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Potential role of avibactam in restoring susceptibility in *Escherichia coli* with two copies of bla_{KPC-3} and PBP3 mutations

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The emergence of carbapenemase-producing *Enterobacterales* represents a significant clinical challenge. Resistance mechanisms involve carbapenemase production, porin and efflux pump alterations, penicillin-binding protein (PBP) modifications, and biofilm formation. This study characterizes a KPC-*producing Klebsiella pneumoniae* and a concurrent *Escherichia coli* isolate harboring the same resistance genes, with *E. coli* also exhibiting PBP3 mutations. Whole-genome sequencing and plasmid analysis identified an IncFII(K) plasmid carrying the bla_{KPC-3} gene. Both strains shared two resistance genes (bla_{KPC-3}, bla_{OXA-9}). *K. pneumoniae* contained a single copy of bla_{KPC-3} on Tn*4401a*, whereas *E. coli* carried two copies on separate Tn*4401a* transposons. Plasmid reconstruction revealed high homology (99.85%) with *E. coli* plasmid pECAZ147_KPC and *K. pneumoniae* plasmid pKPC. This suggests that transposon-mediated bla_{KPC-3} transfer between the two strains may have occurred via the same plasmid. Moreover, *E. coli* harbored PBP3 mutations (A233T, I332V), which have been previously linked to increased ceftazidime MICs and two missense mutations in *marR*. Despite carrying two copies of the bla_{KPC-3} gene, along with *marR* and PBP3 mutations, our *E. coli* isolate did not exhibit a significant increase in ceftazidime/avibactam MIC. This phenomenon could be explained by avibactam's ability to bind to *E. coli* PBP2, potentially compensating for the reduced binding of ceftazidime to the mutated PBP3.

Keywords *Escherichia coli*, *Klebsiella pneumoniae*, KPC, PBP3, Avibactam, Conjugation, Transposon.

Antibacterial resistance is one of the most pressing public health challenges worldwide, further exacerbated by the rapid spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria[1]. Among Gram-negative pathogens, carbapenem-resistant Enterobacterales (CRE) are of critical importance and have been classified as a priority group in the WHO's list of critical pathogens[2]. The Enterobacterales order includes a diverse range of bacteria, such as *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp. From an antimicrobial resistance perspective, these pathogens are particularly significant as they are major etiological agents of both community-acquired and healthcare-associated infection[3]. Carbapenems are considered last-resort antibiotics and have traditionally been used to treat infections caused by extended-spectrum β-lactamase (ESBL)-producing *E. coli* and *Klebsiella pneumoniae*. However, resistance to these agents has risen more rapidly than

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to other antibiotic classes, leading to increased mortality and the urgent need for alternative treatments[4,5]. Few antibiotics remain effective against CRE due to carbapenemase production, which hydrolyze most β-lactams, and the frequent coexistence of resistance mechanisms to other antibiotic classes, including fluoroquinolones and aminoglycosides. In response, new β-lactam/β-lactamase inhibitor (BL/BLI) combinations, such as ceftazidime/avibactam (CAZ/AVI), meropenem/vaborbactam, imipenem/relebactam and aztreonam/avibactam, have been introduced into clinical practice in recent years[6–8]. In this study, we characterized the whole genome of a KPC-producing *K. pneumoniae* and a concurrent *E. coli* isolate from a hospitalized patient who had received multiple antibiotic treatments, including CAZ/AVI. The two isolates shared three common resistance genes, including bla_{KPC-3}. Furthermore, we sequenced the PBP3 of the *E. coli* strain to identify mutations and their potential correlation with resistance to BL/BLI therapies.

