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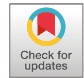
Original article

High-risk international clones ST66, ST171 and ST78 of *Enterobacter cloacae* complex causing blood stream infections in Spain and carrying bla_{OXA-48} with or without *mcr-9*



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ABSTRACT

Background: In the last years, *Enterobacter cloacae* complex has become an important threat associated with nosocomial infections (including bacteraemia). These bacteria have the ability to acquire mobile genetic elements with antimicrobial resistance genes, reducing the number of therapies available for treatment of the infections they cause. Multidrug resistant isolates of the *E. cloacae* complex have been causing blood stream infections in a hospital in northern Spain. The aim of this study was to report the spread of *E. cloacae* complex isolates carrying bla_{OXA-48} with or without *mcr-9* which were involved in blood stream infections, in a Spanish hospital.

Methods: All *Enterobacter* spp. isolates recovered from blood cultures of patients admitted to a tertiary Spanish hospital, over a five-year period were recovered. Of those, OXA-48-producing isolates were selected for further analysis (19 E. xiangfangensis isolates and a single E. hoffmannii). Bacterial identification, antimicrobial susceptibility, DNA sequencing, molecular typing, resistome analysis and plasmid characterization was performed.

Results: 20 isolates were positive for bla_{OXA-48}, harbored by IncL/M plasmids. They belonged to the international high-risk clones ST66, ST171 and ST78. They produced the extended-spectrum β-lactamases CTX-M-15 and/or CTX-M-9 and 40 % of them (n = 8) also carried the *mcr-9* gene, located on IncHI2 plasmids. However, they were susceptible to colistin.

Conclusion: The presence of bla_{OXA-48}, together with at least one bla_{CTX-M} gene in our multidrug resistant high-risk *E. cloacae* complex clones is worrisome. Also, the additional presence of *mcr-9* in some of them is of concern as it could potentially be transferred into other hosts or acquire mutations that might led to emerging colistin resistance. Surveillance systems are essential to detect these difficult-to-treat bacteria which, apart from causing live-threatening infections, can spread important resistance threats.

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Introduction

Members within the *Enterobacter cloacae* complex are facultative anaerobic, rod-shaped, non spore-forming bacteria belonging to the family *Enterobacterales.* They are common opportunistic nosocomial pathogens, able to cause a wide variety of infections such as bacteraemia, pneumonia, or urinary tract infections. This species

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harbors a chromosomal *ampC* gene which confers resistance to several betalactam antibiotics including aminopenicillins and 1st to 3rd cephalosporins [1]. Besides, in the past few years, there has been an extensive clinical use of carbapenems which, together with the ability of the *E. cloacae* complex to acquire mobile genetic elements [2], has helped to the emergence of carbapenem-resistant strains [3]. Several cases and outbreaks of carbapenemase-producing bacteria of the *E. cloacae* complex have been reported worldwide, being OXA-48-producers predominant in Europe and the Middle East [1,4].

Bloodstream infections caused by carbapenemase-producing *Enterobacterales* (CPE) have low therapeutic options and frequently affect patients who are severely ill [5]. They have been reported throughout the European continent and represent a threat to the patient's safety [6–8]. However, despite the increase of CPE in Europe in the last years, the number of invasive isolates of *Klebsiella pneumoniae* and *Escherichia coli* resistant to carbapenems is less than 5 % and 1 %, respectively, except in a few countries [9]. Unfortunately, *E. cloacae* complex is not under the scope of the antimicrobial resistance surveillance in Europe, so reliable data about the epidemiology of carbapenem resistance among invasive isolates within this complex is unknown.

In order to overcome the challenge of CPE infections, the use of polymyxins B and E (colistin) has been gaining strength as one of the last resort therapies. Colistin was administered in the past, but its use has to be discontinued because of its nephrotoxicity and neurotoxicity [10,11]. Despite this, its use has been recently resumed, on account of the lack of other effective antibiotics [12]. Due to its clinical importance in recent years, resistance to this drug is of matter of concern and hence subjected to surveillance nowadays. Several studies have reported a prevalence of colistin resistance among human clinical isolates higher in *Enterobacter* spp. than in *E. coli* and *Klebsiella* spp., being of 0.7 % in a global surveillance program [13].

