Emergence of a multiresistant KPC-3 and VIM-1 carbapenemase-producing *Escherichia coli* strain in Spain

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**Objectives:** To characterize the mechanisms involved in carbapenem resistance, as well as the genetic elements supporting their mobilization, in a multidrug-resistant *Escherichia coli* isolate.

**Methods:** The *E. coli* isolate was obtained from a patient with fatal urinary sepsis. Antimicrobial susceptibility testing was performed by the disc diffusion and agar dilution methods. The *E. coli* molecular type and phylogroup were determined using multilocus sequence typing and the triple PCR technique, respectively. PCR and sequencing were used for virulence and resistance genotype characterization. Plasmid content and gene location were analysed by S1-PFGE, I-Ceu1-PFGE and hybridization experiments. Transformation assays were performed.

**Results:** The *E. coli* strain, typed as ST448 and phylogroup B1, was resistant to all tested antibiotics except fosfomycin, tigecycline and tetracycline. The following resistance and virulence genetic structures were obtained: ISKpn7 + *bla*<sup>KPC-3</sup> + ISKpn6 linked to Tn4401; tnpR + aac(6')-Ib'-9 + aadA1 + *bla*<sup>OXA-9</sup> + tnpR + *bla*<sup>TEM-1</sup> + tnpB + strB + strA + sul2; intI1 + *bla*<sup>VIM-1</sup> + aac(6')-Ib' + aphA15 + aadA1 + catB2 + *qacE*<sup>Δ1</sup>-sul1 + *orf5*; ISepC1 + *bla*<sup>CMY-2</sup>; IS26 + *bla*<sup>SHV-12</sup>; *aph*(3');-I; *aac*(3);-IV; *floR*; *catA*; and *fimA*. Mutations in the *ampC* promoter (−18, −1 and +58) and substitutions in the *gyrA* (Ser-83→Leu and Asp-87→Asn) and ParC (Ser-80→Ile) proteins were observed. IncFII (ST2), IncA/C and ColE<sub>TP</sub> plasmids of 145.5, 87 and <2 kb, respectively, were found. The *bla*<sup>VIM-1</sup> gene was located in a non-typeable plasmid of >300 kb, and the *bla*<sup>KPC-3</sup> gene in the 145.5 kb IncFII plasmid. Transformant strains carried the IncFII and ColE<sub>TP</sub> plasmids, and the *bla*<sup>KPC-3</sup>, *bla*<sup>TEM-1</sup>, *bla*<sup>OXA-9</sup>, aadA1, aac(6')-Ib'-9, *aac*(3);-IV and *floR* genes.

**Conclusions:** This is the first report of the co-production of KPC-3, VIM-1, SHV-12, OXA-9 and CMY-2 in a unique clinical multiresistant *E. coli* isolate. The dissemination of these genes on mobile genetic elements is alarming and complicates antimicrobial therapies.

**Keywords:** carbapenems, ST448, plasmids, aac(6')-Ib', CMY-2

**Introduction**

The alarming rise in the incidence of bacteria resistant to today’s antibiotics is becoming a major public health issue worldwide. In recent years, the therapeutic options for treating some infections have been reduced. The case of carbapenem-resistant Enterobacteriaceae is one of the most shocking. Carbapenems are a therapeutic option against some nosocomial or hospital-acquired infections caused by Gram-negative pathogens. The increase in carbapenem-resistant bacteria is mostly linked to the widespread dissemination of acquired carbapenemases, which are able to hydrolyse nearly all β-lactam antibiotics, including carbapenems. Additionally, carbapenemase-encoding genes are usually located in mobile genetic elements that may carry other resistance determinants, aggravating the problem. The spread of resistance determinants by horizontal genetic mobilization processes are involved in bacterial evolution, resulting in bacteria adapted to different environments.

The aim of this study was to characterize the mechanisms involved in carbapenem resistance, and the genetic elements supporting their mobilization, in a multidrug-resistant *Escherichia coli* isolate obtained from a patient with fatal urinary sepsis.
Methods

Case report
An elderly Spanish woman attended an emergency department with a 4 day history of persistent oliguria and fever, despite empirical treatment with trimethoprim/sulfamethoxazole. Her medical history included renal polycystosis, chronic renal disease and recurrent urinary tract infection, among other diseases. Explorations revealed signs of renal lithiasis with hydronephrosis and pyelonephritis. A mono J catheter was placed, and an E. coli susceptible to all routine antibiotics was isolated. The patient was admitted, and antimicrobial treatment was switched to ertapenem. After 1 week, the patient developed hypercapnic encephalopathy requiring mechanical ventilation. Mild improvement was observed for a few days; however, aggravated symptoms (including fever, sepsis signs and renal failure) appeared. Meropenem therapy was initiated. A new urine culture yielded significant growth of a multidrug-resistant E. coli (identified as W1058). One day later the patient died.

