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RESEARCH

Evolution of ceftazidime/avibactam resistance and plasmid dynamics in OXA-48-producing *Klebsiella* spp. during long-term patient colonization

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Abstract

Purpose To prospectively monitor the evolution of the resistome of OXA-48-producing *Klebsiella* species in a patient with long-term colonization, with a particular focus into the plasmid dynamics and the evolution of ceftazidime/avibactam resistance.

Methods All OXA-48-producing *Klebsiella* spp. isolates from a single patient admitted to a hospital during seven months were prospectively collected. MICs were determined through reference broth microdilution. Multilocus sequence types, SNPs analysis, resistance mechanisms, genetic context of β-lactamases and plasmid dynamics were determined by WGS and bioinformatic analysis. The impact of β-lactamase variant obtained after ceftazidime/avibactam exposure was determined via cloning experiments.

Results Four isolates, two before (one OXA-48-producing *K. pneumoniae* and one CTX-M-15-like-producing *K. pneumoniae*) and two after treatment with ceftazidime/avibactam (one OXA-48- and CTX-M-15-like-producing *K. pneumoniae* and one OXA-48- and CTX-M-15-like-producing *K. aerogenes*) were collected. The plasmid dynamics analysis demonstrated that the IncL and IncFIIK plasmids, in which $bla_{\text{OXA-48}}$ and $bla_{\text{CTX-M-15-like}}$ genes were located, respectively, exhibited a high degree of conservation indicating a potential for both intra- and interspecies transmission. The *K. pneumoniae* isolate obtained after treatment, which differed from the previous isolate by just six SNPs, exhibited resistance to ceftazidime/avibactam through P167S substitution in CTX-M-15, which is now designated CTX-M-273. Cloning experiments demonstrated enhanced resistance to ceftazidime/avibactam.

Conclusion The transfer of plasmid-borne β-lactamase resistance genes between intra- and interspecies bacterial populations enables the rapid diversification of the bacterial genome. The emergence of ceftazidime/avibactam resistance through the modification of CTX-M-enzymes represents a mechanism by which OXA-48-producing Enterobacterales may evolve toward ceftazidime/avibactam resistance in vivo.

Keywords OXA-48 · CTX-M-15 · Ceftazidime/avibactam · *Klebsiella* spp. · Evolution · Plasmid dynamics · β-lactamase · Antimicrobial resistance

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Introduction

The spread of antimicrobial-resistant organisms, mainly carbapenemase-producing Enterobacterales (CPE), is considered one of the most significant threats to human health by the World Health Organization (WHO). Patients affected by these microorganisms have few treatment options, and the incidence is increasing, particularly in the hospital setting [1–3]. The intestinal tract provides an important reservoir for CPE which may subsequently cause life-threatening infections, particularly in immunosuppressed patients or those admitted to intensive care units [4, 5]. Indeed, as demonstrated in several studies, patients colonised by multidrug-resistant *K. pneumoniae* (i.e., OXA-48-producing), exhibit an increased risk of infection in comparison to patients who are not colonised by such microorganisms [6, 7]. These microorganisms frequently coexist in the gastrointestinal tract, thereby creating an optimal environment for the horizontal transfer of genes or complete plasmids that encode antibiotic resistance genes [7, 8].

In this regard, the OXA-48-like β-lactamases assume particular significance, given that they represent one of the most frequently documented carbapenemases worldwide in *Enterobacteriaceae*, including in Spain, where the $bla_{\text{OXA-48}}$ was identified in a majority of CPEs. Indeed, recent reports have documented the emergence of nosocomial outbreaks caused by OXA-48-like carbapenemases in regions where such pathogens are not endemic [9, 10]. Furthermore, this enzyme is widely distributed among *Enterobacteriaceae* and has been shown to be transmitted between them via plasmid, predominantly belonging to the L incompatibility group (IncL) [11]. OXA-48 enzymes exhibited a low level of resistance to carbapenem, rendering them challenging to detect in clinical laboratories and contributing to treatment failures in patients, as well as the lack of control over the spread of the $bla_{\text{OXA-48}}$ [12, 13]. Although rates vary by country, up to 80% of OXA-48- producing isolates also produce extended-spectrum β-lactamases (ESBLs) [9], with $bla_{\text{CTX-M-14-like}}$ and $bla_{\text{CTX-M-15-like}}$ being the most common genes [14, 15].

In accordance with the recently published guidelines of the Infectious Disease Society of America (IDSA), ceftazidime/avibactam is the recommended treatment option for infections caused by OXA-48-producing Enterobacterales [16]. Avibactam is a non-β-lactam β-lactamase inhibitor belonging to the diazabicyclooctane (DBO) family [17]. It was approved by the FDA in 2015 in combination with ceftazidime and has the ability to inhibit class A, class C and some class D β-lactamases, most notably OXA-48 [18, 19]. Unfortunately, since the approval of this novel combination, several mechanisms of resistance have emerged, predominantly driven by point mutations. Available data on this phenomenon predominantly concern KPC-producing isolates [16, 18], but little is known about the emergence of resistance to this compound in enzymes other than KPC in CPE.

