Whole genome sequencing, molecular typing and *in vivo* virulence of OXA-48-producing *Escherichia coli* isolates including ST131 H30-Rx, H22 and H41 subclones

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Carbapenem-resistant *Enterobacteriaceae*, including the increasingly reported OXA-48 *Escherichia coli* producers, are an emerging public health threat worldwide. Due to their alarming detection in our healthcare setting and their possible presence in the community, seven OXA-48-producing, extraintestinal pathogenic *E. coli* were analysed by whole genome sequencing as well as conventional tools, and tested for *in vivo* virulence. As a result, five *E. coli* OXA-48-producing subclones were detected (O25:H4-ST131/PST43-fimH30-virotype E; O25:H4-ST131/PST9-fimH22-virotype D5, O16:H5-ST131/PST506-fimH41; O25:H5-ST83/PST207 and O9:H25-ST58/PST24). Four ST131 and one ST83 isolates satisfied the ExPEC status, and all except the O16:H5 ST131 isolate were UPEC. All isolates exhibited local inflammatory response with extensive subcutaneous necrosis but low lethality when tested in a mouse sepsis model. The *bla*<sub>OXA-48</sub> gene was located in MOB<sub>P131</sub>/IncL plasmids (four isolates) or within the chromosome (three ST131 H30-Rx isolates), carried by Tn1999-like elements. All, except the ST83 isolate, were multidrug-resistant, with additional plasmids acting as vehicles for the spread of various resistance genes. This is the first study to analyse the whole genome sequences of *bla*<sub>OXA-48</sub>-positive ST131, ST58 and ST83 *E. coli* isolates in conjunction with experimental data, and to evaluate the *in vivo* virulence of *bla*<sub>OXA-48</sub> isolates, which pose an important challenge to patient management.

*Escherichia coli* is a common member of the intestine microbiota of warm-blooded vertebrates including humans. It can cause a range of conditions, from diarrheagenic diseases to extraintestinal infections. Extraintestinal pathogenic *E. coli* (ExPEC) lineages are involved in the latter. Within them, the clonal group ST131 and its H30-R and H30-Rx subclones (clades C1 and C2), are associated with antimicrobial resistance and have successfully spread, creating a global epidemic of multidrug-resistant (MDR) *E. coli* infections. Treatment of MDR *E. coli* infections has become a serious clinical issue, with carbapenems being one of the last therapeutic options. Unfortunately, carbapenemase production is increasingly being reported in *E. coli*, as well as in other *Enterobacteriaceae*. The *bla*<sub>OXA-48</sub> gene, which encodes the OXA-48 carbapenem-hydrolysing class D β-lactamase, has been detected in several *E. coli* clonal groups, including ST131<sup>1,2</sup>. The *bla*<sub>OXA-48</sub> gene is usually located on self-transferable plasmids.

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**Table 1.** Origin, resistance properties and molecular typing of OXA-48 β-Lactamase *Escherichia coli* isolates from a Spanish hospital. ¹F, female; M, male. ²years old. ³GSU, general surgery unit-HUCA; ICU, intensive care unit-HUCA; PCC, primary-care center; RU, reanimation unit-HUCA; EU, emergency unit-HUCA; GU, geriatric unit of a long-term care facility; HUCA, Hospital Universitario Central de Asturias. ⁴AMP, ampicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; CTX, cefotaxime; ETP, ertapenem; IPM, imipenem; MER, meropenem; CHL, chloramphenicol; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; STR, streptomycin; ERY, erythromycin; NAL, nalidixic acid; CIP, ciprofloxacin; SUL, sulfonamides; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; I, intermediate resistance. eAll resistance genes were *in silico* determined; *bla*<sub>CTX-M-15</sub> and *bla*<sub>OXA-48</sub> were also experimentally detected by PCR amplification. Plasmid genes are underlined (see Table 2 for details). fST, sequence type according to Achtman; PST, sequence type according to the Pasteur Institute. ²The virotype was determined by PCR based on the presence or absence of 13 virulence genes. gThe virotype was determined by PCR based on the presence or absence of 13 virulence genes. hIsolates partially characterized in a previous study. iMIC of erythromycin: 256 µg/ml, higher than that obtained for isolates lacking *mph*(A): 128 µg/ml (Ec-HUCA 3, Ec-HUCA 5, Ec-HUCA 6, Ec-HUCA 7) or 64 µg/ml (Ec-HUCA 4).
other carbapenems was low. In fact, all isolates except *E. coli*-HUCA-4 were susceptible to meropenem, so this drug could have been included in the therapy regimen for the treatment of the affected patients (Table 1). In addition, all isolates were positive in the modified Hodge and the Carba NP tests, and contained the \( \text{bla}_{\text{OXA-48}} \) gene (Table 1). In *E. coli*-HUCA 1 to 4 the \( \text{bla}_{\text{OXA-48}} \) gene was carried by conjugative plasmids of about 60 Kb, experimentally assigned to the IncL group. The \( \text{bla}_{\text{OXA-48}} \) gene was chromosomally located in *E. coli*-HUCA 5 to 7. All isolates except *E. coli*-HUCA 3 were also resistant to other antimicrobial agents, including fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole and broad spectrum cephalosporins (Table 1). The latter resistance was detected in four isolates (*E. coli*-HUCA 2, 5, 6 and 7), which tested positive for \( \text{bla}_{\text{CTX-M-15}} \) by PCR amplification and sequencing.