Plasmid-mediated colistin resistance (PMCR) due to transferable *mcr* genes has recently emerged among *Enterobacterales*. To date there have been identified up to ten slightly different *mcr* variants (*mcr-1* to *mcr-10*). Since its first discovery *mcr* genes have showed an extraordinary spreading ability, as the first variant discovered, *mcr-1*, was reported in more than twenty countries within the first months after its discovery [14]. Nowadays *mcr* genes have been widely reported worldwide. This was most probably prompted by the recent increased use of this drug in the clinical setting but also due to its wide use in food-producing animals [13]. Colistin has been continuously used in the global livestock production for prophylactic, therapeutic and even for growth promotion purposes, which has already been prohibited in Europe since 2006. PMCR is a matter of concern since it can spread very rapidly among different bacteria by horizontal gene transfer [15,16]. Although *mcr*-carrying isolates were first reported in farm animals, they were later collected from foods, humans and the environment, and belonged to different bacterial species [14].

The aim of this study is to report the spread of carbapenem-resistant bacteria of the *E. cloacae* complex carrying $bla_{OXA\text{-}48}$- with or without *mcr-9*, causing blood stream infections between 2016 and 2019 in a single Spanish hospital.

Material and methods

Bacterial isolates and screening of carbapenemases

All *Enterobacter* spp. isolates recovered from blood cultures of different patients admitted to a tertiary hospital in northern Spain, "Hospital Universitario Central de Asturias" (HUCA), over a five-year period (2014–2019) were recovered (a total of 167 isolates). Blood cultures had been obtained from each patient after inoculation in two separate sets of Standard Aerobic (SA) and Standard Anaerobic (SN) BacT/ALERT®® blood culture bottles (bioMérieux Inc., Durham, NC, USA) which were processed in a BacT/ALERT®® VIRTUO™ machine (VIRTUO ™, bioMérieux Inc., Hazelwood, MO, USA). The bottles were incubated 5 days, and, if positive, standard subcultures were performed according to IDSA guidelines (https://www.idsociety.org/practice-guideline/laboratory-diagnosis-of-infectious-diseases).

A screening of carbapenemase-producing isolates was performed by means of a previously described algorithm based on a disc diffusion assay using ertapenem and temocillin and a lateral flow immunochromatographic assay [17] over this 167 strains, and 20 of them (11.98 %) were classified as carbapenemase-producing isolates and further analyzed.

Identification, susceptibility testing

Bacterial identification was performed by MALDI-TOF/MS (Bruker Daltonics, Billerica, MA). Antimicrobial susceptibility was determined by the Microscan system (Beckman Coulter, Brea, CA) for ampicillin (AMP), amoxicillin/clavulanic acid (AMC), piperacillin/tazobactam (PIP/TZ), cefotaxime (CTX), ceftazidime/avibactam, cefepime (FEP), meropenem, ertapenem (ERT), ciprofloxacin (CIP), trimethoprim-sulphamethoxazole (SXT), gentamicin (GEN), tobramycin (TOB), amikacin (AK) and tigecycline (TIG). Broth microdilution for colistin was carried out according to the EUCAST guidelines, which were also followed for minimal inhibitory concentration (MIC) interpretation (https://www.eucast.org/clinical_breakpoints/).

Molecular characterization, plasmid analysis and phylogenetic SNP-based tree construction

Total DNA from the isolates was extracted using the NZY Microbial gDNA Isolation kit (NZYTech, Lisbon, Portugal). Libraries were prepared using the TruSeq PCR-free DNA Sample Preparation Kit and then sequenced by Illumina technology to generate 125 bp paired-end reads in a HiSeq 1500. Quality control of the reads was accomplished using FastQC software (Babraham Bioinformatics, Cambridgeshire, UK) and *de novo* assembly was carried out with Velvet Optimiser [18], a script implemented within PLACNETw (https://castillo.dicom.unican.es/upload/). After genome sequencing bacterial identification was achieved *in silico* with KmerFinder, available at the Center for Genomic Epidemiology's (CGE) website (https://www.genomicepidemiology.org/). Multi-locus sequence typing (MLST) and detection of relevant resistance genes were performed *in silico* by the MLST 2.0 and ResFinder tools, respectively, also available at the CGE website. To further characterize and inquire the *mcr-9* gene location and for plasmid identification we used the PLACNETw interface, which differentiates contigs derived from the chromosome or plasmids, together with PlasmidFinder 2.1, pMLST 2.0 (https://www.genomicepidemiology.org/) and COPLA, which classifies plasmids into plasmid taxonomic units (PTUs) (https://castillo.dicom.unican.es/copla/). The estimated size and coverage of chromosome and plasmids, as well as the number of contigs of each genetic element are detailed on Supplementary Table SI. A comparison of the *mcr-9*-carrying plasmids was constructed with the Blast Ring Image Generator (BRIG) [19], using their concatenated contigs identified by PLACNETw, and the entire sequence of a closely related plasmid from *Enterobacter hormaechei* strain CPO052 2 (accession number NZ_JAETVM010000002.1) as reference. A phylogenetic SNP-based tree was constructed and visualized using CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/), MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/) and Phylo.io (https://phylo.io/).