Here, we provide insights into the evolution of the resistome of OXA-48 producing *Klebsiella* species in a patient with long-term colonization, with a particular focus into the plasmid dynamics and the evolution of ceftazidime/avibactam resistance.

Materials and methods

Patient case report

Four isolates from a 72-year-old woman with a medical history of hypertension, type 2 diabetes mellitus, and dyslipidaemia, were subjected to characterization (Fig. 1). In December 2018, she was admitted to the intensive care unit (ICU) with a diagnosis of necrotising pancreatitis. Multiple courses of antibiotics, including meropenem among others, were used to treat several complications, such as abdominal collection and pulmonary oedema. In February 2019, OXA-48 producing *K. pneumoniae* was isolated from a rectal sample during weekly active screening for carbapenem-resistant Enterobacterales (CRE); previously, these studies had been negative. Two different isolates, one OXA-48-producing *K. pneumoniae* (KpHUPM-97), phenotypically identical to that previously isolated from a rectal sample, and one ESBL-producing *K. pneumoniae* (KpHUPM-98), were recovered from a blood sample in April 2019. The patient was treated with ceftazidime-avibactam (2/0.5 g/8 h) for a period of 14 days, followed by meropenem (2 g/8 h) for an additional seven days. Three months later (July 2019), an OXA-48-producing *K. pneumoniae* (KpHUPM-106) showing resistance to ceftazidime/avibactam was isolated from a rectal swab. In September 2019, OXA-48- and ESBL-coproducing *K. aerogenes* (KaHUPM-81) was isolated from a rectal swab. No further isolates of OXA-48 producing have been recorded since September 2019. The patient was finally discharged in November 2019. The complete therapeutic regimen and isolates from the patient during her admission are shown in Fig. 1.

Bacterial strains and phenotypic characterization

In accordance with our laboratory protocols, active rectal screening for CPE is conducted twice a week during the course of hospitalisation in the ICU. For this purpose, rectal swab samples are cultured in ChromID Carba Smart

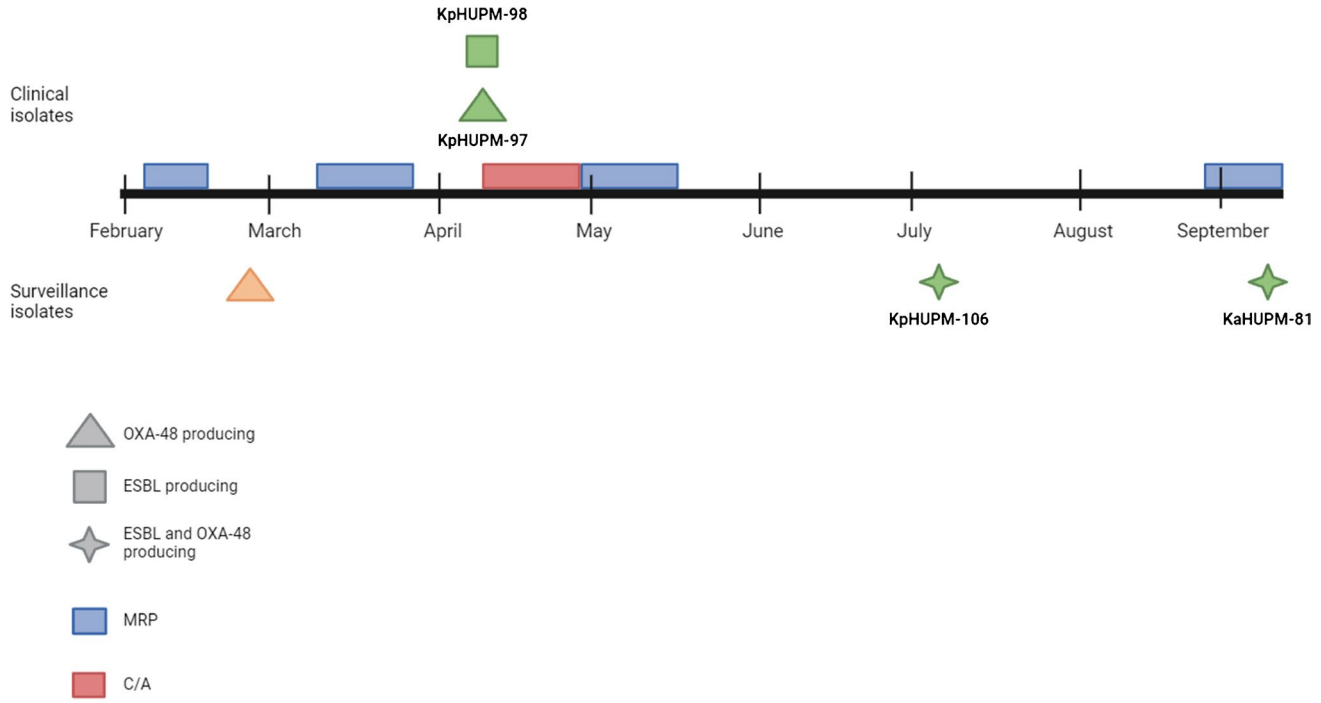

Fig. 1 Culture results and chronology of the patient's treatment. The isolates highlighted in green have been sequenced, whereas the one in orange has not been sequenced. MRP, meropenem; C/A, ceftazidime/avibactam

