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Survival of infection with TEM β-lactamase-producing *Escherichia coli* with Pan-β-lactam resistance

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SUMMARY

Background: Antimicrobial resistance is a critical global health issue, significantly contributing to patient mortality. Recent antibiotic developments have aimed to counteract carbapenemase-producing Enterobacterales; however, the impact of their use on the emergence of antibiotic resistance is unknown. This study investigates the first case of a non-carbapenemase-producing, pan-β-lactam-resistant *Escherichia coli* strain from a patient previously treated with ceftolozane–tazobactam and cefiderocol.

Methods: This study describes the clinical progression of a 39-year-old ICU patient who developed multiple infections, culminating in the isolation of a pan-β-lactam-resistant *E. coli* strain (EC554). The resistance profile was characterised through MIC determination, whole-genome sequencing, the use of the β-lactam inactivation method, RT-qPCR, efflux pump inhibition assays, outer membrane protein analysis, and bla_{TEM} transformation.

Findings: The EC554 isolate displayed resistance to all tested β-lactams and β-lactam–β-lactamase inhibitor combinations. Whole-genome sequencing revealed four plasmids in EC554, with the only β-lactamase gene being blaTEM-252 on the pEC554-PBR-X1-X1 plasmid. We found that the extremely resistant phenotype was attributable to a combination of different mechanisms: a high expression of TEM-252, efflux pump activity, porin loss, and PBP3 mutations.

Interpretation: The findings illustrate the complex interplay of multiple resistance mechanisms in *E. coli*, highlighting the potential for high-level resistance even without carbapenemase production. This study underscores the importance of comprehensively characterising resistance mechanisms in order to inform effective treatment strategies and mitigate the spread of resistant strains.

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Research in context

Evidence before this study

Antimicrobial resistance poses a significant global health threat. We searched PubMed for papers published between Jan 1, 2010, and July 17, 2024, using the search terms "*Escherichia coli*" AND "pan-beta-lactam" AND "resistance". This search yielded four results, three based on an *E. coli* isolate with pan-β-lactam susceptibility, as well as resistance only to piperacillin–tazobactam. The remaining article reported an NDM-producing *E. coli* with resistance to all β-lactams. However, there are no studies on non-carbapenemase-producing Enterobacterales exhibiting pan-β-lactam resistance.

Added value of this study

This study presents a detailed account of the characterisation of a non-carbapenemase-producing *E. coli* strain (EC554) that exhibited resistance to all tested β-lactams. It identifies the combination of resistance mechanisms, including the novel blaTEM-252, high-level expression of TEM-252, efflux pump activity, porin loss, and PBP3 mutations, that contribute to this extremely resistant phenotype. This research provides new insights into the complex interactions of these mechanisms in the development of antibiotic resistance.

Implications of all the available evidence

The findings from this study significantly advance our understanding of resistance mechanisms in *E. coli* and underscore the need for the continuous monitoring of resistance patterns. Detailed resistance characterisation is key to developing new therapeutic strategies, preventing the spread of highly resistant strains and therefore improving patient management and treatment outcomes in clinical settings.

Introduction

Antimicrobial resistance is a global health concern that impacts patient mortality (4.95 million deaths in 2019).[1] Carbapenem-resistant Enterobacterales are now disseminated worldwide, limiting the treatment options for patients with infections caused by these microorganisms.[2]

New antibiotics have been marketed in recent years.[3] These novel combinations, including ceftazidime–avibactam (KPC and OXA-48), meropenem–vaborbactam (KPC), imipenem–relebactam (KPC), and cefepime–taniborbactam (MBL), are specifically addressed for certain types of carbapenemases. Additionally, cefiderocol apparently has stable activity against a broad spectrum of β-lactamases.[3,4]

It is known that previous exposure to antibiotics is a risk factor for developing antibiotic resistance.[5] This exposure contributes to generating or selecting variants resistant to antibiotics. Resistance to new antibiotics has already been reported in Enterobacterales, mainly associated with carbapenemases.[4,6–10]

In this study, we report the case of a non-carbapenemase-producing, pan-β-lactam-resistant *Escherichia coli* isolated from a patient previously treated with ceftolozane–tazobactam and cefiderocol. Therefore, we aimed to characterise the bacterial resistance mechanisms contributing to this resistance profile.