Results

Clinical case

A 77-year-old patient with chronic obstructive pulmonary disease (COPD), idiopathic dilated cardiomyopathy, heart failure with preserved ejection fraction, and paroxysmal atrial fibrillation on direct oral anticoagulants with an implantable cardioverter-defibrillator was admitted to Ischia Hospital due to an acute exacerbation of COPD. He was managed with inhaled glucocorticoids, bronchodilators, and a 12-day course of piperacillin/tazobactam (PIP/TAZO). Four months later, the patient was readmitted to the Surgery Department with sepsis secondary to acute calculous cholecystitis. Blood cultures performed during hospitalization yielded negative results, while a rectal swab tested positive for the bla_{KPC} gene, and the swab culture yielded *K. pneumoniae*. The patient underwent cholecystectomy and was again treated with PIP/TAZO, leading to clinical stabilization. Five days after discharge, the patient was admitted to the Medicine Department with sepsis. Blood cultures from this admission tested positive for carbapenemase-producing *E. coli*. Antimicrobial susceptibility test results for *E. coli* are reported in Table 1. Given the patient's clinical deterioration, empirical therapy was escalated to meropenem and linezolid. Following susceptibility testing, treatment was adjusted to CAZ/AVI (2.5 g 8-hourly) for seven days, leading to clinical improvement and normalization of laboratory findings. A subsequent rectal swab for CRE remained positive for the bla_{KPC} gene, and swab culture again identified *K. pneumoniae.*

Strains characterization

The multi-locus sequence typing (MLST) analysis showed the presence of Sequence Type (ST) 252, K locus KL51 and O locus O1ab for *K. pneumoniae* and ST131, phylogroup B2, serotype O25:H4 and *fum*C40/*fim*H30 for *E. coli*. Resistance genes analysis showed that *K. pneumoniae* and *E. coli* shared two resistance genes: bla_{KPC-3} and bla_{OXA-9}. *K. pneumoniae* harbors one copy of the bla_{KPC-3} gene on Tn*4401a*, while *E. coli* carries two copies on two Tn*4401a* transposons.

PBP3 analysis

Alignment of the *E. coli* reads to the reference genome (RefSeq NC_002695.2) identified two mutations in the PBP3 gene (also known as *ftsI*), specifically A233T and I332V.

Antimicrobial agent	MIC (mg/L)	Susceptibility
Amoxicillin/clavulanic acid	> 16	R
Ampicillin	> 16	R
Cefepime	> 16	R
Cefotaxime	> 32	R
Ceftazidime	32	R
Cefuroxime	> 32	R
Piperacillin/tazobactam	> 64	R
Ceftolozane/tazobactam	> 16	R
Ceftazidime/avibactam	< = 0.12	S
Ertapenem	> 4	R
Meropenem	> 8	R
Imipenem/relebactam	< = 0.25	S
Meropenem/vaborbactam	< = 0.5	S
Cefiderocol	< = 2	S
Amikacin	4	S
Gentamicin	< = 1	S
Ciprofloxacin	> 2	R
Levofloxacin	> 4	R
Eravacycline	< = 0.12	S

Table 1. Antimicrobial susceptibility testing result of *Escherichiacoli* strain isolated from the patient. MIC: minimum inhibitory concentration.

Strain	Gene	Amino acid change	Mutation type	Annotation/comment
E. coli	*acrB*	p.His596Asn	Missense	Transmembrane domain (predicted)
E. coli	*acrB*	p.Ser1043Asn	Missense	Periplasmic region (unknown role)
E. coli	*acrA*	p.Thr104Ala	Missense	Helical region (unknown role)
E. coli	*marR*	p.Gly103Ser	Missense	Quinolone resistance in animals[9]
E. coli	*marR*	p.Tyr137His	Missense	Carbapenem resistance[10]
E. coli	*marA*	p.Ser127Asn	Missense	C-terminal (DNA-binding domain)
K. pneumoniae	*acrA*	p.Val313Ile	Missense	Conservative change
K. pneumoniae	*acrA*	p.Ala188Thr	Missense	Reported in tigecycline-resistant strain[11]

Table 2. Summary of missense mutations identified in efflux-related antimicrobial resistance (AMR) genes in *E. coli* and *K. pneumoniae*. For each variant, the gene, amino acid change, and a brief annotation are reported.