Antimicrobial susceptibility testing
Testing for susceptibility to ampicillin, amoxicillin/clavulanate, piperacillin, piperacillin/tazobactam, cefalotin, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, doripenem, ertapenem, streptomycin, gentamicin, kanamycin, tobramycin, amikacin, netilmicin, nalidixic acid, ciprofloxacin, chloramphenicol, tetracycline, tigecycline, colistin, trimethoprim, sulphonamides, trimethoprim/sulfamethoxazole, fosfomycin, rifampicin and fusidic acid was performed by the disc diffusion and/or agar dilution methods. The metallo-β-lactamase, extended-spectrum β-lactamase, class A carbapenemase and AmpC phenotypes were tested using inhibitor-based double-disc methods.

Multilocus sequence typing (MLST) and phylogroup characterization
MLST was performed according to the E. coli MLST Database (http://mlst.ucc.ie/mlst/dbs/Ecoli) and the phylogenetic group was determined using triple PCR (see Table S1, available as Supplementary data at JAC Online).

Antimicrobial resistance genes and virulence factors
The presence of metallo-β-lactamase, class A carbapenemase, extended-spectrum β-lactamase, AmpC and other β-lactamase genes, in addition to their surrounding regions, was determined using PCR, primer-walking PCR and sequencing. Mutations in the ampc promoter/attenuator and in the gyrA, gyrB and parC genes were analysed by PCR and sequencing. Aminoglycosides, chloramphenicol, quinolone and sulphonamide resistance genes, virulence factors and the presence of class 1, 2 and 3 integrons were tested by PCR. The contained gene cassettes and the integron promoter (Pc) were characterized by PCR and sequencing.

The primers used in all these PCRs are detailed in Table S1.

Plasmid and hybridization analysis
The plasmids were classified by PCR-based replicon typing and those belonging to IncF were subtyped by plasmid MLST (Table S1). The plasmid number and size were determined using S1-PFGE analyses (Takara Inc., Japan). The gels obtained from the plasmid extraction (Qiagen Midi Kit, Hilden, Germany), S1-PFGE and I-Ceu1-PFGE (New England Biolabs Inc., USA) were analysed by Southern blot and hybridization using blaKPC-3, blaVIM-1, CoIE1, IncFI, IncA/C and 165 rDNA specific probes (PCR Dig Probe Labelling Mix; Roche Applied Science, Barcelona, Spain).

Transformation assay
Electroporation of E. coli DH10B cells and plates with imipenem (32 mg/L), imipenem (8 mg/L) + chloramphenicol (32 mg/L) or aztreonam (64 mg/L) were used to select the transformants, which were confirmed by repetitive extragenic palindromic sequence PCR (REP-PCR) (Table S1).

Results and discussion
The clinical E. coli strain W1058 belonged to phylogroup B1, was ascribed to sequence type ST448 (clonal complex 448) and, among the virulence factors tested, only the fimA gene was detected.

Phenotype, genotype and their correlation
E. coli W1058 was resistant to all tested antibiotics except fosfomycinn, tigecycline, colistin and tetracycline. The MICS of ampicillin, ceftaxime, ceftazidime, cefoxitin, aztreonam, imipenem and meropenem were alarming (≥128 mg/L). Related to this phenotype, the blaVIM-1, blaKPC-3, blaShv-12, blaOxa-9, blaTEM-1-4 and blaCMY-2 β-lactamase genes and mutations in the chromosomal ampC promoter/attenuator region (−18, −1 and +58) were detected.

Among the other antimicrobial resistance genes investigated, floR, catB2 and catA related to chloramphenicol resistance, sul1 and sul2 related to sulphonamide resistance and aph(3′)-I, aac(3)-IV, aac(6′)-Ib′, aac(6′)-Ib′-9, adaA1 and strA-strB related to aminoglycoside resistance were found. Regarding quinolone resistance (MIC of ciprofloxacin, 32 mg/L), the substitutions Ser-80 → Leu and Asp-87 → Asn were found in GyrA, and Ser-80 → Ile in ParC, whereas neither mutations in GyrB nor transferable quinolone resistance genes were detected.

The presence of two aac(6′)-Ib′ variants was noted. Compared with AAC(6′)-Ib′, AAC(6′)-Ib′ is identical with the exception of the Leu-119 → Ser substitution, and AAC(6′)-Ib′-9 has Gin-118 → Leu, Leu-119 → Ser and Asp-200 → Val substitutions. The aac(6′)-Ib′-9 gene has previously been found associated with blaVIM-2, blaKPC-3 or blaOXA-48 genes in Klebsiella pneumoniae carriers.

To our knowledge, this is the first description of the coexistence of blaVIM-1, blaKPC-3 genes in an E. coli strain. Co-producers of KPC and VIM have previously been described in K. pneumoniae isolates in other countries, but never in Spain or in E. coli strains.

To determine the genetic environment of the β-lactamase genes, and the coexistence of several resistance genes in the same mobile structures, several studies were carried out.

