Dissemination of multiresistant \textit{Enterobacter cloacae} isolates producing OXA-48 and CTX-M-15 in a Spanish hospital

Javier Fernández\textsuperscript{a,b}, Ignacio Montero\textsuperscript{a}, Óscar Martínez\textsuperscript{b}, Ana Fleites\textsuperscript{b}, Laurent Poirel\textsuperscript{c}, Patrice Nordmann\textsuperscript{c,d}, M. Rosario Rodicio\textsuperscript{a,*}

\textsuperscript{a} Department of Functional Biology, Section of Microbiology, University of Oviedo, Oviedo, Spain
\textsuperscript{b} Service of Microbiology, Hospital Universitario Central de Asturias, Oviedo, Spain
\textsuperscript{c} Medical and Molecular Microbiology, Emerging Antibiotic Resistance Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland
\textsuperscript{d} HFR – Hôpital Cantonal, Fribourg, Switzerland

Twenty-one multiresistant \textit{Enterobacter cloacae} isolates producing OXA-48 (n = 10), CTX-M-15 (n = 7) or both (n = 4) \(\beta\)-lactamases were detected in a Spanish hospital during a 1-year period (June 2013 to June 2014). The isolates were also resistant to non-\(\beta\)-lactam antimicrobials, further complicating the therapeutic options. Genotyping of the isolates identified two major clones (ST74 and ST66) that caused prolonged outbreaks in different buildings of the hospital as well as some sporadic isolates (ST78, ST45 and ST295). Isolates belonging to clone 1 (n = 7) were carbapenem-resistant and carried the \textit{bla}\textsubscript{OXA-48} gene on a conjugative IncI/M plasmid of ca. 65 kb. Clone 2 isolates (n = 11) were resistant to cefepime and harboured the \textit{bla}\textsubscript{CTX-M-15} gene on an 150-kb, non-conjugative plasmid of the IncF group, co-harbouring the \textit{qnrB} and \textit{aac(6’)-Ib-cr} genes encoding quinolone resistance. Four clone 2 isolates were also resistant to carbapenems owing to the co-production of OXA-48. Most of the isolates were recovered from critically ill patients and were admitted to intensive care units; a single patient was transferred from another Spanish hospital. Intrahospital and interhospital dissemination of multiresistant \textit{E. cloacae} isolates is of major clinical concern as it could lead to endemic nosocomial situations.

1. Introduction

\textit{Enterobacter cloacae} is an opportunistic pathogen frequently involved in nosocomial infections [1]. This enterobacterial species is intrinsically resistant to aminopenicillins, amoxicillin/clavulanic acid (AMC) and cephalosporins of early generations owing to a chromosomally encoded AmpC \(\beta\)-lactamase. Isolates overproducing AmpC are also resistant to broad-spectrum cephalosporins. Furthermore, \textit{E. cloacae} has the ability to acquire additional resistance mechanisms, including plasmid-encoded extended-spectrum \(\beta\)-lactamases (ESBLs) and carbapenemases [1]. Phenotypic detection of ESBL production may be difficult since it can be masked by overexpression of AmpC. Some \textit{E. cloacae} isolates may also exhibit permeability defects due to the absence of porins or porin mutations, further increasing the minimum inhibitory concentrations (MICs) of \(\beta\)-lactams and carbapenems, particularly ertapenem [1].

In members of the \textit{Enterobacteriaceae} family, CTX-M-15 and OXA-48 are among the most common enzymes involved in resistance to broad-spectrum cephalosporins and carbapenems, respectively [2,3]. CTX-M-15 is a class A ESBL encoded by the \textit{bla}\textsubscript{CTX-M-15} gene, which has been identified in plasmids of varying size (85–200 kb) and structure, and often belonging to the FI incompatibility (Inc) group [4]. OXA-48 is an Ambler class D carbapenemase that confers resistance to penicillins and reduced susceptibility to carbapenems but does not significantly hydrolyse broad-spectrum cephalosporins [3]. The \textit{bla}\textsubscript{OXA-48} gene has been almost always located on a conjugative plasmid of ca. 62 kb assigned to the IncI/M group and has been identified only in enterobacterial species [5,6]. Both CTX-M-15 and OXA-48 have been detected in several enterobacterial species, being particularly frequent in \textit{Klebsiella pneumoniae}, whilst they are relatively uncommon in \textit{E. cloacae} [2,3]. Here we report the clinical and genetic features of 21 \textit{E. cloacae} isolates producing CTX-M-15, OXA-48 or both, which were mainly recovered from critically ill patients, in a Spanish hospital during a 1-year period.
2. Patients and methods

