–68 days), including one patient (designated as patient no. 7 in Table 2) admitted to the emergency room who had recent periods of hospitalization.
KPC-producing *K. pneumoniae* clones in Spain

Conclusions

The emergence of Tn4401- bla<sub>KPC-3</sub> located in a pKPN3/4-like plasmid within ST384 and ST388 carbapenem-resistant *K. pneumoniae* clones in Madrid (Spain) is described. Interestingly, both clones were newly recognized as being associated with KPC-3, ST388 being a persistent clone found in our institution since 1998 and harbouring the bla<sub>CTX-M-10</sub> gene. Although KPC-3 producers have scarcely been reported in Europe, the location of Tn4401a- bla<sub>KPC-3</sub> on widespread IncFII plasmids from Klebsiella, which can contain similar sequences to other enterobacterial plasmids also frequently identified among Klebsiella isolates, might fuel its future inter-clonal dissemination among isolates of the same or other genera by different horizontal gene processes.36,41,62 This study also documents the penetration of bla<sub>KPC-3</sub> genes within clinical *K. pneumoniae* clones in the central region of Spain that may become endemic as occurred in other European areas. Finally, this work highlights the role of endemic multiresistant clones as substrates of new antibiotic resistance genes, such as bla<sub>KPC-3</sub>.

Acknowledgements

We thank Christian G. Giske for providing the KPC-3-producing *K. pneumoniae* positive control. We are grateful to Fernando de la Cruz and Maria del Pilar García-Llorca (Universidad Cantabria, Spain) for continuous support in the classification of plasmid conjugation modules.

Funding

This work was partially funded by research grants from Instituto Carlos III, Spanish Ministry of Science and Innovation (PI-06/0806) and the European Commission (HEALTH-F3-2008-223031). T. C. was supported by research grant PI-06/0806 and is currently supported by a Research fellow contract (FI09/00901) from Instituto Carlos III, Spanish Ministry of Science and Innovation.

Transparency declarations

None to declare.

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