Five out of the seven isolates were identified as ST131 by the Achtman scheme and further differentiated into PST43 (*E. coli*-HUCA 5 to 7), PST9 (*E. coli*-HUCA 4) and PST506 (*E. coli*-HUCA 1) according to the Pasteur Institute scheme (Table 1). All ST131 isolates belonged to serotype O25:H4, except *E. coli*-HUCA 1 which was O16:H5. The three O25:H4-ST131/PST43 isolates carried \( \text{fimH30} \), were resistant to fluoroquinolones and positive for \( \text{bla}_{\text{OXA-48}} \) and \( \text{CTX-M-15} \) (Table 1). *E. coli*-HUCA 1 to 4 presented different serotypes (O9:H25 and O25:H5), STs (ST58 and ST83), PSTs (PST24 and PST207) and \( \text{fimH} \) alleles (H27 and H21) (Table 1). ST58 has been identified in isolates of different origins (human, animal and environment), and was frequently associated with CTX-M production, particularly CTX-M-1. However, besides *E. coli*-HUCA-2, only one other ST58 OXA-48-producer has been reported. In contrast to ST131 and ST58, little information exists on ST83, which was previously found in uropathogenic *E. coli* from cats. In addition, a query of the Enterobase database (version 6th May 2017) identified 22 ST83 entries from different sources (human, companion animals, wildlife and environment) and geographical regions. The presence of \( \text{bla}_{\text{CTX-M-15}} \) in the otherwise susceptible *E. coli*-HUCA 3, demands further surveillance of the ST83 lineage. By PCR, the ST131 and ST83 isolates were assigned to phylgroup B2, while the ST58 isolate belonged to phylgroup B1.

The isolates were also analysed by XbaI-PFGE electrophoresis. The dendrogram generated from comparison of the macrorestriction profiles was consistent with the genetic diversity of the isolates. Thus, only the three ST131/PST43 H30-Rx isolates of virotype E clustered with similarity ≥85%. The remaining two ST131 isolates (*E. coli*-HUCA 1 and 4) presented 76.7% and 65% similarity with that cluster (Supplementary Figure S1). Although the high diversity of the ST131 clonal group has been widely reported, it was unexpected for this specific group of five ST131 OXA-48-producing isolates, since they were obtained in the same health area over a relatively short period of time.

### Sequencing of OXA-48-producing isolates and plasmid reconstruction.

The draft genomes of the seven *E. coli* isolates yielded 22 to 135 contigs larger than 1 Kb, with assembly sizes ranging from 4.9 Mb (*E. coli*-HUCA 3) to 5.4 Mb (*E. coli*-HUCA 4); (average of 5.2 ± 0.137 Mb). This is the first time that the genomes of OXA-48-producing *E. coli* isolates of virotype E clustered with similarity ≥85%. The remaining two ST131 isolates (*E. coli*-HUCA 1 and 4) were related with similarity ≥76.7% and 65% similarity with that cluster (Supplementary Figure S1). The high diversity of the ST131 clonal group has been widely reported. It was unexpected for this specific group of five ST131 OXA-48-producing isolates, since they were obtained in the same health area over a relatively short period of time.

In order to establish the plasmid content of the seven *E. coli* genomes, the PLACNET protocol was used for plasmid reconstruction (Supplementary Figure S2). As shown in Table 2, plasmids were found in all isolates in numbers ranging from one (*E. coli*-HUCA 5 to seven (*E. coli*-HUCA 4). In the genomes of *E. coli*-HUCA 2 and 4, PLACNET identified two plasmids that could not be separated and accounted for a total of 348 Kb [IncF (F2/51/40:A-:B1)] and 253 Kb [IncF (F2:A-:B1) plus IncI1 (ST48)], respectively. Plasmid extraction followed by visualization on agarose gels resolved two plasmids of ca. 140 and 110 Kb in *E. coli*-HUCA 2 and *E. coli*-HUCA 5 which was O16:H5.