2.1. Setting, patients and bacterial isolates

‘Hospital Universitario Central de Asturias’ (HUCA) (Oviedo, Spain) is a 1100-bed university hospital providing care for a population of ca. 342,000 inhabitants in the Asturias region of northern Spain. During the 1-year period from June 2013 to June 2014, all E. cloacae isolates recovered at the hospital (n = 448) were analysed phenotypically for production of carbapenemases and ESBLs. A total of 21 multiresistant isolates, all recovered from different patients, produced a carbapenemase, an ESBL or both. Most E. cloacae isolates were obtained from critically ill patients admitted to two intensive care units (ICU1 and ICU2) located in different buildings or to other hospital wards. Relevant features regarding the patients are shown in Table 1.

2.2. Susceptibility testing

All E. cloacae isolates underwent antimicrobial susceptibility testing by the disc diffusion assay (Becton Dickinson, Sparks, MD). The MicroScan® system (Neg Combo Panel Type 53; Siemens Healthcare Diagnostics, Deerfield, IL) was used for determination of MICs and for bacterial identification, and the latter was confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (microflexTM; Bruker Daltonik GmbH, Bremen, Germany). Results of disc diffusion assays and MICs were interpreted according to Clinical and Laboratory Standards Institute (CLSI) breakpoints updated in January 2014 [7]. Etest strips (bioMérieux, Marcy-l’Etoile, France) were used for determining the MICs of ertapenem and imipenem in those isolates suspected of carbapenemase production. The Carba NP test recommended for detection of carbapenemases [8] was also performed for isolates with elevated MICs of ertapenem and imipenem. All E. cloacae suspected of ESBL production (double-disc synergy test positive and/or elevated MIC to cefepime) were tested with the cefepime/cefepime + clavulanic acid (PM/PML) Etest (bioMérieux).

2.3. Identification of genes encoding extended-spectrum β-lactamases and carbapenemases, and plasmid analysis

Genes encoding resistance to broad-spectrum cephalosporins (blaTEM, blaSHV and blaCTX-M) and carbapenems (blaOXA-48) were identified by PCR amplification [5] followed by sequencing. Transposon Tn1999 (known to carry the blaOXA-48 gene) and plasmid-mediated quinolone resistance genes [qnrA, qnrB, qnrS and aac(6′)-Ib-cr] were searched by PCR amplification followed by sequencing of the obtained products [3,9]. Plasmid DNA was extracted by the Kado and Liu method [5] and was visualised by agarose gel electrophoresis.

Plasmid Inc groups were determined by PCR-based replicon typing [5]. Conjugation experiments were performed using donor isolates representative of three antibiotic resistance phenotype groups, namely resistance to carbapenems but susceptibility to cefepime (Ec110), resistance to cefepime but susceptibility to carbapenems (Ec8 and Ec19), or resistance to both (Ec115). A rifampicin-resistant Escherichia coli J53 strain was used as recipient for conjugation assays. Transconjugants were selected on eosin–methylene blue agar (Oxoid, Madrid, Spain) containing rifampicin (100 mg/L) plus ertapenem (0.5 mg/L) or rifampicin plus ceftriaxone (10 mg/L). Plasmid DNA extracted from each of the donor E. cloacae and their transconjugants was transferred to a nylon membrane and was then hybridised with probes specific for the blaCTX-M-15 and blaOXA-48 genes. Probes were labelled using the PCR DIG Labeling Mix (Roche Applied Science, Penzberg, Germany), followed by gel extraction with the GFX™ DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont, UK).

2.4. Pulsed-field gel electrophoresis (PFGE) analysis

Total DNA from the 21 E. cloacae isolates was digested with XbaI (Takara Bio Europe, Saint-Germain-en-Laye, France; 30U, 4h at 37°C). The generated fragments were separated by PFGE using a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA) under the conditions recommended by PulseNet (http://www.pulsenetinternational.org). Similarity between XbaI profiles was evaluated by the Jaccard’s coefficient (S), and cluster analysis was performed by the unweighted pair-group method with arithmetic averages (UPGMA) using the software program MVSP (Multivariate Statistical Package for PCs; RockWare Inc., Golden, CO).