Analysis of antimicrobial resistance-associated gene mutations

Whole-genome variant calling of Illumina sequencing revealed several mutations in antimicrobial resistance-associated genes in both isolates. In *E. coli*, we detected two missense mutations in *acrB* (p.His596Asn and p.Ser1043Asn), one in *acrA* (p.Thr104Ala), two in *marR* (p.Gly103Ser and p.Tyr137His) and one in *marA* (p.Ser127Asn). In *K. pneumoniae*, two missense mutations were identified in *acrA* (p.Val313Ile and p.Ala188Thr) (Table 2).

Study of mobile genetic elements

PlasmidFinder identified several plasmids, including an IncFII(K) plasmid carrying the bla_{KPC} gene. Assembly and contig analysis using MOB_suite detected a transposon harboring the bla_{KPC} gene within a plasmid homologous to pECAZ147_KPC from *E. coli* strain Ecol_AZ147 (GenBank accession number CP018992) and pKPC from *K. pneumoniae* strain D1 (GenBank accession number CP043971). Comparative analysis revealed a high degree of homology (99.85%) between these plasmids. Due to the limitations of short-read sequencing with Illumina technology, we were unable to fully reconstruct the plasmid. Oxford Nanopore long-read sequencing allowed for full plasmid characterization in both strains. In *K. pneumoniae*, three plasmids were identified (ranging from 50 to 150 kb), while *E. coli* carried two plasmids of approximately 60 kb and 120 kb. In both cases, bla_{KPC-3} was located on ~ 120 kb plasmids, which also included replicase genes, confirming their co-localization on the same contig. MOB-suite analysis assigned these plasmids to the IncFII incompatibility group, frequently linked to antimicrobial resistance, and detected MOBP-type relaxases, supporting their mobilization potential. Furthermore, both plasmids were classified as conjugative, and Mash distance analysis showed high similarity (distance < 0.01) between the bla_{KPC-3}-carrying plasmids of *E. coli* and *K. pneumoniae*, strongly suggesting horizontal plasmid transfer between the two species. (Supplementary Tables 1 and 2).

Discussion

The susceptibility of bacteria to β-lactam antibiotics depends on a complex interplay of factors, including target binding affinity, resistance to β-lactamase degradation, and, in Gram-negative bacteria, outer membrane permeability[12]. Bacterial antibiotic resistance can arise through intrinsic or acquired mechanisms[13]. Intrinsic resistance involves genes naturally present on the host's chromosome, such as AmpC β-lactamase in Gram-negative bacteria and efflux pump systems[13]. Acquired resistance, on the other hand, may result from mutations in antibiotic target genes or the horizontal transfer of resistance elements via plasmids, bacteriophages, transposons, and other mobile genetic elements[13]. Conjugation is the primary mechanism by which bacteria acquire external genetic material, with plasmids and transposons playing a crucial role in this process[14]. These resistance mechanisms often coexist within the same pathogen, contributing to multidrug resistance or increasing resistance levels to specific antibiotic classes[14]. Another key resistance mechanism involves mutations in penicillin-binding proteins (PBPs), which are essential for peptidoglycan synthesis and serve as primary targets for β-lactam antibiotics[15]. Structural modifications in PBPs, resulting in reduced affinity for β-lactams, are well-documented in Gram-positive bacteria but are increasingly reported in Gram-negative species[16]. Specifically, penicillin-binding protein 3 (PBP3), a high-molecular-weight transpeptidase, is a major component of *E. coli* and plays a key role in catalyzing peptide cross-bridges between the glycan chains of the peptidoglycan[17,18]. Mutations in PBP3, often driven by antibiotic pressure, can decrease its affinity for β-lactams, including carbapenems, complicating treatment strategies[19]. Ceftazidime and aztreonam preferentially inhibit PBP3, meaning that mutations in this protein can significantly impact bacterial susceptibility to these agents, potentially leading to increased minimum inhibitory concentration (MIC) values[20]. Additionally, the production of β-lactamase enzymes, particularly extended-spectrum β-lactamases (ESBLs) and carbapenemases, further contributes to multidrug resistance, severely limiting therapeutic options for Enterobacterales infections[21]. *Klebsiella pneumoniae* carbapenemases (KPCs) belong to class A serine β-lactamases and are the most widespread carbapenemases globally, predominantly found in *K. pneumoniae* but also in other Gram-negative species[22,23]. These enzymes hydrolyze penicillins, cephalosporins, aztreonam, and carbapenems and are frequently located on mobile plasmids within the Tn*4401* transposon[24]. Consequently, in addition to clonal expansion, the bla_{KPC} gene can spread through plasmid- and transposon-mediated horizontal gene transfer[25]. With the introduction of new beta-lactamase inhibitors, the emergence of KPC variants resistant to these therapies has been observed. Among them, the most common determinants are KPC-2 and KPC-3, which exhibit point mutations, insertions,