Results

Isolate recovery and species identification

The 20 carbapenemase-producing *Enterobacter* spp. isolates selected for the present study were recovered from blood cultures of patients admitted to different hospital wards physically located far away from each other. They were identified as members of the *Enterobacter cloacae* complex by MALDI-TOF, and as *E. hormaechei* subsp. *xiangfangensis* (19 isolates) and *E. hormaechei* subsp. *hoffmannii* (a single isolate) by KmerFinder. Relevant properties of the isolates under study, such as species, date of isolation and hospital ward where they were detected are compiled in Table SII.

Susceptibility and resistance-encoding genes

Antibiotic resistance profiles and antibiotic resistance-encoding genes found in each isolate are detailed in Table SII. All of them were multidrug resistant according to Magiorakos criteria [20], being resistant to at least three categories of antibiotics. All proved to be non-susceptible to ertapenem, with minimal inhibitory concentrations (MICs) ranging from 1 to > 4; however they were susceptible to meropenem with MICs ranging from 0.25 to 1 mg/L. Regarding other beta-lactams, all isolates were resistant to AMP, AMC, CTX (which could be expected due to production of their intrinsic AmpC-beta-lactamase) but also to FEP, which suggested the additional presence of an extended-spectrum-beta-lactamase (ESBL). In fact, all of them harbored at least an ESBL-encoding gene ($bla_{CTX-M-9}$ and/or $bla_{CTX-M-15}$) and nineteen the oxacillinase OXA-1. All isolates were resistant to piperacillin/tazobactam (expected due to OXA-48 production) and susceptible to ceftazidime/avibactam with MICs ranging from 0.5 to 1 mg/L.

Moreover, some of the isolates were resistant to drugs of other families, like quinolones, aminoglycosides and SXT. However, all of them remained susceptible to colistin, with MICs lower or equal to 0.125 mg/L, even the eight that harbored the *mcr-9* gene (Table 1).

mcr-9 location and plasmid analysis

The *mcr-9* was located on large plasmids of the incompatibility group IncHI2; IncHI2A (typed as ST1), often accompanied by $bla_{CTX-M-9}$ and bla_{OXA-1}. The *mcr-9*-carrying plasmids detected in the present study were classified as PTU-HI2 with host-range Grade IV by COPLA. However, as previously reported for many other Enterobacterales, bla_{OXA-48} was carried by IncL/M plasmids of about 62 kb [4,21–23].

It is of note that the lack of full-length plasmid sequences, due to the Illumina technology used, is a limitation of the study. However, a BRIG comparison of the IncHI2 plasmids found in our *Enterobacter* isolates, reconstructed from the contigs identified by PLACNETw, and a closely related plasmid from *E. hormaechei* strain CPO052 2, revealed the limited differences existing between them and the very high number of antimicrobial resistance genes they carry (Fig. 2). The *mcr-9* was flanked by IS*903* (located upstream of the gene) in all isolates, and by IS*1* (located downstream) in seven isolates. The *mcr-9* gene of the remaining isolate was interrupted by IS*26* in position 1587, altering the last 17 amino acid residues of the C-terminal region of the protein (not shown).

Molecular epidemiology

The 20 isolates under study belonged to three different sequence types: ST66 (n = 11) and ST171 (n = 8), identified as *E. xiangfangensis*; and ST78 (n = 1), assigned to *E. hoffmannii*.

All non-*mcr-9*-carrying isolates (n = 12) belonged to ST66, except one that was ST171, while those positive for both bla_{OXA-48} and *mcr-9* were also ST171 (seven isolates) or belonged to ST78 (a single isolate).

SNP-based tree

To investigate how similar the HUCA isolates were to each other, we constructed a SNP tree including the 20 bla_{OXA-48} isolates detected in the HUCA and three additional ST78 isolates reported in the literature [24,25], (Fig. 1). The resulting tree distributed the isolates into two clusters, A and B, the latter divided in two subclusters, with each grouping associated with a different ST.

Discussion

During a five-year period, all carbapenemase-producing *Enterobacter* spp. isolates from blood cultures in our hospital were recovered (20/167; ca. 12 %). These 20 isolates were identified as *E. hormaechei* subsp. *xiangfangensis* and *E. hormaechei* subsp. *hoffmannii* by KmerFinder. However, these subspecies have been recently reclassified as *E. xiangfangensis* and *E. hoffmannii*, respectively, both within the *E. cloacae* complex [26].