2.5. Multilocus sequence typing (MLST)

MLST was performed for representative isolates with different PFGE profiles (Ec110, Ec13, Ec9, Ec18, Ec121, Ec15 and Ec17) as described previously [10]. The database available at http://pubmlst.org/ecloaca was used for assigning sequence types (STs).

3. Results

3.1. Antimicrobial susceptibility and genetic basis for resistance to broad-spectrum cephalosporins and carbapenems

As shown in Table 2, 20 of the 21 E. cloacae isolates were resistant to cefotaxime and 11 were also resistant to cefepime. Moreover, 14 isolates displayed intermediate susceptibility or resistance to carbapenems. In addition to β-lactam antibiotics, all E. cloacae isolates were variably resistant to other antimicrobial groups, including quinolones (nalidixic acid and ciprofloxacin), aminoglycosides (gentamicin and tobramycin) and trimethoprim/sulamethoxazole.

The blaCTX-M-15 gene was detected in all isolates resistant to cefepime and positive for the PM/PML test (n = 11), whilst the blaOXA-48 gene was found in all isolates with elevated MICs of imipenem and positive for the Carba NP test (n = 14). Both blaCTX-M-15 and blaOXA-48 were identified in four isolates. Overall, isolates harbouring blaCTX-M-15, blaOXA-48 or both accounted for 1.6%, 2.2% and 0.9% of the total number of E. cloacae recovered in HUCA over the 1-year study period (n = 448).

The genetic location of blaCTX-M-15 and blaOXA-48 was established by plasmid analysis, hybridisation and conjugation experiments. As shown in Fig. 1A and Table 2, all ESBL-producers carried a plasmid of ca. 150 kb hybridising with the blaCTX-M-15 probe and belonging to the IncF group. Furthermore, all imipenem-resistant isolates harboured a common plasmid of ca. 65 kb that gave a positive hybridisation signal with the blaOXA-48 probe and belonged to the IncL/M group. Noteworthily, the blaOXA-48 gene was always identified in transposon Tn1999.I.

To investigate the potential of transfer of the IncF and IncL/M plasmids carrying the blaCTX-M-15 and blaOXA-48 genes, conjugation experiments were performed using Ec18 and Ec9 (blaCTX-M-15), Ec110 (blaOXA-48) and Ec115 (blaCTX-M-15 and blaOXA-48) as donors. When selection was performed with ertapenem, E. coli transconjugants were readily obtained both from Ec110 and Ec115 donor strains. Three independent E. coli transconjugants analysed from each mating experiment showed resistance to ampicillin, AMC and ertapenem, were positive by PCR for the blaOXA-48 gene and carried an ca. 65 kb plasmid (Fig. 1A; Table 2). By contrast, no transconjugants were obtained after three independent conjugations using Ec18 and Ec9 as donors and ceftriaxone as selective agent, and only two transconjugants after the same number of
matings and ceftriaxone selection using Ecl15 as donor. Both Ecl15 transconjugants were resistant to ampicillin, AMC, broad-spectrum cephalosporins and aminoglycosides, had an elevated MIC of ciprofloxacin and carried two plasmids, one of ca. 150 kb and the other slightly larger than 65 kb, which hybridised with the blaCTX-M-15 and blaOXA-48 probes, respectively (Fig. 1A; Table 2). The latter could have derived from the ca. 65 kb plasmid through acquisition of additional DNA. Taken together, these results strongly suggest that the ca. 150 kb IncF plasmid carrying blaCTX-M-15 is not self-transferable, but it could be mobilised by the ca. 65 kb plasmid though at a rather low frequency. The qnrB and aac(6’)-Ib-cr genes were detected in the Ecl15 transconjugants as well as in the E. cloacae isolates carrying the IncF plasmid, all with decreased susceptibility to ciprofloxacin.