In four out of the seven *E. coli* isolates (*E. coli*-HUCA 1 to 4; Table 2), the MOB_{131}/IncL plasmids were experimentally and in silico linked with the \( \text{bla}_{\text{CTX-M-15}} \) gene. Their genetic relationship was assessed by building a phylogenetic tree, which also included other IncL and IncM plasmids present in the GenBank-NCBI database (Fig. 1). Assignment to one or the other Inc group was corroborated in silico, or newly determined using the primers reported by Carattoli et al. The tree separated IncL and IncM plasmids in two clusters, the latter with two subclusters, IncM1 and IncM2, as previously observed. Interestingly, \( \text{bla}_{\text{CTX-M-15}} \) was not only carried by IncL plasmids but also by IncM1 plasmids, formerly classified as IncM1C. Besides, it was found also on IncC plasmids. The backbone of the IncM1 plasmids from the HUCA isolates were identical to each other and to nine other IncM plasmids, originating in *Klebsiella pneumoniae*, *Citrobacter freundii*, *Raoultella planticola* and *E. coli*. These results underline the prevalence of an IncM1 plasmid lineage which plays a major role in the horizontal spread of \( \text{bla}_{\text{CTX-M-15}} \) between members of the *Enterobacteriaceae*. BRIG comparison of the IncL plasmids is shown in Fig. 2. The p*E. coli*-HUCA and pE71T plasmids are highly similar to pOXA-48. Accession Number LN864820 showed a large gap at the mobilization-transfer region (24–39 Kb); whereas pCTX-M3 and pNDM-HK lack the \( \text{bla}_{\text{OXA-48}} \) region (25–8 Kb), as expected.