Methods

Background

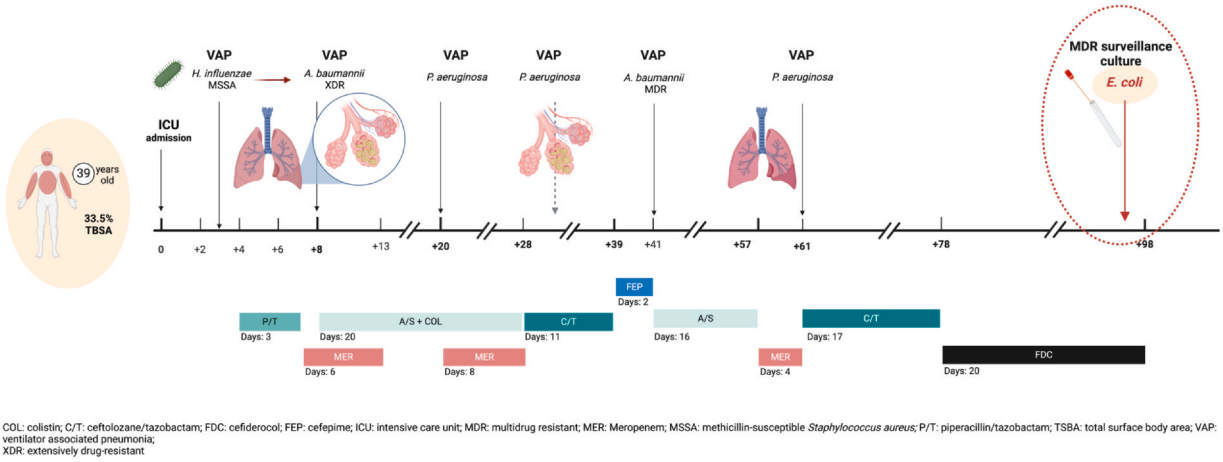
A 39-year-old male patient was admitted to the Intensive Care Unit (ICU) at the University Hospital Virgen del Rocío, a 1534-bed tertiary care teaching hospital covering a population of 564,399 in Seville, Spain, following a deflagration incident that resulted in burns covering 33·5% of his body's surface area. The patient initially developed ventilator-associated pneumonia (VAP) caused by *Haemophilus influenzae* and methicillin-susceptible *Staphylococcus aureus*, treated initially with piperacillin–tazobactam (3 days), which was further complicated by a superinfection with an extensively drug-resistant *Acinetobacter baumannii*, leading to septic shock and multiorgan failure. This condition was managed with a 15-day course of colistin and ampicillin–sulbactam, resulting in symptomatic improvement. The patient later experienced another episode of VAP due to *Pseudomonas aeruginosa*. Initial treatment with meropenem for seven days was followed by treatment with ceftolozane–tazobactam for 25 days after the development of carbapenem resistance. Subsequently, a new episode of pneumonia with septic shock and multiorgan failure was associated with the presence of *P. aeruginosa* isolates in respiratory samples, which exhibited resistance to ceftolozane–tazobactam. Consequently, the treatment regime was changed to cefiderocol administration for 22 days. In the following days, the patient's clinical condition improved, leading to the withdrawal of ventilatory support, and the patient was transferred to the hospital ward. Throughout his ICU stay, routine microbiological surveillance was carried out to detect multidrug-resistant organisms. Eight previous weekly screenings for MDR *Enterobacterales* were negative until the final surveillance, which yielded a pan-β-lactam-resistant *E. coli* isolate (Fig. 1).

Bacterial isolate and resistance profile

E. coli (EC554) was isolated from rectal swabs in agar ESBL Brilliance/agar CRE Brilliance (Thermo Scientific, USA). The isolate was identified via MALDI-TOF (Bruker Daltonik, GmbH, Germany), and the susceptibility profile was initially ascertained via broth microdilution using the semiautomatic method on the MicroScan WalkAway system (Beckman Coulter, USA). Minimal Inhibitory Concentrations (MICs) for the following antibiotics were subsequently confirmed using the broth microdilution reference method: ampicillin, amoxicillin–clavulanic acid, piperacillin–tazobactam, ceftazidime, cefotaxime, cefepime, aztreonam, imipenem, meropenem, ceftazidime–avibactam, imipenem–relebactam, meropenem–vaborbactam, and cefepime–taniborbactam. For ceftolozane–tazobactam, the MIC was determined using the gradient strip method (Liofilchem, Italy). On the other hand, for cefiderocol, the disc diffusion test was performed, conducted according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). All the MICs were interpreted according to the EUCAST clinical breakpoints 2024. [11] *E. coli* ATCC 25922 and *E. coli* HB4 strains were used as standard controls. Additionally, three *E. coli* [*E. coli* PT4, *E. coli* AEC-51, and *E. coli* AEC-24] TEM-1 hyperproducers were also used in the MIC and RT-qPCR assays.[12]