(bioMérieux, Spain). The identification of species is confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (MALDI Biotyper System, Bruker Daltonics GmbH&Co, KG, Bermen, Germany). The presence of carbapenem-resistance enzymes is detected using NG-Test CARBA 5 (NG Biotech, Guipry, France). CPE from active surveillance and from extraintestinal infections are stored at −80 °C for further analysis.

The minimum inhibitory concentrations (MICs) of piperacillin/tazobactam, aztreonam, cefotaxime, ceftazidime, ceftazidime/avibactam, cefepime, cefiderocol, ertapenem, imipenem, imipenem/relebactam, meropenem and meropenem/vaborbactam were determined for clinical isolates and transformants in triplicate experiments with reference broth microdilution assays. The MICs were determined using cation-adjusted Müeller-Hinton (MH) broth in all cases, with the exception of cefiderocol, which was assessed using iron-depleted cation-adjusted MH broth prepared according to CLSI M100 guidelines [20]. Tazobactam, avibactam and relebactam were tested at a fixed concentration of 4 mg/L, whereas vaborbactam was tested at 8 mg/L. EUCAST v14.0 clinical breakpoints and guidelines (https://www.eucast.org/clinical_breakpoints) were used for reference purposes. The reference strains *E. coli* ATCC 25922, *E. coli* NCTC 13353 and *K. pneumoniae* ATCC BAA-2814 were used as controls.

Whole-genome sequencing (WGS)

DNA extraction was performed with MagCore HF16 Plus (RBCBioScience Corp., Taipei, Taiwan). Paired-end libraries were prepared using a DNA prep library preparation kit (Illumina Inc., San Diego, CA, USA) and were sequenced with MiSeq (Illumina Inc., San Diego, CA, USA) with 600 cycle MiSeq Reagent Kit v3. The isolates were also sequenced using a MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) with an R9.4.1 flow cell. The WGS data described here were deposited in the GenBank database under accession numbers JANFHS020000000 (genome sequence data for strain KpHUPM-97), JANFHT020000000 (genome sequence data for strain KpHUPM-98), JAQIVP020000000 (genome sequence data for strain KpHUPM-106) and JANFGU020000000 (genome sequence data for strain KaHUPM-81). The sequence of the new CTX-M-15 variant described is available in the GenBank database under accession number PQ324802 ($bla_{\text{CTX-M-273}}$).

Bioinformatics analysis

The raw reads from Illumina MiSeq were quality filtered using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Trimmomatic v0.39 [21], and long reads were quality filtered with NanoPlot v1.42.0

[22] and Porechop v0.2.4 (https://github.com/rrwick/Porechop). Unicycler v0.4.8 was used for de novo assembly [23]. Multi Locus Sequence Typing (MLST) and genome annotation were performed using MLST v2.0.9 (https://cge.food.dtu.dk/services/MLST/) and Bakta v1.9.1 [24], respectively. CARD v3.2.3 was used to obtain β-lactamase resistance genes while ResFinder v4.1 was used to identify other resistance determinants, including chromosomal point mutations. To identify mutations in outer membrane proteins (OMPs) that result in a loss of function, the OMPs were manually aligned against *K. pneumoniae* ATCC 13883 and *K. aerogenes* ATCC 13048. Typing of plasmid incompatibility groups and transposable elements (including transposons and insertion sequences) was performed using PlasmidFinder v2.1 and ISfinder (https://www-is.biotoul.fr/index.php), respectively. Snippy v4.6.0 [25] was used to perform SNP analysis, with the previous isolate used as a reference to call SNPs in subsequent isolates. MOB-Suite v3.1.9 [26] software was used to predicting the mobility of the plasmids. BRIG v0.95 [27] was used to demonstrate the similarity between plasmid sequences from each isolate. Linear comparison figures were generated using Easyfig v2.2.5 [28].

Molecular cloning

To determine the specific contribution of the P167S substitution to ceftazidime/avibactam resistance, $bla_{\text{CTX-M-15}}$ genes from the KpHUPM-98 and KpHUPM-106 isolates were amplified in parallel with PCR using the primers 5'-GAA TTCCTTCGTGAAATAGTGATTTT-3' and 5'-CGGGAT CCCGGCTATTACAAACCGTCGG-3'. The PCR products were ligated to the pUCP-24 plasmid using SacI and BamHI restriction sites to obtain recombinant plasmids, which were subsequently electroporated into the *Escherichia coli* TOP10 strain, according to previously described protocols [29]. The transformants were selected on LB agar plates containing 10 mg/L gentamicin. The recombinant isolates were characterised with MIC determination following the methodology described previously.