### Genetic environment of the \( \text{bla}_{\text{OXA-48}} \) gene.

As already indicated, \( \text{bla}_{\text{OXA-48}} \) was carried by MOB_{131}/IncL plasmids in four *E. coli* isolates, but chromosomally located in another three (Table 2). Although the DNA surrounding \( \text{bla}_{\text{OXA-48}} \) could not be assembled from the short illumina reads, the location of the gene within Tn1999-like transposons and the genetic environment of the transposons was established by PCR mapping. Specifically, \( \text{bla}_{\text{OXA-48}} \) was carried by Tn1999.2 in *E. coli*-HUCA 4; an inverted Tn1999.2 in *E. coli*-HUCA 1 to 3; and an
| Isolate | Plasmid Chromosome | Size (bp) | Contigs | Relaxase protein** | Replication rotein*** | Inc group (pMLST)** | Resistance gene** | Virulence gene** |
|---------|-------------------|-----------|---------|-------------------|----------------------|---------------------|------------------|-----------------|
| Ec-HUCA 1 | pEc-HUCA 1_1 | 131330 | 26 | MOBp12 | RepFII, RepFIB | IncF (F29:A-B10) | bla135a-3, mph(A), tet(B) | senB, fmsO, traT |
| pEc-HUCA 1_2 | 61395 | 6 | MOBp121 | IncF_RepA_superfamily (pfam02387) | IncL/M | blaOCA-6 | nd |
| pEc-HUCA 1_3 | 40230 | 6 | MOBp111 | Rep3_superfamily (pfam01051) | IncN (ST9) | nd | nd |
| pEc-HUCA 1_4 | 34530 | 4 | MOBp11 | Rep3_superfamily (pfam01051) | IncX1 | nd | nd |
| ICE_Ec-HUCA 1 | 201132 | 1 | MOBp12 | nd | nd | nd |
| Chr_Ec-HUCA 1 | 4903773 | 62 | na | na | na | gyra-S83L | gat, fmsABCDEF,GHI, ideBC, iha, pdhA, uap, matB, betaA, trp2, sitA, fyuA/psi |
| Ec-HUCA 2 | pEc-HUCA 2_1 + 2 | 347908 | 346 | MOBp12 | RepFII (c3), RepFIB | IncF (F2/51/40:A-B1) | bla135a-3, blaOCA-6, mph(A), tet(A) | trbN, mchf, ampT, sitC, cba, hlyF, hlyB, hlyC, sitC, sitD, fyuA/psi |
| pEc-HUCA 2_3 | 61304 | 6 | MOBp11 | IncF_RepA_superfamily (pfam02387) | IncL/M | blaOCA-6 | nd |
| pEc-HUCA 2_4 | 6187 | 1* | MOBp112 | Replicase (pfam03090) + priCT1 (pfam08708) | nd | nd | ceb |
| pEc-HUCA 2_5 | 3003 | 1* | MOBp11 | RNAI-II replication system | ColE-like | nd | nd |
| Chr_Ec-HUCA 2 | 4741812 | 134 | na | na | na | nd | gat, lpaA, fmsABCDEF,GHI, ideBC, matB, betaA, trp2, fyuA/psi, int |
| Ec-HUCA 3 | pEc-HUCA 3_1 | 111201 | 1* | no-MOB | nd | nd | nd |
| pEc-HUCA 3_2 | 61879 | 6 | MOBp11 | IncF_RepA_superfamily (pfam02387) | IncL/M | blaOCA-6 | nd |
| Chr_Ec-HUCA 3 | 4747878 | 64 | na | na | na | ampC2, ampH | gat, pic, fmsABCDEF,GHI, ideBC, fhaX, ampB, matB, betaA, trp2, fyuA/psi, vat, pabPCDFHJK |
| pEc-HUCA 4_1 + 2 | 253046 | 24 | MOBp113 + MOBp14 | RepFII, RepFIB | IncF_RepA_superfamily (pfam02387) | IncF (F2/A-B1) + IncI1 (ST48) | bla135a-3, cmrA1, tet(A), sul3 | trbN, mchf, ampT, sitC, cba, hlyF, hlyB, hlyC, sitC, sitD, fyuA/psi |
| pEc-HUCA 4_3 | 62423 | 5 | MOBp11 | IncF_RepA_superfamily (pfam02387) | IncL/M | blaOCA-6 | nd |
| pEc-HUCA 4_4 | 33543 | 1* | MOBp13 | Rep3_superfamily (pfam01051) | IncX4 | nd | nd |
| pEc-HUCA 4_5 | 6544 | 1 | MOBp11 | RNAI-II replication system | ColE-like | nd | nd |
| pEc-HUCA 4_6 | 4502 | 1 | MOBp11 | RNAI-II replication system | ColE-like | nd | nd |
| pEc-HUCA 4_7 | 1546 | 1* | no-MOB | RepA_HTH36, pfam13730 | nd | nd | nd |
| Chr_Ec-HUCA 4 | 4996329 | 48 | na | na | na | gyra-S83L | gat, cmfA, iss, cnf1, pdr, fmsABCDEF,GHI, ideBC, fhaX, ampB, matB, betaA, trp2, fyuA/psi, pabPCDFHJK, hlyABC |
| Ec-HUCA 5 | pEc-HUCA 5_1 | 5251 | 1* | MOBp11 | RNAI-II replication system | ColE-like | nd | nd |
| Chr_Ec-HUCA 5 | 5187005 | 184 | na | na | na | gyra-S83L | gat, cmfA, iss, cnf1, pdr, fmsABCDEF,GHI, ideBC, fhaX, ampB, matB, betaA, trp2, fyuA/psi, pabPCDFHJK, hlyABC |
| pEc-HUCA 6_1 | 45832 | 14 | MOBp11 | Rep3_superfamily (pfam01051) | IncN (ST7) | qnrS1, difA14 | nd | nd |
| pEc-HUCA 6_2 | 5251 | 1* | MOBp11 | RNAI-II replication system | ColE-like | nd | nd |
| Chr_Ec-HUCA 6 | 5218580 | 123 | na | na | na | gyra-S83L | gat, cmfA, iss, cnf1, pdr, fmsABCDEF,GHI, ideBC, fhaX, ampB, matB, betaA, trp2, fyuA/psi, pabPCDFHJK, hlyABC |

Continued
bioinformatic methods. The genetic bases of most other resistances could be also established by analysis in silico (Tables 1 and 2), being of note the presence of a W30R mutation in the E E84V) previously reported for the 30 subclone 32. The S83L mutation was also found in the not

| Isolate | Plasmids | Chromosome | Size (bp) | Contigs | Relaxase protein | Replication protein | Inc group (pMLST) | Resistance genes | Virulence genes |
|---------|-----------|------------|----------|---------|-----------------|---------------------|------------------|------------------|----------------|
| Ec-HUCA 7 | pEc-HUCA 7_1 | 48671 | 14 | MOB31 | Rep3_superfamily (pfam01651) | IncN (ST7) | blaCTX-M-15, qnrS1, dfrA14 | nd |
| pEc-HUCA 7_2 | 5251 | 1* | MOB311 | RNAI-II replication system | ColE-like | nd |
| chr_Ec-HUCA 7 | 5186594 | 125 | na | na | na | blaOXA-48-gyrA-S83I, E87N, parC-S80I + E84V, parE-I529L | gud, enfl, ptc, fimABCDEFGH, iebBC, iha, aap, matB, betA, tcp2, ntc, fnuA/psn, sat, papBCDFEGHIK, cnj1, hlyA/BCD, isi, incABC, intA |