Susceptibility testing assays revealed that all the isolates were multidrug resistant, and positive for bla_{OXA-48} harbored by IncL/M plasmids. Interestingly they were resistant to ertapenem but susceptible to meropenem. This is common in OXA-48-producing *Enterobacterales*, due to the weak hydrolyzing activity of OXA-48 against carbapenems and its predilection for ertapenem over meropenem [27,28]. The fact that all isolates carried at least one ESBL-encoding gene ($bla_{CTX-M-15}$ and/or $bla_{CTX-M-9}$), together with bla_{OXA-48}, is a matter of concern, as they will be resistant to cefepime and carbapenems, reducing even more the therapeutic arsenal available for treatment as these bacteria which are already intrinsically resistant to several clinically used antimicrobials, such as penicillins as well as first- and second-generation cephalosporins, due to the constitutive expression of AmpC beta-lactamase, and to third-generation cephalosporins under overexpression conditions of AmpC [2,29].

The *mcr-9* gene of our isolates was located on IncHI2 plasmids, which are the main vectors contributing to the spread of this gene worldwide [30]. However, this gene has also been found in hybrid IncHI2-IncR (mainly in China), hybrid IncHI2-IncA/C2 and IncFII plasmids [15,31,32]. Due to their high host-range grade (IV), PTU-HI2 plasmids, like those reported herein, are able to colonize bacteria not only from different genus or families but also from different orders [33]. Plasmids with such wide host-range are definitely a matter of concern, as dissemination of the resistance genes they carry can further affect the limited effective therapies available to treat serious infections caused by Gram negative bacteria in hospitals. The *mcr-9* gene of our isolates was flanked upstream by IS*903*, and by IS*1* downstream or interrupted by IS*26*. However, the most common core structure of the *mcr-9* cassettes consists of IS*903-mcr-9-wbuC* [15,34].

The presence of the two-component regulatory system QseBC downstream *mcr-9* has been linked to the induction of *mcr-9* expression by subinhibitory concentrations of colistin, leading to increased MICs in *Escherichia coli* [35]. In our *Enterobacter* isolates the *qseB* and *qseC* genes were not found on the IncHI2 plasmids downstream *mcr-9* (see below) but they were located on the chromosome. Nonetheless, reduced colistin susceptibility was not detected, as previously reported for other *mcr-9*-positive isolates not only of *Enterobacter* [24,36,37], but also of *E. coli* and *Salmonella enterica* from the United States, independently on the presence or absence of the *qseBC* genes [38]. Despite these results, detection of *mcr-9* is still a cause of concern due to the potential transfer of the gene into hosts or genetic environments where its expression can be achieved [35], and to the possible accumulation of additional mutations that may

Table 1
Clinical information and molecular features of $bla_{\text{OXA-48}}$ and *mcr-9* -carrying *Enterobacter cloacae* complex isolates recovered from blood cultures in a Spanish hospital from 2014 to 2019.