Results

Resistance phenotypes

Table 1 shows the MICs of the clinical isolates included in the study. All the isolates were resistant to piperacillin/tazobactam, cefotaxime and cefepime and were susceptible to cefiderocol. The OXA-48-producing isolate (KpHUPM-97) was susceptible to aztreonam and ceftazidime, and resistant to ertapenem and imipenem-relebactam. The ESBL-producing isolate (KpHUPM-98) demonstrated susceptibility to all carbapenems (ertapenem, imipenem and meropenem)

Table 1 Date of isolation, sequence type and antimicrobial susceptibility testing, β-lactam resistance genes and plasmids of K. *pneumoniae* and K. *aerogenes* clinical isolates. Positives isolates are colored orange

Strain	Species	Isolation date	Sample	ST	MIC (mg/L) [a]										β-lactam resistance genes						Plasmid (Inc Types)					
					P/T (R>8)	AZT (R>4)	CTX (R>2)	CAZ (R>4)	CZA (R>8)	FEP (R>4)	FDC (R>2)	ERT (R>0.5)	IPM (R>4)	I/R (R>2)	MEM (R>8)	M/V (R>8)	*bla*OXA-48	*bla*OXA-1	*bla*CTX-M-15-like	*bla*SHV-27	*bla*SHV-40	*bla*TEM-1	IncFIIK	IncL	Col440I	Col440II	ColRNAI
KpHUPM-97	*K. pneumoniae*	01/04/2019	Blood	4975	>64	1	16	4	4	16	≤0.06	64	4	4	4	4											
KpHUPM-98	*K. pneumoniae*	01/04/2019	Blood	716	64	32	64	32	0.125	16	≤0.06	≤0.06	0.125	≤0.06	≤0.06	≤0.06											
KpHUPM-106	*K. pneumoniae*	09/07/2019	Rectal swab	4975	>64	>64	>64	1024	16	>64	0.5	>64	32	32	32	32											
KaHUPM-81	*K. aerogenes*	10/09/2019	Rectal swab	186	>64	>64	>64	128	2	>64	0.25	>64	64	64	64	64											

ST sequence type, *P/T* piperacillin/tazobactam, *AZT* aztreonam, *CTX* cefotaxime, *CAZ* ceftazidime, *CZA* ceftazidime/avibactam, *FEP* cefepime, *FDC* cefiderocol, *ERT* ertapenem, *IPM* imipenem, *I/R* imipenem/relebactam, *MEM* meropenem, *M/V* meropenem/vaborbactam

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and the novel combinations (ceftazidime-avibactam, imipenem/relebactam and meropenem/vaborbactam) tested in this study. The OXA-48- and ESBL-coproducing isolates, KpHUPM-106 and KaHUPM-81, displayed resistance to all carbapenems and their combinations, and KpHUPM-106 was also resistant to ceftazidime-avibactam.

Resistance genomics and genetic context of β-lactamases

K. pneumoniae isolates were assigned to ST4975 (KpHUPM-97 and KpHUPM-106) and ST716 (KpHUPM-98), and the *K. aerogenes* isolate was assigned to ST186 (KaHUPM-81). SNP analysis conducted to KpHUPM-97 and KpHUPM-106 revealed only six SNPs of difference between them (the genes affected by these 6 SNPs are detailed in Supplementary Table S1).

The distribution of plasmids and resistance genes among the isolates is shown in Table 1**.** The IncFIIK, IncL, ColRNAI, Col440I and Col440II plasmids were identified. The IncL and IncFIIK plasmids were classified as conjugative upon examination of the results obtained from MOB-Suite software. $bla_{\text{OXA-48}}$ was carried by IncL plasmids and was located in a Tn*1999.2* transposon. $bla_{\text{CTX-M-15}}$ was carried by IncFIIK plasmids, which also carry $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$. In addition, other β-lactam-resistance genes, corresponding to bla_{SHV} variants, were found (Table 1). A deletion at position 26 of OmpK37 was identified in all the clinical *K. pneumoniae* isolates, but no changes were observed in OmpK35 or OmpK36 when the *K. pneumoniae* clinical isolates and the reference strain were compared. In the KaHUPM-81 isolate, a stop codon was found at position 102 in OmpK36.

To facilitate a comprehensive comparison between different plasmids, BRIG was employed to align and visually represent other genomic variations (Fig. 2). SNP analysis of the IncL plasmids of KpHUPM-97, KpHUPM-106, and KaHUPM-81 isolates, which carry $bla_{\text{OXA-48}}$, revealed no SNP variation between them.