Table 2. Plasmid content of OXA-48-producing Escherichia coli isolates and location of resistance and virulence genes. aRelaxase and replication proteins were identified by the use of homemade databases. bIncompatibility groups were determined according to the PBRT scheme and pMLST subtypes according to the allele scheme http://pubmlst.org/plasmid/. cAntimicrobial resistance genes were detected using the ARG-ANNOT and ResFinder databases. dVirulence genes were detected according to VirulenceFinder and homemade databases. *na, not applicable; nd, not detected. fLength of the ICE (integrative and conjugative element)-containing contig. *Closed plasmids.

inverted and deleted version of Tn1999.2 in Ec-HUCA 5 to 7 (Fig. 3). Tn1999.2 differs from Tn1999.1 by insertion of IS199 over the IS199 copy upstream of blaOXA-48 while in the deleted version, the IS199-containing copy of IS199 is truncated at the 5' end. IS199 enhances transcription of the downstream blaOXA-48 gene by providing an efficient promoter – 35 box with an optimal 17-bp spacing with regard to the –10 box supplied by IS199. This fact could explain the more frequent detection of Tn1999.2 and variants in comparison with Tn1999.1. Recently, the complete sequences of the first two ST131 blaOXA-48 plasmids have been reported; in both, the gene was carried by Tn1999.2, like in Ec-HUCA 4.

To identify the insertion sites of blaOXA-48 within the chromosomes of Ec-HUCA 5 to 7, assembled contigs were analysed. Three contigs containing sequences homologous to pOXA-48a (NC_019154.1) and pRA35 (LN864821.9) were identified, and two of them were flanked by E. coli chromosomal genes. The three isolates shared an identical 21.9 Kb fragment in which the inverted and deleted version of TnOXA-48 was found, followed by genes previously detected in pOXA-48a and pRA35. This fragment, which is flanked by IS199, constitutes an IS199 composite transposon, termed Tn6237. With the single excision of an isolate from Lebanon, this is the first report of a Tn6237 insertion into the chromosome of ST131. The precise insertion site of the transposon was established by PCR mapping (Supplementary Figure S4). In Ec-HUCA 6 and 7, Tn6237 was placed between the tdcA (DNA-binding transcriptional activator) and tdcB (threonine dehydratase) genes, which are contiguous in other E. coli genomes. In the case of Ec-HUCA 5, Tn6237 was followed by tdcB and morA (putative dehydrogenase) was placed upstream. This organization may have resulted from inversion of the chromosomal segment spanning tdcA to morA. Consistent with its expected lack of specificity, IS199-flanked Tn6237, was also found in three other insertion sites within the E. coli chromosome, in isolates from the United Kingdom, Czech Republic and Lebanon.

Resistance genes for other β-lactam and non-β-lactam antimicrobials. According to their phenotypes (Table 1), additional resistance genes were found in all OXA-48 isolates, contained in MOB311/IncF, MOB311/IncI and MOB311/IncN plasmids or in the chromosome (Table 2). Thus, diverse plasmids act as vehicles for the spread of resistance genes between E. coli clones/subclones circulating in the same health area. Remarkably, blaCTX-M-15 appeared in different locations within the four ST131 isolates carrying it. Specifically, blaCTX-M-15 was chromosomally located in three of the three isolates belonging to the H30-Rx subclone (Ec-HUCA 5 and 6), whereas in Ec-HUCA 7 it was carried by a MOB311/IncN (ST7) plasmid of 48.7 Kb, together with qnrS1 and dfrA14, for plasmid-mediated quinolone resistance (PMQR) and trimethoprim resistance, respectively. Interestingly, a slightly smaller MOB311/IncN (ST7) plasmid of 45.8 Kb contained qnrS1 and dfrA14, but not blaCTX-M-15, in Ec-HUCA 6. The blaCTX-M-15 gene of the non-ST131 isolate (Ec-HUCA 2), was carried by a conjugative MOB311/IncF plasmid of 150 Kb.