Isolate	Patient (Age/ Gender)	Hospital ward	Type of infection	Isolation date	Final therapy /outcome	Sequence type	Chromosome Plasmids	Inc group/MOB	Host-range	Resistance genes	Antimicrobial resistance
Eclo_HUCA_Bac_96	56/M	Urology	Urinary tract infection	18/06/2016	Meropenem/Survival	171	Chr (4.6 Mb)	na	na	$bla_{\text{ACT-16}}$, $bla_{\text{CTX-M-15}}$, *fosA*	AMP, AMC, PIP/TZ, CTX, FEP, ERT, CIP, SXT, GEN, TOB
							p1 (273.4 Kb)	IncHI2;IncHI2A/ MOBH	IV	$bla_{\text{OXA-1}}$, *mcr-9*, *qnrA1*, *aac(6')-Ib-cr*, *aadA2b*, *ant(2'')-Ia*, *aac(3)-IIa*, *sul1*, *dfrA14*	
							p2 (61.8 Kb)	IncL/M/MOBP	IV	$bla_{\text{OXA-48}}$	
							p3 (110.2 Kb)	IncFIB(pHCM2)	III	nd	
							p4 (2.6 Kb)	oriColE	nd	nd	
							p5 (2.4 Kb)	oriColE	nd	nd	
Eclo_HUCA_Bac_97	48/M	Hematology	CVL infection	18/07/2016	Meropenem/Survival	171	Chr (4.6 Mb)	na	na	$bla_{\text{ACT-16}}$, $bla_{\text{CTX-M-15}}$, *fosA*	AMP, AMC, PIP/TZ, CTX, FEP, ERT, CIP, SXT, GEN, TOB
							p1 (315Kb)	IncHI2;IncHI2A/ MOBH	IV	$bla_{\text{CTX-M-9}}$, $bla_{\text{OXA-1}}$, *mcr-9*, *qnrA1*,*aac(6')-Ib-cr*, *aadA2b*, *ant(2'')-Ia*, *aac(3)-IIa*, *sul1*, *dfrA14*, *drfA16*, *catA1*, *tet(A)*	
							p2 (62 Kb)	IncL/M/MOBP	IV	$bla_{\text{OXA-48}}$	
							p3 (2.4 Kb)	oriColE	V	nd	
Eclo_HUCA_Bac_98	63/F	Gastroenterology	Abdominal/ urinary infection	30/06/2016	Meropenem+amikacin /Survival	171	Chr (4.6 Mb)	na	na	$bla_{\text{ACT-16}}$, *fosA*	AMP, AMC, PIP/TZ, CTX, FEP, ERT, CIP, SXT, GEN, TOB
							p1 (289.7 Kb)	IncHI2;IncHI2A/ MOBH	IV	$bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{OXA-1}}$, *mcr-9*, *aac(6')-Ib-cr*, *aadA2b*, *ant(2'')-Ia*, *aac(3)-IIa*, *sul1*, *dfrA14*	
							p2 (61.8 Kb)	IncL/M/MOBP	IV	$bla_{\text{OXA-48}}$	
							p3 (2.6 Kb)	oriColE	nd	nd	
							p4 (2.4 Kb)	oriColE	nd	nd	
Eclo_HUCA_Bac_99	41/M	General surgery	CVL infection	01/08/2016	Meropenem/Survival	171	Chr (4.6 Mb)	na	na	$bla_{\text{ACT-16}}$, *fosA*	AMP, AMC, PIP/TZ, CTX, FEP, ERT
							p1 (283.5 Kb)	IncHI2;IncHI2A/ MOBH	IV	$bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{OXA-1}}$, *mcr-9*, *qnrA1*, *aac(6')-Ib-cr*, *aadA2b*, *ant(2'')-Ia*, *aac(3)-IIa*, *sul1*, *dfrA14*	
							p2 (61.7 Kb)	IncL/M/MOBP	IV	$bla_{\text{OXA-48}}$	
							p3 (2.6 Kb)	oriColE	nd	nd	
							p4 (2.4 Kb)	oriColE	nd	nd	
Eclo_HUCA_Bac_100	50/M	ICU	Abdominal infection	17/08/2016	Imipenem /Exitus	171	Chr (4.6 Mb)	na	na	$bla_{\text{ACT-16}}$, *fosA*	AMP, AMC, PIP/TZ, CTX, FEP, ERT, CIP, SXT, GEN, TOB
							p1 (291.1 Kb)	IncHI2;IncHI2A/ MOBH	IV	$bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{OXA-1}}$, *mcr-9*, *qnrA1*, *aac(6')-Ib-cr*, *aadA2b*, *ant(2'')-Ia*, *aac(3)-IIa*, *sul1*, *dfrA14*	
							p2 (61.7 Kb)	IncL/M/MOBP	IV	$bla_{\text{OXA-48}}$	
							p3 (9.4 Kb)	ColRNAI/MOBC	III	nd	
							p4 (2.6 Kb)	oriColE	nd	nd	
							p5 (2.4 Kb)	oriColE	nd	nd	

(*continued on next page*)

Table 1 (*continued*)