Study of regions surrounding β-lactamase genes
E. coli W1058 contained a new class 1 integron with the blaVIM-1 + aac(6′)-Ib′ + aphA15 + adaA1 + catB2 gene cassette arrangement regulated by a PcS promoter (Figure 1). This integron, named as In916 by the INTEGRALL database, was deposited in GenBank under accession number KF856617. The insertion sequences IS26 and IScep1 were found upstream of the blavim-12 and blacmy-2 genes, respectively. None of the previously described structures was found downstream of blacmy-2 or the expected b1c gene conserved from the chromosomal blacmy-2 origin (Figure 1).
The ISKpn7-bla<sub>KPC-3</sub>-ISKpn6 structure associated with Tn4401 was detected, as previously reported. Additionally, the bla<sub>OXA-9</sub> and bla<sub>TEM-1</sub> genes were both located in the previously reported Tn1331-associated structure: tnpR+aac(6')-Ib'-9+aadA1+bla<sub>OXA-9</sub>+tnpR+bla<sub>TEM-1</sub>+tnpB+strB+strA+ sul2 (Figure 1). Tn1331 includes the aac(6')-Ib, aadA1, bla<sub>OXA-9</sub> and bla<sub>TEM-1</sub> antibiotic resistance genes, although aadA1/bla<sub>OXA-9</sub> is a fused gene cassette that can be excised in the presence of an IntI1 integrase, allowing recombination events.

**Plasmid characterization and transformation assay**

More than four plasmids of between <2 and 388 kb were detected in *E. coli* W1058. The bla<sub>KPC-3</sub> gene was located in an IncFII (F2:A-A-B-) plasmid of 145.5 kb, the bla<sub>TEM-1</sub> gene was located in a non-typeable plasmid of 388 kb, and the IncA/C and ColE<sub>Tp</sub> probes hybridized to plasmids of ~87 and ~2 kb, respectively.

The ColE<sub>Tp</sub>-like plasmid was completely sequenced, named as pNPO1, and included in GenBank under accession number KF992024. Among its 1917 nucleotides, neither antimicrobial resistance genes nor mobilization (mob) elements were detected. An open reading frame of 100 amino acids was identified, which showed a zinc ribbon domain and a transmembrane TM2 domain, and 99% identity with a hypothetical protein detected in a *Salmonella enterica* strain (GenBank no. ESF63298). Interestingly, this protein had previously been detected (but interrupted by the Tn4401-bla<sub>KPC-2</sub> element) in plasmid pBC633 of a carbapenem-resistant *K. pneumoniae* strain (GenBank no. EU176012). Tn4401 is a transposon capable of mobilizing bla<sub>KPC</sub> genes at high frequency, and the location of this Tn4401-bla<sub>KPC</sub> in plasmids increases the rapid dissemination of bla<sub>KPC</sub> genes. Indeed, the high degree of plasticity of KPC-carrying plasmids has recently been demonstrated.

To determine the transferability of the plasmids detected in *E. coli* W1058, transformation assays were performed. Class A carbapenemase-positive transformants resistant to penicillins, carbapenemases, aminoglycosides and chloramphenicol were obtained from imipenem-containing plates. Additionally, the genes bla<sub>KPC-3</sub>, bla<sub>TEM-1</sub>, bla<sub>OXA-9</sub>, aadA1, aac(6')-Ib'-9, aac(3)-IV and flbR were detected, as well as the replications IncFII and ColE<sub>Tp</sub>. None of the remaining genes, the integron or the IncA/C plasmid was amplified in the transformant strains. Considering all these results, the coexistence of these resistance genes in the same IncFII plasmid could be deduced. Similar results showing the coexistence of Tn4401 and Tn1331 in the same plasmid have previously been reported.

KPC-type carbapenemases are largely disseminated worldwide among *K. pneumoniae* isolates, but less frequently among other *Enterobacteriaceae* species. In Spain, the clonal dissemination of Tn4401-bla<sub>KPC-3</sub> on widespread IncFII plasmids in *Klebsiella* isolates has been reported, but our work documents not only the presence but also the transformation ability of this type of bla<sub>KPC-3</sub>-harbouring plasmid in an *E. coli* strain. Indeed, the transmission of KPC-carrying plasmids among *Enterobacteriaceae* species has been previously demonstrated.

No bla<sub>VIM-1</sub>-positive transformant was obtained in our study. The location of this gene in an integron, which is not a self-mobile element, and inside a large plasmid (388 kb), could explain the lack of successful transfer, as has been reported by other studies. In previous studies, the bla<sub>VIM-1</sub> gene has been detected in...
different class 1 integrons, such as In-e541, Inl113 and Inl110, whose arrangements included more than three gene cassettes related to aminoglycoside and/or chloramphenical resistance. Moreover, these large integrons were located inside plasmids of type IncI1 or IncHI2 of 60kb and 300–435kb, respectively.2,21

Conclusion
To the best of our knowledge, this is the first report of the coexistence of blaKPC-3, blaVIM-1, blaSHV-12, blaOXA-13, and blaOXY-2 β-lactamase genes in a unique multidrug-resistant E. coli strain. The spread of this strain and its resistance elements are of great concern for public health. Therefore, early detection, characterization and surveillance of these resistance elements are extremely important to avoid their dissemination and consequent treatment failures.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Oxford (http://jac.oxfordjournals.org/).

References