In the three ciprofloxacin resistant ST131 H30-Rx-OXA-48 isolates (Ec-HUCA 5 to 7; MIC > 32 mg/L), in silico analysis of the genomes revealed the distinct gyrA/parC allele combination (gyrA-S83I/D87N; parC-S80I/E84V) previously reported for the H30 subclone. The S83L mutation was also found in the gyrA gene of Ec-HUCA 1 and 4, which were resistant to nalidixic acid but susceptible to ciprofloxacin. Apart from qnrS1 found in Ec-HUCA 6 and 7, other PMQR genes, including additional qnr genes and qep or oqX genes, tested negative by bioinformatic methods. The genetic bases of most other resistances could be also established by in silico analysis (Tables 1 and 2), being of note the presence of a W30R mutation in the folA gene (dihydrofolate reductase) of Ec-HUCA 5, which was resistant to trimethoprim-sulfamethoxazole but lacked sul and dfr genes.

Virulence gene content and studies of virulence “in vivo”. Based on PCR-screening of 50 genes/alleles characteristic of ExPEC, Ec-HUCA 2 and 4 showed the lowest and highest virulence scores, with 16 and 33 virulence factors (VF), respectively. Of the five ST131 isolates, Ec-HUCA 4 was virotype D5 while Ec-HUCA 5 to 7 were virotype E (Supplementary Figure S1; Supplementary Table S1). The virulence profile of the O16:H5-ST131 isolate did not match any of the 12 virotypes included in the scheme. Four out of the five ST131 (Ec-HUCA 4 to
7) and the ST83 (Ec-HUCA 3) isolates showed the ExPEC status, and all isolates except Ec-HUCA 1 were classified as UPEC.

PCR-screening results were complemented by in silico analysis of the genome sequences, which allowed the identification of additional VFs, mainly encoded by genes located in the chromosome but also on plasmids (Table 2). Like before, the highest VF score was obtained for Ec-HUCA 4, followed by the ST131 H30-Rx-OXA-48 isolates (Ec-HUCA 5 to 7), which shared the same profile consisting of four operons and 15 individual genes. In contrast, a score of 16 was obtained for Ec-HUCA 1, the ST131 isolate with undefined virotype.

With regard to non-ST131 isolates, 27 VFs were detected in Ec-HUCA 2 and only 13 in Ec-HUCA 3. Interestingly, the plasmid virulence genes of Ec-HUCA 2 and 4 were nearly identical, and some virulence genes have chromosomal and plasmid copies (iss in Ec-HUCA 2, and mchF and iss in Ec-HUCA 4), providing regions of homology for interaction between the two replicons.

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**Figure 1.** Phylogenetic tree of IncL plasmids from OXA-48-producing isolates of *Escherichia coli*. The tree is based on SNPs found in the core genome (19,995 bp +/− 45 bp; 26 CDS with ≥80% identity, ≥60% pairwise alignment coverage), common to 31 IncL (18), IncM1 (seven) and IncM2 (six) plasmids. Bootstrap support values of 1,000 replicates are shown at the nodes. Clusters corresponding to each group/subgroup are enclosed in yellow, pink and blue boxes. IncL plasmids are more closely related to IncM2 than to IncM1 plasmids.
Finally, the intrinsic extraintestinal virulence of the OXA-48-producing *E. coli* was assessed in a mouse sepsis model. Within a seven day experiment, all mice challenged with *E. coli* CFT703 (positive control) died, compared with none of the mice challenged with *E. coli* MG1655 (negative control), which remained healthy. All OXA-48 isolates showed lethality as low as ≤10% and only three (*Ec-HUCA 2, 6 and 7*) caused the death of one out of the ten inoculated mice. In contrast, all isolates caused local inflammatory response, with extensive subcutaneous necrosis, in the surviving mice (Supplementary Table S2). Previously, we observed different virulence patterns in the final lethality, the rapidity in causing death and the inflammation-causing ability of ST131 isolates in correlation with the virotype, with the highest lethality (≥80% of mice challenged killed) shown by virotypes A, B, C and D1. By contrast, isolates within virotypes D2, D3 and D4 led to different outcomes, and isolates of virotype E showed the lowest final lethality, varying from 10 to 40% of the challenged mice. We also observed that certain ST131 isolates of virotypes C, D, and E induced an acute inflammatory response in the inoculation region22, like those in this study. There are few comparable *in vivo* studies and this is the first one assaying OXA-48-producing isolates. Results derived from the sepsis model might be consistent with the clinical nature of the isolates, not involved in severe disease but recovered from surgical wounds and UTIs. Future studies would be necessary to investigate the mechanisms responsible for the differences in lethality within ST131 virotypes.