SNP analysis was also conducted between the three IncFIIK plasmids found in KpHUPM-98, KpHUPM-106 and KaHUPM-81. Only one SNP was detected between the IncFIIK plasmid of KpHUPM-106 and those from KpHUPM-98 and KaHUPM-81. This SNP was found to correspond to a nucleotide mutation, specifically C508T, which was identified in the $bla_{\text{CTX-M-15}}$ gene. A further genomic comparative analysis was conducted, which revealed that this mutation corresponds to an amino acid exchange at position 167 of CTX-M-15 β-lactamase from the KpHUPM-106 isolate. This position is designated according to the classification system proposed by Ambler et al. for class A enzymes and corresponds to the Ω-loop region of the β-lactamase [30]. Notably, this specific substitution was not detected in either the KpHUPM-98 or KaHUPM-81 isolates.

In both the KpHUPM-98 and KaHUPM-81 isolates, IS*Ecp1* was identified upstream of $bla_{\text{CTX-M-15}}$ gene. However, in the KpHUPM-106 isolate, the genetic environment of the $bla_{\text{CTX-M-15}}$ was found to differ, with the gene surrounded by IS26 elements. To analyse this discrepancy,

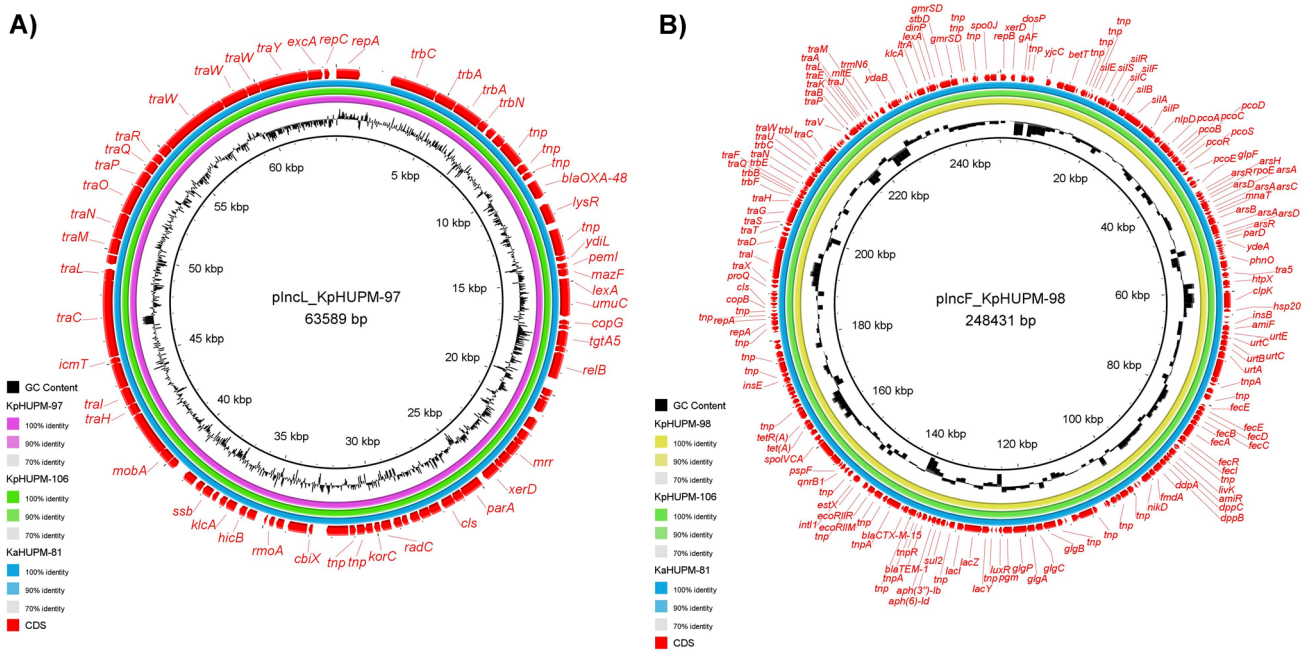

Fig. 2 Comparison of plasmids using BRIG. **A**) Comparison of IncL plasmids obtained from KpHUPM-97, KpHUPM-106 and KaHUPM-81 clinical isolates. **B**) Comparison of IncFIIK plasmids obtained from KpHUPM-98, KpHUPM-106 and KaHUPM-81 clinical isolates

EasyFig was employed. The resulting alignment revealed a recombinant position within the plasmid. The complete structures and recombinant sites are illustrated in Figs. 3 and 4.

Impact of the P167S substitution on ceftazidime/avibactam resistance

Comparative MIC values for the recombinant *E. coli* TOP10 transformants expressing the respective wild-type (P167) and mutated (S167) CTX-M-15 β-lactamases are shown in Table 2. As shown, expression of the S167 variant significantly increased resistance to ceftazidime and ceftazidime/avibactam, resulting in an eightfold increase in the MIC of this β-lactam/β-lactamase inhibitor combination (MIC shift from 0.25 to 2 mg/L). In addition, slight increases in the MICs of piperacillin/tazobactam and cefiderocol were also observed. In contrast, it conferred lower MIC values for aztreonam and cefotaxime, while no differential effects were observed for carbapenems.