Isolate	Patient (Age/ Gender)	Hospital ward	Type of infection	Isolation date	Final therapy /outcome	Sequence type	Chromosome Plasmids	Inc group/MOB	Host-range	Resistance genes	Antimicrobial resistance
Eclo_HUCA_Bac_108	72/M	Hematology	Febrile neutropenia/ CVL infection	16/11/2017	Meropenem/Exitus	171	Chr (4.6 Mb)	na	na	*bla*$_{ACT-16}$, *bla*$_{CTX-M-15}$, *fosA*	AMP, AMC, PIP/TZ, CTX, FEP, ERT, CIP, SXT, GEN, TOB, TIG
							p1 (330.4 Kb)	IncHI2;IncHI2A/ MOBH	IV	*bla*$_{CTX-M-9}$, *bla*$_{OXA-1}$, *mcr-9, qnrA1, aac(6')-Ib-cr, aadA2b, ant(2'')-Ia, aac(3)-IIa, sul1, dfrA14, dfrA16, catA1, tet*(A)	
							p2 (61.8 Kb)	IncL/M/MOBP	IV	*bla*$_{OXA-48}$	
							p3 (2.6 Kb)	oriColE	nd	nd	
							p4 (2.5 Kb)	oriColE	nd	nd	
Eclo_HUCA_Bac_110	60/M	Hematology	Bacteremia of unknown origin	22/01/2018	Piperacillin tazobactam +amikacin /Survival	78	Chr (4.8 Mb)	na	na	*bla*$_{ACT-5}$, *fosA*	AMP, AMC, PIP/TZ, CTX, FEP, ERT, CIP, SXT, TIG
							p1 (368 Kb)	IncHI2;IncHI2A/ MOBH	IV	*bla*$_{CTX-M-9}$, *mcr-9, qnrA1, aadA2b, ant (2'')-Ia, sul1, dfrA15, dfrA16, catA1, tet*(A)	
							p2 (142.4 Kb)	IncFIA/MOBH	nd	nd	
							p3 (62.1 Kb)	IncL/M/MOBP	IV	*bla*$_{OXA-48}$	
							p4 (10 Kb)	oriColE/MOBP	nd	*aph(6)-Id, aph(3'')-Ib*	
							p5 (2.6 Kb)	oriColE	nd	nd	
Eclo_HUCA_Bac_111	60/M	Urology	Urethral perforation	31/01/2018	Amikacin /Survival	171	Chr (4.6 Mb)	na	na	*bla*$_{ACT-16}$, *fosA*	AMP, AMC, PIP/TZ, CTX, FEP, ERT, CIP, SXT, GEN, TOB
							p1 (306.5 Kb)	IncHI2;IncHI2A/ MOBH	IV	*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-9}$, *bla*$_{OXA-1}$, *mcr-9, qnrA1, aac(6')-Ib-cr, aadA2b, ant(2'')-Ia, aac(3)-IIa, sul1, dfrA14*	
							p2 (61.7 Kb)	IncL/M/MOBP	IV	*bla*$_{OXA-48}$	
							p3 (9.4 Kb)	ColRNAI/MOBC	III	nd	
							p4 (2.6 Kb)	oriColE	nd	nd	
							p5 (2.4 Kb)	oriColE	nd	nd	

M, male; F, Female; CVL, central venous line; na, not applicable; nd, not detected; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; PIP/TZ, piperacillin/tazobactam; CTX, cefotaxime; FEP, cefepime; ERT, ertapenem; CIP, ciprofloxacin; SXT, trimethoprim-sulphamethoxazole; GEN, gentamicin; TOB, tobramycin; TIG, tigecycline.