or deletions that alter their resistance levels to those molecules[26]. The presented clinical case may represent an example of plasmid-mediated conjugation. Although Illumina sequencing did not allow for the complete reconstruction of the plasmid, the high homology (99.85%) between the plasmid harbored in *K. pneumoniae* and that in *E. coli* strongly suggests that the transposon carrying the bla_{KPC-3} gene was transferred between the two strains via the same plasmid. The bla_{KPC} gene is typically located within the mobile transposon *Tn4401*[27]. In our study, the *K. pneumoniae* strain harbored a single copy of the bla_{KPC-3} gene within Tn*4401a*. This transposon variant, designated as 'isoform a', is characterized by a 100 bp deletion between bla_{KPC} and *istB*. While this isoform is commonly associated with bla_{KPC-2}, it has also been identified in certain bla_{KPC-3}-producing isolates from Israel and Italy[28]. In contrast, the *E. coli* strain carried the same bla_{KPC-3} variant in two copies, each located on separate instances of Tn*4401a*. A similar occurrence was previously reported in an *E. coli* strain of the same sequence type (ST) in the United States[29]. Additionally, a study demonstrated that *E. coli* DH5α transformants harboring an IncX3 plasmid with two copies of $_{bla}$KPC-3:Tn*4401a* exhibited significantly MICs of carbapenems, cephalosporins, and BL/BLIs[30]. The Tn*4401a* transposon has been linked to increased carbapenem resistance due to modifications in the promoter region that enhance bla_{KPC-3} expression[31]. Compared to other transposon variants, Tn*4401a* exhibits higher bla_{KPC} expression, likely due to a 99-bp deletion in the intervening sequence between the P1 and P2 promoters. This deletion may contribute to a more stable RNA structure, thereby enhancing gene expression[27]. The increased promoter activity of Tn*4401a* correlates with elevated carbapenem resistance, as demonstrated by higher meropenem and cefepime MICs in *E. coli* transformants compared to those harboring other transposons[27]. Consistently, our *E. coli* isolate exhibited high MIC values of carbapenems, cephalosporins and traditional BL/BLIs further supporting the role of Tn*4401a* in enhanced resistance to these agents. The two amino acid substitutions in the PBP3 of our *E. coli*, namely A233T and I332V, have been previously reported[19]. The presence of these PBP3 mutations, which reduce the affinity of ceftazidime for PBP3, together with other genetic alterations such as mutations in *acrR* and *marR*, and overexpression of *sdiA*, contribute to a significant increase in ceftazidime MIC in *E. coli* mutants[19]. Beta-lactam antibiotics mimic a transpeptidase substrate and bind to the catalytic domain of PBP, where they function as suicide inhibitors by forming a covalent adduct with the catalytic serine[37]. It can be hypothesized that the A233T mutation, which involves the substitution of alanine (a non-polar residue) with threonine (a polar residue), may induce conformational changes in the active site of PBP3, leading to reduced β-lactam binding due to altered electrostatic interactions. Similarly, the I332V mutation, where isoleucine is replaced by valine (a smaller residue), could impact PBP3 interactions with other cell division components, potentially influencing its function and antibiotic susceptibility[19]. In addition to these point mutations, recent literature highlights the significant role of PBP3 insertions in conferring reduced susceptibility to BL/BLI combinations. Specifically, the tetrapeptide duplication of amino acids 334–337 (YRIN) and its single-amino acid variant YRIK have been associated with decreased susceptibility to both aztreonam-avibactam and cefiderocol; furthermore, duplications