Discussion

In accordance with the IDSA guidelines, ceftazidime/avibactam is regarded as the primary treatment option for multidrug-resistant (MDR) bacteria, including KPC- and OXA-48-producing *Enterobacteriaceae* [16]. Although resistance to this compound in Enterobacterales remains uncommon, the evolutionary trajectory of the resistome in patients who have been long-term colonised by OXA-48-producing *Klebsiella* spp. and treated with ceftazidime/avibactam remains poorly understood. The objective of this study is to gain insights into plasmid dynamics in the context of long-term colonization by OXA-48-producing *Klebsiella* spp. and into the evolution of the resistome during treatment with ceftazidime/avibactam.

Two *K. pneumoniae* isolates, one OXA-48-producing (KpHUPM-97) and one ESBL- and OXA-48-coproducing (KpHUPM-106) isolates, were assigned to ST4975, an infrequently reported clone, although it was previously identified by our group in a previous work including patients with OXA-48-producing *K. pneumoniae* long-term colonisation

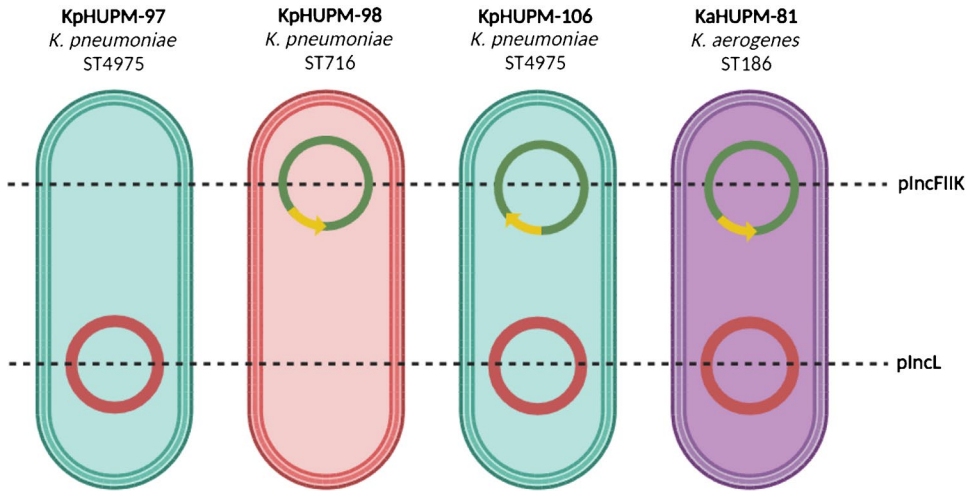

Fig. 3 Two plasmid backbones are associated with three microbial populations isolated from a single patient. The genus, species, and sequence type are indicated above each strain, along with the strain name. Plasmid types are listed on the right. The IncFIIK recombinant region is highlighted in yellow

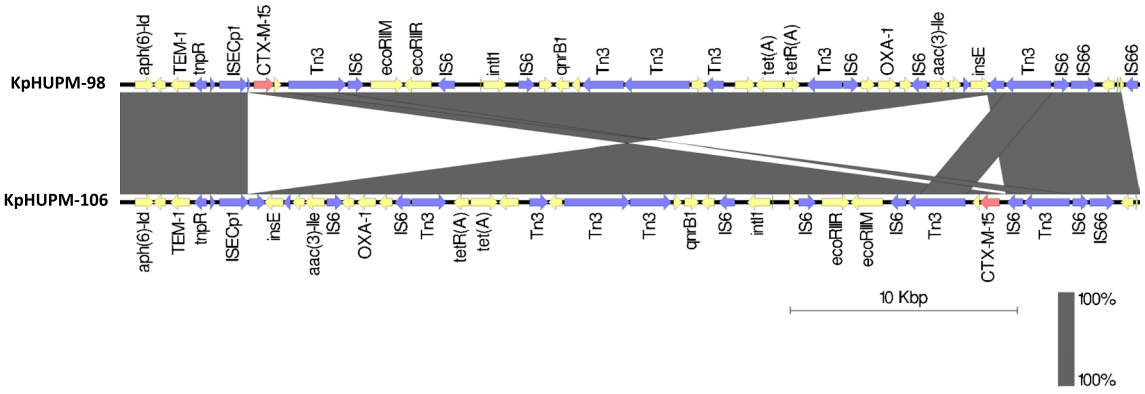

Fig. 4 The alignment of region found to be different between KpHUPM-98 and KpHUPM-106 isolates using Easyfig. Elements associated with gene mobility and $bla_{\text{CTX-M-15}}$ genes are marked in blue and red, respectively

Table 2 Susceptibility testing of *E. coli* transformants expressing CTX-M-15 and CTX-M-273 (CTX-M-15 P167S) β-lactamases