Whole-genome sequencing and bioinformatic analysis

Whole-genome sequencing (WGS) was performed with two different techniques—short-read Illumina sequencing and long-read Oxford Nanopore technology—to obtain a consensus sequence. Total DNA was extracted from colonies using a Magcore® extractor system H16 (RBC Bioscience Corp., Taiwan). A DNA library was prepared by using either (i) an NEB Ultra II FS DNA library prep kit for Illumina (NEB, France), which was then run on the MiSeq sequencer (Illumina, France), or (ii) an R9.4 flow cell and a 1D native barcoding genomic DNA kit (SQK-LSK109) on a MinION sequencer (Oxford Nanopore Technologies, UK). The base calling of Nanopore reads was



COL: colistin; C/T: ceftolozane/tazobactam; FDC: cefiderocol; FEP: cefepime; ICU: intensive care unit; MDR: multidrug resistant; MER: Meropenem; MSSA: methicillin-susceptible *Staphylococcus aureus;* P/T: piperacillin/tazobactam; TSBA: total surface body area; VAP: ventilator associated pneumonia;
XDR: extensively drug-resistant

Fig. 1. Clinical progression of the patient from whom the *Escherichia coli* 554 was isolated.

performed using Guppy Basecaller v.6.5.7 in high-accuracy mode.[13] Quality control and filtering of Illumina and Nanopore reads were conducted using Fastp v.0.23.4.[14] We employed high-quality Nanopore reads and the Flye v.2.9.2 assembler for genome assembly.[15] This was followed by long-read polishing with Medaka v.1.11.1 (https://github.com/nanoporetech/medaka) and four rounds of Illumina short-read polishing using Pilon v.1.24.[16]

The EC554 genome was also annotated using the RAST server.[17] MLST was obtained using MLST v.2.0 (Center for Genomic Epidemiology). The acquired and intrinsic antimicrobial resistance genes and plasmids were identified using ResFinder v.3.0 and PlasmidFinder v.2.1 (Center for Genomic Epidemiology) (https://www.genomicepidemiology.org/). Additionally, coverage data obtained after the assembly of Nanopore reads were used to estimate the plasmids' relative copy number (RCN) through the coverage ratio of the plasmid contig to the chromosomal contig.

BLAST analysis and sequence comparison using BRIG

Consensus sequences of the plasmid harbouring the bla_{TEM} gene were blasted with the most similar plasmid found in the NCBI by using BLAST Ring Image Generator (BRIG) software (version 0.95-dist). The blastn algorithm was used to generate a circular BLAST between both plasmids.

β-lactam inactivation method (βLIM)

To determine whether a hydrolytic mechanism underlies this pan-β-lactam resistance phenotype, a modified carbapenem inactivation method [18] was applied as follows: starting from a full 10 µL suspension, inoculation of EC554 in five NaCl 0·9% tubes was performed. Then, five susceptibility testing discs containing 10 µg meropenem, 10 µg imipenem, 30 µg ceftazidime, 30 µg cefepime, and 30 µg cefiderocol (Oxoid, UK) were immersed in each tube independently and then incubated for two hours at 35ºC. After incubation, the discs were placed on a Mueller–Hinton agar plate previously inoculated with *E. coli* ATCC 25922 reference strain. Additionally, the same *E. coli* ATCC 25922 was used as a negative control for hydrolysis.

RNA extraction and RT-qPCR

Bacterial RNA was extracted using an RNeasy Mini Kit (Qiagen, Netherlands) from cultures in the exponential phase. Then, reverse transcription was carried out using a SuperScript IV Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions. We used specific primers to amplify the bla_{TEM} gene (FW 5′-GAGGACCGAAGGAGCTAACC-3′; RV 5′-TTGCCGGGAAGCTAGAG TAA-3′) and the housekeeping *rpoB* gene (FW 5′-GAATTCGATCCGAAG GACAA-3′; RV 5′-AAAAGATGCGGTTTCACCAC-3′). A quantitative real-time PCR assay was carried out with FastStart SYBR Green Master (Roche, USA) in a QuantStudio (Applied Biosystems, USA). Gene expression was relatively quantified using the Comparative CT (ΔΔCt) Method (Applied Biosystems Guide, USA). The obtained cycle threshold (CT) values were analysed using the 2−ΔΔCT method.[19] The values were corrected with the appropriate reference gene (rpoB). Relative expression levels were calculated through a comparison with three *E. coli* strains overexpressing blaTEM-1 via different mechanisms.[12]