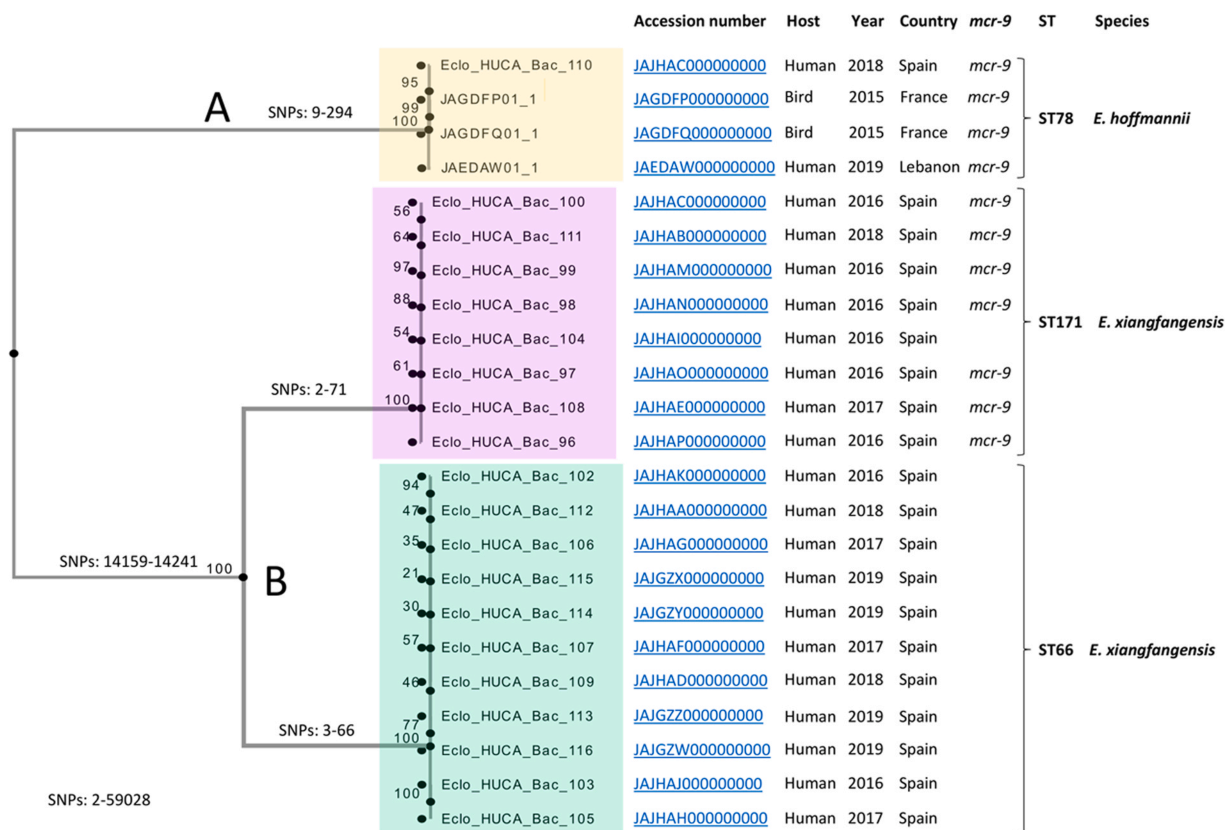

Fig. 1. Single Nucleotide Polymorphism (SNP) tree of 20 *bla*$_{OXA-48}$-carrying *Enterobacter cloacae* complex isolates causing bacteraemia in a Spanish hospital from 2014 to 2019, and three additional *E. hoffmannii* strains from the literature. Minimum and maximum values of SNP differences within and between groups are detailed. Bootstrap values based on 1000 replicates are indicated at the nodes. There are two different clusters: A and B, the latter comprising two subclusters. Isolates within cluster A, with ST78, were identified as *E. hoffmannii*, while ST171 and ST66 isolates, separated into two subclusters within cluster B, belonged to *E. xiangfangensis*.

lead to emerging colistin resistance [38]. Interestingly, *mcr-9* was first reported by Carroll et al., in 2019 in *S. enterica* [39], but here we found out that *Enterobacter* isolates carrying this gene have been infecting patients in our hospital at least since 2016.

Isolates from this study belonged either to ST66, ST171 or ST78, all of them considered as high-risk clones within the *Enterobacter cloacae* complex, since they have been previously reported globally and are responsible for the spread of clinically important resistance genes [40]. Clinical isolates identified as *E. cloacae*, assigned to these STs and resistant to critically important antibiotics have been reported in different countries. For instance ST66 and ST78 were associated with *bla*$_{OXA-48}$ and/or *bla*$_{CTX-M-15}$ (our hospital in 2013–2014; [4]); ST78 with *bla*$_{VIM-1}$ and *mcr-9* (the Netherlands; [41]); *bla*$_{KPC-2}$ (Israel and China), *bla*$_{CTX-M-15}$ (France), *bla*$_{CTX-M-9}$ (Latvia), *bla*$_{NDM-1}$ and *bla*$_{CTX-M-3}$ (China), *bla*$_{NDM-7}$ (Spain), *bla*$_{VIM-1}$ (Greece, Italy and Spain) and *bla*$_{OXA-48}$ (Turkey) [42–45]; and ST171 with *bla*$_{KPC-3}$ (widely spread in the United States), *bla*$_{NDM-1}$ (China, Guatemala, Brazil and South Africa), *bla*$_{KPC-2}$ (Colombia) and *bla*$_{KPC-4}$ (United Kingdom) [42,43].

When analyzing the SNP-based tree it is clear that two clusters are formed: cluster A (comprising ST78 isolates) and cluster B (englobing ST171 and ST66 isolates). According to the number of SNP differences (with minimum and maximum values within and between groups detailed in the Fig. 1), all isolates from the same cluster or subcluster were closely related to each other, and fairly different from those in other groupings. It stands out how the two sub-clusters comprising the ST171 and ST66 strains, which belong to the same species: *E. xiangfangensis*, are clearly separated from the cluster comprising ST78 isolates, which correspond to *E. hoffmannii*.

The spread of *bla*$_{OXA-48}$-positive bacteria of the *E. cloacae* complex, carrying *mcr-9* or not, is a cause of concern, as they have been originating blood stream infections in our hospital for several years. It is very important to control and study these bacteria, since they have a great capacity to cause dangerous outbreaks that, if not controlled, can cause a serious situation and the death of many patients. This was for instance the case reported for a neonatal intensive care unit where 86 % of the newborns diagnosed with bacteraemia caused by the *E. cloacae* complex, died [46].

In conclusion, surveillance systems in hospitals, especially in regions with high carbapenem resistance rates, are highly relevant in order to detect and handle troublesome difficult-to treat bacteria like the isolates of the *E. cloacae* complex reported herein. In addition to causing live-threatening infections, these isolates have the potential to spread important resistance threats. When such isolates display an elevated MIC to carbapenems, a carbapenemase screening should be performed to detect them promptly, since this will condition the therapeutic approach and allow the early instauration of infection control measures. In addition, due to the presence of *mcr-9* in isolates of this complex, as demonstrated in this and previous studies, a screening should be performed to avoid its silent propagation. Complementarily, One Health approaches must be designed in order to control the dispersion of relevant resistances and pathogens, like the ones here described, in other scenarios such as animals and the environment.