Strain	MIC (mg/L) [a]											
	P/T (R > 8)	AZT (R > 4)	CTX (R > 2)	CAZ (R > 4)	C/A (R > 8)	FEP (R > 4)	FDC (R > 2)	ERT (R > 0.5)	IPM (R > 4)	I/R (R > 2)	MEM (R > 8)	M/V (R > 8)
***E. coli* TOP10**	2	0.125	≤ 0.06	0.125	0.125	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06
***E. coli* TOP10 + pUCP-CTX-M-15**	2	64	> 64	64	0.25	16	0.125	≤ 0.06	0.125	0.125	≤ 0.06	≤ 0.06
***E. coli* TOP10 + pUCP-CTX-M-273 (CTX-M-15 P167S)**	4	32	32	256	2	8	0.25	≤ 0.06	0.125	0.125	≤ 0.06	≤ 0.06

P/T piperacillin/tazobactam, *AZT* aztreonam, *CTX* cefotaxime, *CAZ* ceftazidime, *C/A* ceftazidime/avibactam, *FEP* cefepime, *FDC* cefiderocol, *ERT* ertapenem, *IPM* imipenem, *I/R* imipenem/relebactam, *MEM* meropenem, *M/V* meropenem/vaborbactam

[a]2024 EUCAST BREAKPOINTS INDICATED

[31]. The SNP analysis conducted between the two isolates revealed that they exhibited nearly identical and clonally related profiles, with a distance of only six SNPs, which is below the threshold proposed by some studies [32, 33]. The *K. pneumoniae* ESBL-producing isolate (KpHUPM-98) was assigned to ST716, which has only been detected in China, where it has been linked to carbapenemase-producing isolates [34, 35]. Furthermore, no isolate of this sequence type has been identified in the most recent multicentre studies conducted in Spain [10, 36].

IncL plasmids are associated with a broad host range, with sizes between 50 and 80 kb, and low copy number [37]. The $bla_{\text{OXA-48}}$ gene is almost always located on a conjugative plasmid of 62 kb (pOXA-48a) assigned to the IncL group and inserted in Tn1999 [38]. In our study, $bla_{\text{OXA-48}}$ was detected in the Tn1999.2 structure, characterised by the presence of one copy of IS*1R* inserted into IS*1999* [39, 40]. This Tn*1999* variant has been described as one of the most frequently detected in our geographical region [41]. We detected the IncL plasmid in three clinical isolates (KpHUPM-97, KpHUPM-106 and KaHUPM-81), and no SNPs or additional genetic mobilisations were identified between them, including the IncL plasmid detected in *K. aerogenes* isolate (KaHUPM-81). These results indicate that the OXA-48-IncL plasmid is highly conserved, and it may have been transmitted between *K. pneumoniae* and *K. aerogenes*. Plasmid transmission intra- and interspecies has been reported in previous studies [32, 42].

The IncF group consists of plasmids with low copy numbers and varying sizes from 45- > 200 kb [37]. These plasmids are more commonly found in *E. coli*, although they have also been identified in other *Enterobacteriaceae* species. Notably, this group is closely related to certain resistance genes, including the most frequent genes, i.e. the ESBL and carbapenemase genes [43]. In our study, $bla_{\text{CTX-M-15}}$ was identified in IncFIIK plasmids from three clinical isolates, comprising two *K. pneumoniae* strains assigned to different STs (KpHUPM-98 and KpHUPM-106) and one *K. aerogenes* (KaHUPM-81). No modifications were identified between the IncFIIK plasmids from KpHUPM-98 and those from KaHUPM-81, again suggesting plasmid transmission between these two species. Moreover, it seems reasonable, considering the chronology of the isolates, that the IncFIIK plasmid was transmitted from ST716 (KpHUPM-98) to ST4975 (KpHUPM-106). However, the IncFIIK plasmid from KpHUPM-106 presented a nucleotide substitution within the $bla_{\text{CTX-M-15}}$ gene and an inverted fragment of 32,450 pb compared with the other two plasmids. The aforementioned fragment was delimited to $bla_{\text{CTX-M-15}}$ and IS6. Consequently, the region upstream of $bla_{\text{CTX-M-15}}$ in KpHUPM-106 was observed to differ from that detected in KpHUPM-98 and KaHUPM-81. The IncFIIK rearrangement probably occurred, according to the dates of the isolations, following treatment with meropenem and ceftazidime/avibactam.

Self-transmissible plasmids by conjugation are required to encode a complete set of genes responsible for transfer. These include the replicase, the relaxase (Mob protein), the type IV coupling protein (T4CP) and the type IV secretion system (T4SS) [44]. Upon examination of these elements, the IncL and IncFIIK plasmids described here were classified as conjugative. However, the precise direction of plasmid transmission between the isolates included in our study remains uncertain. It is also plausible that further populations may be present, as evidenced by other studies [45].