### 3.2. Genotyping of the CTX-M-15- and/or OXA-48-producing Enterobacter cloacae isolates

Typing of the 21 E. cloacae isolates by PFGE yielded seven profiles (X1–X7) [Fig. 1B]. A dendrogram of similarity based on these profiles revealed two main clusters of E. cloacae (clusters I and II) that were strongly associated with the two separate buildings of HUCA (Fig. 1C). Cluster I comprised two profiles (X1 and X5) with seven and one isolates, respectively. A representative X1
Table 2
Microbiological characteristics of clinical Enterobacter cloacae isolates and their transconjugants.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Antimicrobial resistance phenotype</th>
<th>ESBL/CARB gene</th>
<th>ERT/IPM MIC (mg/L)</th>
<th>Corresponding plasmids</th>
<th>PFGE profile/cluster/building</th>
<th>MLST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ec11, Ec12, Ec14, Ec10.</td>
<td>AMX-AMC-FOX-CTX-IPM-NAL-CIP-SXT</td>
<td>blaCTX-M-15</td>
<td>4/2</td>
<td>65 kb</td>
<td>X1/I/1</td>
<td>ST74</td>
</tr>
<tr>
<td>Ec11, Ec17, Ec18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tc-Ec10-ERT (1, 2 and 3)</td>
<td>AMX-AMC-ERT</td>
<td>blaCTX-M-15</td>
<td>2/0.5</td>
<td>65 kb</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TcEc13</td>
<td>AMX-AMC-FOX-CTX-GEN-ERT-IPM-NAL-CIP-SXT</td>
<td>blaCTX-M-15</td>
<td>3/2</td>
<td>65 kb</td>
<td>X5/I/2</td>
<td>ST78</td>
</tr>
<tr>
<td>Ec6, Ec12, Ec14, Ec16, Ec120</td>
<td>AMX-AMC-FOX-CTX-FEP-GEN-TOB-NAL-CIP</td>
<td>blaCTX-M-15</td>
<td>≤0.5/≤1</td>
<td>150 kb</td>
<td>X2/I/2</td>
<td>ST66</td>
</tr>
<tr>
<td>Ec9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ec19, Ec21</td>
<td>AMX-AMC-FOX-CTX-FEP-ERT-IPM-GEN-TOB-NAL-CIP</td>
<td>blaCTX-M-15</td>
<td>2/0.5</td>
<td>65 kb</td>
<td>X3/I/2</td>
<td></td>
</tr>
<tr>
<td>Ec13, Ec15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tc-Ec15-ERT (1, 2 and 3)</td>
<td>AMX-AMC-ERT</td>
<td>blaCTX-M-15</td>
<td>2/0.5</td>
<td>65 kb</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Tc-Ec15-CRO (1 and 2)</td>
<td>AMX-AMC-CTX-FEP-ERT-GEN-TOB-CIP</td>
<td>blaCTX-M-15</td>
<td>1/0.38</td>
<td>150 kb, &gt;65 kb</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ec15</td>
<td>AMX-AMC-FOX-CTX-ERT-IPM-NAL-CIP-SXT</td>
<td>blaCTX-M-15</td>
<td>4/4</td>
<td>65 kb</td>
<td>X6/–/1</td>
<td>ST45</td>
</tr>
<tr>
<td>Ec17</td>
<td>AMX-AMC-FOX-CTX-ERT-IPM-NAL-CIP-SXT</td>
<td>blaCTX-M-15</td>
<td>8/4</td>
<td>65 kb</td>
<td>X7/–/1</td>
<td>ST295</td>
</tr>
</tbody>
</table>

ESBL, extended-spectrum β-lactamase; CARB, carbapenemase; MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence type; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; FOX, cefoxitin; CTX, cefotaxime; ERT, ertapenem; IPM, imipenem; TOB, tobramycin; CRO, ceftriaxone; N/A, not applicable.

a Enterobacter cloaceae (Ec) isolates with numbers corresponding to patient numbers in Table 1. Ec isolates used as donors in conjugation experiments are shown in bold.

a See Fig. 1C and text for details.

b Inc/M plasmids.

c Isolates positive for the qnrB and aac(6′)-Ib-cr genes.

d IncF plasmids.

isolate and the X5 isolate belonged to ST74 and ST78, respectively, within the same clonal complex (Fig. 1C; Table 2). All X1 isolates were recovered in building 1, which houses the main ICU of the hospital (ICU1), where five of them were detected. The remaining two came from the internal medicine and neurology services. All isolates within cluster I were resistant to carbapenems owing to production of OXA-48 and all were also resistant to broad-spectrum cephalosporins, consistent with overexpression of the chromosomal bla ampC gene. Cluster II was associated with a second building of the hospital encompassing the cardiac and maxillofacial surgery units and another ICU (ICU2). It included 11 isolates with identical (X2; 7 isolates) or closely related (X3 and X4, with 3 and 1 isolates, respectively) profiles assigned to ST66, and all except one (Ec18; X4 profile) were recovered in building 2 (Fig. 1C). Interestingly, Ec18 was identified from a single patient who had been transferred from a hospital in the nearby region of Santander, where the E. cloacae infection had already been diagnosed. Isolates within cluster II were all resistant to broad-spectrum cephalosporins owing to the production of CTX-M-15, and 4 of the 11 isolates also harboured blaOXA-48 and were therefore resistant to carbapenems. Two distantly related profiles (X6 and X7) were shown by OXA-48-producers belonging to ST45 and ST295, respectively, and found in building 1 (Fig. 1C; Table 2).