**Phylogenomics of the OXA-48-producing isolates.** The phylogenetic context of the OXA-48-producing *E. coli* was assessed by comparison with 28 *E. coli* genomes with different STs, PSTs, virotypes and *fimH* alleles (Fig. 4, Supplementary Table S3). All ST131 isolates grouped in a single cluster which was further divided into subclusters according to clade, virotype and serotype. In agreement with their assignment to phylogroup B2, the ST131 cluster was genetically closer to ST83 (also B2) than to ST58 (phylogroup B1). It is of note that only two SNPs differences
were found between the COG-based core genomes of Ec-HUCA 6 and 7, and that both differed from Ec-HUCA 5 by 14 SNPs. Comparing these three highly similar isolates using SNP analysis and conventional PFGE, we found that both approaches discriminated between Ec-HUCA 5 vs 6 and 7, the latter displaying 100% identity between them and 88.4% with Ec-HUCA 5 by PFGE (Supplementary Figure S1). As shown in Table 1, these clonal isolates were detected in a primary-care centre, in the emergency unit of the HUCA and in the geriatric unit of a long-term facility. Thus, the H30-Rx-OXA-48 subclone, with the chromosomally located carbapenemase gene, is circulating both in the community and health-care institutions, which indicates transmission between the two settings. The number of SNPs between these and other isolates progressively increased according to the ST/PST and phylogroup affiliation, with averages of 3,755, 8334, 19,787 and 79,640, with regard to Ec-HUCA 4, 1, 3 and 2, respectively.

Concerning Ec-HUCA 5, 6 and 7, they are classified as virotype E and belong to clade C (PST43/H30), which comprises previously reported ST131 genomes with virotypes A, B and C. None of these ST131 references carried the blaOXA-48 gene, although BIDMC20B, BWH24 and MNCRE-44 (virotype C) harbour the blaKPC-3 carbapenemase gene, always located on large conjugative plasmids. It is important to note that this is the first study where ST131 virotype E genomes have been sequenced. In the clades A (PST506/H41) and B (PST9/H22-234), containing the Ec-HUCA 1 and 4 genomes, respectively, only Ecol_743 harbours the blaOXA-48 gene (located on a 69 Kb plasmid named pEC743_OXA48), while Ecol_448 contains the closely related class D beta-lactamase blaOXA-163 gene (pEC448_OXA163, 71 Kb plasmid).

According to our results, diverse OXA-48-producing E. coli clones are circulating in Oviedo, a situation justified in part by the conjugative potential of the IncL plasmid carrying the blaOXA-48 gene. Additional plasmids also play a role as vehicles of resistance and/or virulence genes. Treatment of the affected patients represents a serious challenge since all except one isolate were MDR. So, antimicrobial stewardship policies, new antimicrobial therapy approaches and control measures are necessary to combat the infections caused by these bacteria, and to control further dispersal.

**Material and Methods**

**Epidemiological background of the OXA-48-producing isolates.** The isolates were recovered from wound infections or UTIs between 2012 and 2015 (Table 1). Like Ec-HUCA 1 to 3, Ec-HUCA 4 caused a hospital-acquired infection and affected a critical patient exposed to long-term hospitalization and prolonged antimicrobial treatment in the HUCA. The remaining isolates were recovered from patients attended at a primary care centre (Ec-HUCA 5), the emergency unit of the HUCA (Ec-HUCA 6), and the geriatric unit of a long-term care facility associated with the hospital (Ec-HUCA 7).

**Antimicrobial susceptibility testing and plasmid analysis.** For the new isolates, antimicrobial susceptibility was determined by disk (Oxoid, Madrid, Spain or Becton Dickinson, Sparks, MD, USA) diffusion assays and the Microscan system (MicroScan, Beckman Coulter, CA, USA), which also allows bacterial identification.

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**Figure 3.** Genetic environment of the blaOXA-48 gene. (A) Structures of the Tn1999-like transposons and adjacent DNA from the IncL blaOXA-48 plasmids of Ec-HUCA 1, 2, 3 and 4. (B) Structure of the inverted and deleted Tn1999.2 transposon and the adjacent DNA including the insertion sites in the chromosomes of Ec-HUCA 5, 6 and 7. Open reading frames are represented by arrows indicating the direction of transcription and having different fillings: orange, IS1999; black dots, IS1; red, blaOXA-48; purple, lysR; blue, IncL plasmid genes; green, E. coli chromosomal genes. The Tn1999-like structures are highlighted by yellow boxes.
MICs for erythromycin were determined by broth microdilution following CLSI guidelines. MICs for carbapenems (ertapenem, imipenem and meropenem) were obtained with Etest strips (bioMérieux, Marcy-l’Étoile, France). Results were interpreted according to CLSI breakpoints. Carbapenemase production was confirmed by the modified Hodge and Carba NP tests. Identification of genes encoding resistance to carbapenems and broad-spectrum cephalosporins, and plasmid analysis were performed as reported. The genetic context of the bla\textit{OXA-48} gene was determined by PCR mapping (see Supplementary Table S4 and Supplementary Figure S4).