such as PYRI (amino acids 333–336) and TIPY (amino acids 331–334) have similarly been linked to reduced susceptibility to aztreonam-avibactam[38]. We did not identify any missense mutation in *acrR*, while we detected two missense mutations in *acrB* (p.His596Asn and p.Ser1043Asn), one in *acrA* (p.Thr104Ala), two in *marR* (p.Gly103Ser and p.Tyr137His) and one in *marA* (p.Ser127Asn). AcrR is a repressor of the *acrAB* operon[32], and acrA and acrB are key components of an efflux system in *E. coli*, which actively expels a wide range of toxic compounds, including antibiotics, thereby contributing to intrinsic antimicrobial resistance[33]. *MarR* is a repressor of *marRAB* operon and therefore also a repressor of *marA* expression[34]. The *marA* locus is needed for production of a multiple-antibiotic-resistant (Mar) phenotype in *E. coli*, which include resistance to cephalosporins[35,36]. Among the variants we observed, *acrB* p.His596Asn is located in the predicted transmembrane domain and could theoretically affect substrate transport; however, this mutation has not been previously reported and its functional impact remains speculative. The *acrB* p.Ser1043Asn and *acrA* p.Thr104Ala substitutions are located in periplasmic or helical regions, with no known role in antibiotic resistance. Similarly, *marA* p.Ser127Asn lies in the C-terminal region, potentially involved in DNA binding, but no evidence supports an effect on *acrAB* expression or regulatory activity. The two *marR* (p.Gly103Ser and p.Tyr137His) missense mutations are associated with resistance to quinolones and carbapenems, respectively[19,37,38]. Despite the potential overproduction of KPC-3, the presence of PBP3 mutations, and the missense mutations in *marR*, our *E. coli* isolate exhibited a low MIC for CAZ/AVI (Table 1). Given that the β-lactamase inhibitor avibactam is virtually devoid of intrinsic antibacterial activity[39] and its ability to restore ceftazidime activity against KPC-producing Enterobacterales depends on its capacity to inhibit KPC β-lactamase activity[40], the concurrent presence of PBP3 mutations (considering that PBP3 is the primary target of ceftazidime), and mutations in *marR* (which could potentially confer cephalosporin resistance) would theoretically be expected to result in resistance to CAZ/AVI. However, this was not observed in our *E. coli* isolate. There are two possible explanations for the low MIC of CAZ/AVI in this strain. The first is the absence of potential concurrent intrinsic resistance mechanisms[13]. The second, more speculative yet intriguing hypothesis is that avibactam, by binding to *E. coli* PBPs, particularly PBP2[41,42], acts just like a β-lactam and could compensate for the reduced binding of ceftazidime to the mutated PBP3. This interaction may help achieve a critical level of PBP binding, which is determined by both the number of PBPs bound and the affinity of the β-lactam for its targets[43]. Such an effect could potentially restore the full antimicrobial activity of the CAZ/AVI combination and is conceptually similar to the principle of enhanced bacterial killing through complementary target redundancy in Gram-negative bacteria[44]. Our research group proposed this mechanism in a commentary on the ATTACK trial[45,46], suggesting that simultaneous inhibition of multiple PBPs could enhance bacterial killing by overcoming resistance mechanisms. This hypothesis was later confirmed by in vitro time-kill studies conducted by Veeraraghavan et al.[47], which demonstrated a synergistic effect between sulbactam-durlobactam and imipenem against carbapenem-resistant *Acinetobacter baumannii calcoaceticus* complex strains[47]. These findings reinforce the clinical relevance of exploiting