4 Discussion

Enterobacter cloaceae isolates producing OXA-48, CTX-M-15 or both β-lactamas emerged as important pathogens in HUCA, a general university hospital in northern Spain. In fact, they accounted for 4.7% (21/448) of the total E. cloaceae isolates recovered over a 1-year period, although they were not previously reported in this region. Together with some sporadic isolates, two major clones (ST74 and ST66, falling in two different clusters according to PFGE analysis) are causing prolonged outbreaks in separate buildings of the hospital. The ST74 clone consisted of carbapenem-resistant isolates carrying the blaOXA-48 gene. This is the first outbreak caused by OXA-48-producing E. cloacae reported in Europe, but a previous outbreak has been noticed in a neonatal ICU in Jerusalem [11]. In contrast to that observed here, isolates from Israel carried a derivative of the OXA-48 plasmid that had acquired the blaCTX-M-14 gene. The second clone identified (ST66) corresponded to blaCTX-M-15-positive isolates with the ESBL gene located on an ca. 150-kb, non-conjugative plasmid of the IncF group. Some of them co-harboured the IncM plasmid and co-produced OXA-48. Interestingly, one of the clone 2 isolates was apparently transferred from another hospital located in a nearby region of Spain, so in addition to intrahospital dissemination, interhospital spread is contributing to the emergence of CTX-M-15-producing E. cloaceae in HUCA. Additional outbreaks of E. cloacae producing CTX-M-15 have occurred in a newborn ICU in Spain as well as in a children’s hospital in Finland [12,13].

Noticeably, some isolates belonging to clone 2 also carried the blaOXA-48 plasmid, which could have been acquired from other OXA-48-producing Enterobacteriaceae circulating in the hospital [5; our unpublished results]. This might be in accordance with the high frequency of dissemination of the blaOXA-48-carrying plasmid as previously demonstrated [14].
Occurrence of the *qnrB* and *aac(6')-Ib-cr* genes in enterobacterial isolates producing OXA-48 or ESBLs, including some of the CTX-M-15-producing *E. cloacae* isolates causing the outbreak in a neonatal unit in Spain, was previously noticed [12].

Most *E. cloacae* reported in this study were assigned to STs previously associated with ESBL- and carbapenemase-producers and considered as high-risk international clones (ST45, ST66, ST74 and ST78), with the latter two belonging to the same clonal complex [10,15]. Noteworthy, none of these clones had been previously...
associated with OXA-48 production. Since phylogenetic studies on multiresistant *E. cloacae* have been initiated only recently, further studies are required to expand the knowledge of these multiresistant bacteria.

Although the number of *E. cloacae* producing carbapenemases or ESBLs has increased in recent years, their real prevalence could be underestimated because of difficulties in detecting both β-lactamases. In the present study, the Carba NP test proved to be very efficient for accurate identification of carbapenemase-producers.

5. Conclusions

Intrahospital and interhospital dissemination of multiresistant *E. cloacae* isolates producing CTX-M-15, OXA-48 or both β-lactamases is a cause of concern due to the limited options remaining for the treatment of affected patients, most with critical underlying conditions. Interestingly, as observed for *K. pneumoniae*, emergence of both broad-spectrum resistance traits in single *E. cloacae* isolates corresponds to the transfer of genes having disseminated from community pathogens into nosocomial pathogens. Considering that the community reservoir of some of these resistance traits is significantly growing nowadays, the likelihood of witnessing an increased number of nosocomial outbreaks due to multiresistant *E. cloacae* is very high.

**Funding:** This work has been supported by project FIS-P11-00808 (Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Spain), co-funded by the European Regional Development Fund of the European Union: a way to Making Europe, and by the University of Fribourg (Fribourg, Switzerland). A short stay of JF at the University of Fribourg was supported by a grant from the Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). IM was the recipient of a predoctoral grant from the Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y la Tecnología [FICYT BP01-069].

**Competing interests:** None declared.

**Ethical approval:** Not required.

**References**


http://doc.rero.ch