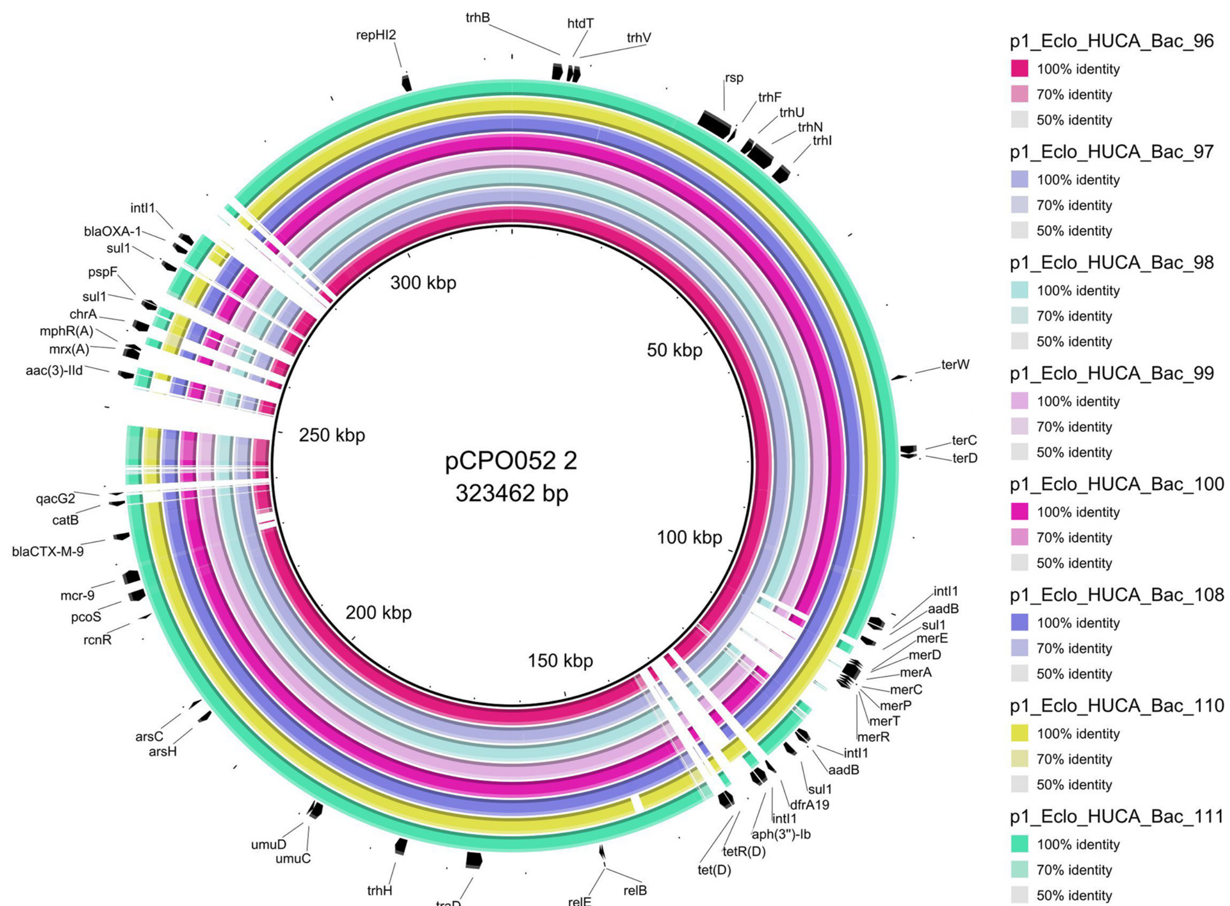

Fig. 2. BRIG (Blast Ring Image Generator) comparison of the IncHI2 plasmids of seven *Enterobacter xiangfangensis* and one *E. hoffmannii* isolates, all carrying *mcr-9*, recovered from blood stream infections in a Spanish hospital from 2014 to 2019. Plasmid from *E. hormaechei* strain CPO052 2 (accession number NZ_JAETVM010000002.1) was used as reference. Each ring corresponds to a plasmid, following the color code indicated to the right of the Figure. The inner black ring represents the reference plasmid. The outer black ring shows the annotation of relevant genes, represented by arrows pointing to the direction of transcription. Please note that the image was obtained from the concatenated contigs of each of the plasmids under study, identified by PLACNETw. Thus, the presence/absence of genes, but not necessary the order, is accurate.

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Conflict of Interest

We have no conflict of interest to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2022.12.015.

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