complementary PBP targeting to restore or enhance the efficacy of β-lactam-based therapies, particularly against multidrug-resistant Gram-negative pathogens but further studies are needed to confirm this hypothesis.

In conclusion, this study underscores the multifactorial nature of antibiotic resistance in the analyzed *E. coli* strain, involving both plasmid-mediated dissemination of the bla_{KPC-3} gene and potential contributions from PBP3 and *marR* mutations. While the exact impact of these mutations on MICs remains unclear due to the lack of comparative data, the findings highlight the need for further studies to clarify their role and better understand the interplay between plasmid dynamics, transposon activity, and target protein modifications in resistance mechanisms.

Materials and methods

Patient and samples

In this study, we extracted demographic and clinical data from the medical records of a patient admitted to Ischia Hospital in June 2024. The study was approved by the local Institutional Review Board and all research was performed in accordance with relevant guidelines and regulations. Informed consent was obtained from the patient for the publication of this information. A KPC-positive *E. coli* isolate from a blood culture and a KPC-positive *K. pneumoniae* isolate from a surveillance swab, both obtained from this patient, were characterized. The strains were provided as cryopreserved stocks, which were streaked onto standard blood agar plates and incubated at 37 °C to obtain single colonies. These colonies were then inoculated into tryptic soy broth and incubated overnight at 37 °C to prepare bacterial cultures for subsequent molecular analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Vitek®2 system (bioMérieux, Marcy-l'Étoile, France). Disk diffusion on agar was used exclusively for cefiderocol.

DNA isolation

Genomic DNA was isolated using the Quick-DNA Fungal/Bacterial Kit (Zymo Research), supplementing the lysis buffer with lysozyme. The DNA concentration and quality were determined using a NanoDrop 2000 spectrophotometer and a Qubit Flex Fluorometer (Thermo Fisher Scientific).

Library preparation and illumina sequencing

Library preparation was conducted starting from 300ng of genomic DNA using the FX DNA Library Preparation Kit (Qiagen) and the NexteraTM XT Index kit (Illumina), following manufacturer's instruction. Sequencing was performed on an Illumina MiSeq System using a 2×500 bp paired-end run.

Illumina data analysis

Data analysis was carried out using a series of specialized tools. Quality control (QC) of raw sequencing reads was performed using *FastQC* and *MultiQC* to evaluate the quality of reads both before and after filtering with fastp. Taxonomic classification was conducted using *Kraken2. De novo* assembly of reads was performed with Shovill and contig annotation was achieved using *Prokka*. The MLST tool was used to determine sequence types from the PubMLST database, while *ABRicate* identified resistance genes (CARD database) and virulence factors (VFDB database), applying coverage and identity thresholds of 90%. *MOB_suite* enabled plasmid reconstruction from contigs, while PlasmidFinder provided plasmid identification and typing. Additionally, after aligning reads to the reference sequence with bwa mem algorithm, *EMBOSS transeq* was used to convert DNA sequences to amino acid sequences in order to identify amino acid mutations.

Long-read whole genome sequencing with nanopore technology and data analysis

Library preparation was performed using the 4bases Microbiome WGS kit, a shotgun-based solution optimized for long-read sequencing. The protocol enables multiplexed library construction compatible with Oxford Nanopore platforms. Sequencing was performed on a GridION system using FLO-MIN114 flow cells, following the manufacturer's instructions. Raw reads quality was assessed using FastQC. The presence of plasmids and the genes necessary for conjugation or mobilization as well as the presence of antimicrobial resistance genes were identified using the MOB-suite pipeline[48–50]. Variations were characterized with respect to a reference assembled genome (*E. coli* = GCA_000005845 and *K. pneumoniae* = GCA_000016305, NCBI) using snippy (https://github.com/tseemann/snippy). Identified SNPs and INDELs were then annotated with respect to gene, effect, and amino acid substitutions using snpEff[51].

Data availability

The datasets generated and/or analysed during the current study are available in the BioProject repository, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1223389, accession code: PRJNA1223389.

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Author contributions

Conception and design of the study, SG, JA, CFP, CT; acquisition of data, CDG, CA, ST, MCS, RM; analysis and interpretation of data, SG, CFP, PB, VF, MB, BK, CP, FC; drafting the article or revising it critically for important intellectual content, SG, SF, LM, CT.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

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Additional information

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