**Typing, subtyping and phylogenetic grouping of the isolates.** \textit{E. coli} isolates were characterized with regard to O:H serotype and \textit{fimH} alleles (for type 1 fimbrial adhesion). The STs were established following the MLST schemes of Achtman (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and the Pasteur Institute (http://bigodb.pasteur.fr/ecoli/ecoli.html). The XbaI-PFGE profiles were determined according to PulseNet protocol (http://www.pulsenetinternational.org/) imported into BioNumerics (Applied Maths, St-Martens-Latem Belgium) and clustered by Dice/UPGMA. Fifty genes/alleles encoding virulence factors (VF) were screened by PCR (Supplementary Table S5). Isolates were presumptively designated as ExPEC if positive for two or more of five markers and as uropathogenic (UPEC) if positive for three or more of four markers (Supplementary Figure 4).
Table S1)^24,5. The virotype of the ST131 isolates was established according to the scheme described by Dahbi et al.5. Assignment to the main phylogroups (A, B1, B2 and D) was based on the protocol of Clermont et al.15.

**Genome sequencing, assembly and analysis.** Total DNA from *E. coli* isolates was extracted with the QIamp DNA Mini Kit (Qiagen). Libraries were prepared using the TruSeq PCR-free DNA Sample Preparation Kit (Illumina) at the sequencing facility of the University of Cantabria. Paired-end 100 bp reads (550 bp insert size) were sequenced in a HiSeq 2500 (Health in Code Facility). Reads were assembled with the Velvet Optimiser. PL script of Velvet software56. Serotype, MLST, fimH alleles and virulence gene profiles were in silico determined with SerotypeFinder v1.1.45,46. and handmade MLST (Achtman and Pasteur schemes), fimH and virotype databases47. Antimicrobial resistance genes were detected using the ARG-ANNOT48 and ResFinder49 databases; virulence gene content was established with VirulenceFinder and handmade databases40.

For phylogenetic analysis, core genome was defined as described by Lanza et al.25, using the genomes of the *E. coli* isolates from the HUCA plus reference full-genomes retrieved from GenBank-NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/bacteria/) and Enterobase (http://enterobase.warwick.ac.uk/species/index/ecoli).

**Plasmid reconstruction from WGS data and analysis.** Plasmid reconstructions were based on the PLACNET method33. Contig analysis was performed against complete bacterial genomes and plasmids from GenBank-NCBI. Relaxase proteins (REL) and Replication Initiation Proteins (RIP) were identified using in-house databases35,51. Incompatibility groups and mPLSM subtypes were experimentally8,52 and/or in silico determined (http://pubmlst.org/plasmid/). Reconstructed *bla*~OXA-48~ IncL plasmids and references belonging to the IncL and IncN groups were compared using BRIG33, and a phylogenetic tree was built from variable positions (SNPs) in genes encoding core proteins.

**Mouse lethality assay.** A mouse sepsis model was used to assess extraintestinal virulence of the isolates22. For each one, 10 outbred female RjOrl:Swiss mice (3–4 weeks old; Janvier Labs, France) received a subcutaneous injection into the nape of the neck of approximately 2 × 10^6 CFU of log-phase bacteria. After inoculation, mice were clinically inspected along one week. Time of death and local presence of lesions (acute inflammation in the region of inoculation) were recorded for each mouse. Surviving mice were euthanatized on day seven by cervical dislocation. In each assay, two control isolates were included: *E. coli* K-12 MG1655 which does not kill mice by seven days post-challenge, and *E. coli* CFT073 which shows a lethality of ≥80% by seven days post-challenge. Results of lethality were indicated as the number of mice killed within 24 h and within seven days post-injection.

**Ethics statement.** All experimental protocols dealing with bacteria from human samples were approved by the ethics committee of the HUCA.

All animal experimentation was conducted following European (Directive 2010/63/EU on the protection of animals used for scientific purposes) and National (RD 53/2013) regulations for transport, housing and care of laboratory animals. The protocol used was approved by the Animal Welfare Committee of the Veterinary Faculty in Lugo, University of Santiago de Compostela (AE-LU-002/14-1).

**Data availability.** This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession numbers NBSZ00000000, NBSY00000000, NBSX00000000, NBSW00000000, NBSV00000000, NBSU00000000, NBST00000000 for *E. coli* HUCA 1 to 7, respectively. All the samples are part of BioProject PRJNA381431 and correspond to BioSample IDs SAMN06676454 to SAMN06676460.

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