Efflux pump inhibition assay

The contribution of the efflux pumps to the resistance profile was evaluated using aztreonam, cefepime, ceftazidime, ceftazidime–avibactam, ceftolozane–tazobactam, meropenem, meropenem–vaborbactam, imipenem, and imipenem–relebactam gradient strips on a Müller–Hinton (MH) agar plate with and without a wide-spectrum efflux pump inhibitor, phenylalanine–arginine β-naphthylamide (PAßN). In the case of cefiderocol, the same procedure, but with the use of an antibiotic disc, was followed. For cefepime/taniborbactam, the MIC was determined by broth microdilution with the addition of the PAβN compound. PAβN was used at 25 mg/L, which is a concentration that has been demonstrated to exhibit high sensitising potency while staying below the intrinsic MIC.[20] Additionally, *E. coli* ATCC 25922 was used as a control.

SDS-PAGE analysis of outer membrane proteins

Bacterial cells were grown in LB to the logarithmic phase and lysed via sonication (5–7 min in 30 s intervals). Outer membrane proteins (OMPs) were extracted with 2% sodium lauryl sarcosinate (Merck, Spain) and recovered via ultracentrifugation as described previously.[21] The OMP profiles were determined via SDS-PAGE analysis using 12% SDS gels and 6 µg of OMPs, followed by Simply Blue SafeStain gel (ThermoFisher, USA).

Isolation and transformation of the plasmid pEC554-PBR-X1-X1

In order to determine the extent to which $bla_{TEM\text{-}252}$ contributes to the resistance, we transformed the plasmid pEC554-PBR-X1-X1 into two reference strains [*E. coli* Top-10 and *E. coli* HB4 (porin-deficient)]. The plasmid for EC554 was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega, USA) and then

Table 1
Minimal inhibitory concentrations (MICs) for all tested *Escherichia coli* strains.

Antibiotics	MIC (mg/L)					
	E. coli 554		*E. coli* HB4 (pEC554-PBR-X1)	*E. coli* HB4	*E. coli* TOP-10 (pEC554-PBR-X1)	*E. coli* TOP-10
	-	PaβN[b]				
Cefepime	>256	>256	>256	1	>256	0.06
Ceftazidime	>256	>256	>256	1	>256	0.25
Ceftazidime-avibactam	64	16	8	0.5	8	0.25
Ceftolozane-tazobactam	>256	>256	>256	1	>256	0.25
Aztreonam	>256	>256	>256	0.5	>256	0.125
Meropenem	>32	4	1	0.5	0.125	0.06
Meropenem-vaborbactam	16	4	0.5	0.5	0.06	0.06
Imipenem	>32	2	0.5	0.5	0.25	0.25
Imipenem-relebactam	6	2	0.25	0.5	0.25	0.25
Cefepime-taniborbactam	64	16	4	1	0.125	<0.06
Cefiderocol[a] (mm)	6	6	18	34	18	35

[a] Susceptibility to cefiderocol was tested through disk diffusion (MIC is expressed in mm).
[b] Phenylalanine-arginine beta-naphthylamide (PAβN) efflux pumps inhibitor at 25 mg/L.

transformed into *E. coli* TOP-10 and *E. coli* HB4. An agarose electrophoresis gel (1%) was used for the plasmid extraction to check whether the pEC554-PBR-X1 plasmid, containing the $bla_{\text{TEM-252}}$ gene, was present. Transformant cells were selected after growth in LB agar plates with 100 μg/mL of ampicillin and a bla_{TEM}-positive PCR.

Data availability

Whole-genome sequences were deposited in GenBank under the BioProject accession number PRJNA1129355. Additionally, the DNA and protein sequence of the new variant of TEM was deposited in the GenBank database under the accession number PP933208.

Statistical analysis

Group data are presented as means ± standard errors of the means (SEM). A one-way analysis of variance (ANOVA) was used to determine differences between means using GraphPad Prism9. A *p*-value < 0.05 was considered significant.