In the course of our investigation, we observed that the KpHUPM-106 isolate exhibited resistance to ceftazidime/avibactam. A comparative genomic analysis revealed that KpHUPM-106 acquired a proline to serine substitution at position 167 in the CTX-M-15 β-lactamase. This novel CTX-M-15 variant, which emerged in vivo, has been designated as CTX-M-273. Both et al. [46] detected CTX-M-14 P167S from in vivo isolates and demonstrated, with cloning experiments, an increase in the MICs of ceftazidime/avibactam. Different amino acid substitutions introduced into CTX-M-15 by site-directed mutagenesis were studied by Compain et al. [47], and the P167S substitution was associated with a fourfold increase in the MICs of ceftazidime/avibactam. To our knowledge, our study reports for the first time the in vivo emergence of the CTX-M-15 P167S variant. According to our results, the expression of the CTX-M-15 S167 variant in *E. coli* TOP10 transformants increased resistance to ceftazidime/avibactam, resulting in an eightfold increase in the MIC of ceftazidime/avibactam (MIC shift from 0.25 to 2 mg/L). This finding is consistent with previous studies that demonstrated the pivotal role of residues within the Ω-loop, including position 167, in determining substrate specificity and the inhibition profile of class A β-lactamases [48–50]. A previous structural study indicated that the P167S substitution in CTX-M-15 cause a *trans* peptide bond at residue 167 and a conformational change in the Ω-loop. This, in turn, results in increased hydrolysis of ceftazidime [51]. These findings are consistent with our own observations, in which the expression of CTX-M-273 in the recombinant isolate resulted in a fourfold increase in the MIC of ceftazidime, with the MIC shifting from 64 to 256 mg/L. Although resistance to this combination remains rare, there have been reports of resistance to ceftazidime/avibactam due to mutations in bla_{KPC}, bla_{GES} and $bla_{CTX\text{-}M\text{-}1\text{-}like}$ genes [46, 52–54]. Substitutions in the Ω-loop region of CTX-M β-lactamases, analogous to those observed in KPC, also result in an increase in MIC values approaching the clinical breakpoint, and in some cases, even lead to non-susceptibility to ceftazidime/avibactam [53]. In this context, the high co-occurrence of CTX-M-like- and OXA-48-producing Enterobacterales represents a significant risk due to development of resistance to ceftazidime/avibactam as a consequence of the emergence of CTX-M variants, such as CTX-M-273, which renders the novel combination ineffective.

The development and approval of ceftazidime/avibactam signalled an end to a period during which several CPEs were without treatment options. Subsequently, novel β-lactam/β-lactamase inhibitor combinations are currently in development or have been approved. The β-lactam/β-lactamase inhibitor combinations imipenem/relebactam and meropenem/vaborbactam have already been approved and have been demonstrated to exhibit potent inhibitory activity against class A and C β-lactamases; however, they display no activity against OXA-48 enzymes [55]. As expected, the MICs of OXA-48-producing clinical isolates from our study (KpHUPM-97, KpHUPM-98 and KaHUPM-81) against imipenem/relebactam and meropenem/vaborbactam were comparable to those obtained with the carbapenem alone, no inhibitory effect was observed. This is of particular concern for the KpHUPM-106 isolate, which was resistant to broad-spectrum cephalosporins, ceftazidime/avibactam and these two novel combinations. Fortunately, all clinical isolates remain susceptible to cefiderocol. However, a slight increase in the MIC of cefiderocol was observed in the KpHUPM-106 isolate. This was also observed in the transformant isolate expressing CTX-M-273, resulting in a twofold increase in the MIC of cefiderocol (MIC shift from 0.125 to 0.25 mg/L). This increase could almost be expected due to the increase in ceftazidime MIC and the closely related structure of these two antibiotics. Furthermore, the cross-effect of cephalosporin resistance substitution with cefiderocol has been documented in KPC Ω-loop mutants and other transferable β-lactamases and intrinsic AmpC enzymes of *Pseudomonas aeruginosa* (PDC) variants [56–58].

In light of the evidence presented, it can be inferred that the observed ceftazidime/avibactam resistance in KpHUPM-106 is largely attributable to the P167S substitution. As evidenced by previous studies, antibiotic resistance is becoming an increasingly complex phenomenon, involving the presence of multiple resistance mechanisms. These include the accumulation of different β-lactamases and different gene expressions, truncated porins and efflux pumps, among others [10]. In addition of these mechanisms, the critical development of mutations such as the P167S substitution further decreases the options for the use of novel compounds.

In conclusion, this study delineates the plasmid dynamics encompassing both intra- and interspecies transfer of conjugative plasmids and rearrangement within IncFIIK plasmid, which can facilitate the rapid generation of genomic diversification. Furthermore, we have described the first in vivo emergence of the CTX-M-15 P167S variant and demonstrated the role of this substitution in resistance to ceftazidime/avibactam. This emphasises the necessity for meticulous observation throughout the course of treatment with novel β-lactam/β-lactamase inhibitor combinations. A comprehensive understanding of the evolutionary dynamics of the resistome of Enterobacterales in long-term colonised patients is indispensable for our knowledge.

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Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval The project was approved by the Research Ethics Committee of Cádiz (PEIBA code 2609-N-21; registry number 225.21).

Competing interest The authors declare no competing interests.

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