Results

Susceptibility profile

The EC554 isolate showed resistance to all tested β-lactams, including both traditional ones (ampicillin, amoxicillin–clavulanic acid, piperacillin–tazobactam, ceftazidime, cefotaxime, cefepime, aztreonam, imipenem, and meropenem) and newer ones, such as ceftazidime–avibactam, imipenem–relebactam, meropenem–vaborbactam, cefepime–taniborbactam, and cefiderocol (Table 1).

Molecular characterisation

The WGS analysis revealed that the EC554 isolate belonged to ST479 and harboured four different plasmids: 1) pEC554-PBR-X1-X1 (IncX1/X1, 36.8Kb), 2), pEC554-PBR-FII1 (Inc FII, 29.8Kb), 3) pEC554-PBR-FII2 (IncFII 72,5Kb), and 4) pEC554-PBR-FII-FIA-FIB (Inc FII/FIA/FIB, 109.1Kb). Detailed information about the content of the plasmid is available in Table 2. Surprisingly, the only β-lactamase found in EC554 was $bla_{\text{TEM-252}}$, encoding in the 36·8Kb IncX1/X1 plasmid (pEC554-PBR-X1-X1). TEM-252 is a point mutation derivative of TEM-10 (Ser to Gly substitution at Ambler position 268). Additionally, the EC554 isolate also harboured genes resistant to tetracyclines (*tet(A), tet(B)*), aminoglycosides (*aph(6)-lc*, *aph(3'')-l*), macrolides (*mph(A)*, *mph(E)/(G)*), *msr(E)*), and chloramphenicol (*cat (A1)/(A4)*) encoding in the pEC554-PBR-FII-FIA-FIB plasmid. The other two plasmids are encoding virulence genes and conjugative transfer genes (Table 2).

Notably, the structure of the pEC554-PBR-X1-X1 plasmid appears to be composed of a fusion of two identical IncX1 (18.4 Kb) plasmid copies (Fig. 2). Thus, this plasmid harbours two sets of replications and, therefore, carries twice the $bla_{\text{TEM-252}}$. A similar phenomenon was reported by Fang JI *et al*. (2023) upon characterising a multiple-replicon plasmid, IncX1-X1, harboured by multidrug-resistant *E. coli* from Malayan pangolin and formed through the fusion of two copies of a smaller plasmid.[22] Our Basic Local Alignment Search Tool (BLAST) results showed only two similar 18 Kb plasmids sequenced in Massachusetts General Hospital, USA, from human samples [pGTEN-24, (CP116484.1)] and the Department of Veterinary Microbiology, Bangkok, Thailand [pCUVET16–321.2], with identities reaching 99·91% and 99·88%, respectively. Both plasmids harbour $bla_{\text{TEM-1}}$ and cover ~50% of the pEC554-PBR-X1-X1 sequence (Fig. 2). Interestingly, the RCN results also showed roughly six to seven copies for pEC554-PBR-X1-X1, differing from the rest of the plasmids, which had only one to two copies.

On the other hand, a mutation in chromosomal genes that could be involved in the antibiotic resistance phenotype [porins, penicillin-binding protein 3 (PBP3), TonB-dependent transporters (TBDTs), gyrA, and ParC] was analysed through protein–BLAST analysis using *E. coli* K-12 as a reference strain. The porins OmpC/OmpF strongly regulate β-lactam resistance in *E. coli* when they are

Table 2
A summary of the contents of the *Escherichia coli* 554 plasmid.

Plasmids	Inc type	Plasmid size (Kb)	RCN[a]	Resistance genes	Other genes
pEC554-PBR-X1	X1	36,8	6–7	$bla_{\text{TEM-10-like}}$ (S268G)	*IS*26, *Tn*3, replication protein, toxin-antitoxin system (RelE/ParE)
pEC554-PBR-FII1	FII	29,8	1–2	-	replication proteins, toxin-antitoxin systems, VirB9 gene
pEC554-PBR-FII2	FII	72,5	1–2	-	Conjugative transfer proteins, replication proteins
pEC554-PBR-FII-FIA-FIB	FII/FIA/FIB	109,1	1–2	*tet(A), tet(B), aph(6)-lc, aph(3")-l, mph(A), mph(E)/(G), cat(A1)/(A4), msr(E)*	Conjugative transfer proteins, mobile genetic elements (IS and transposons), replication proteins, toxin-antitoxin systems

[a] RCN: Relative copy number calculated by the coefficient of the plasmid coverage and the chromosomal coverage after assembly assay.

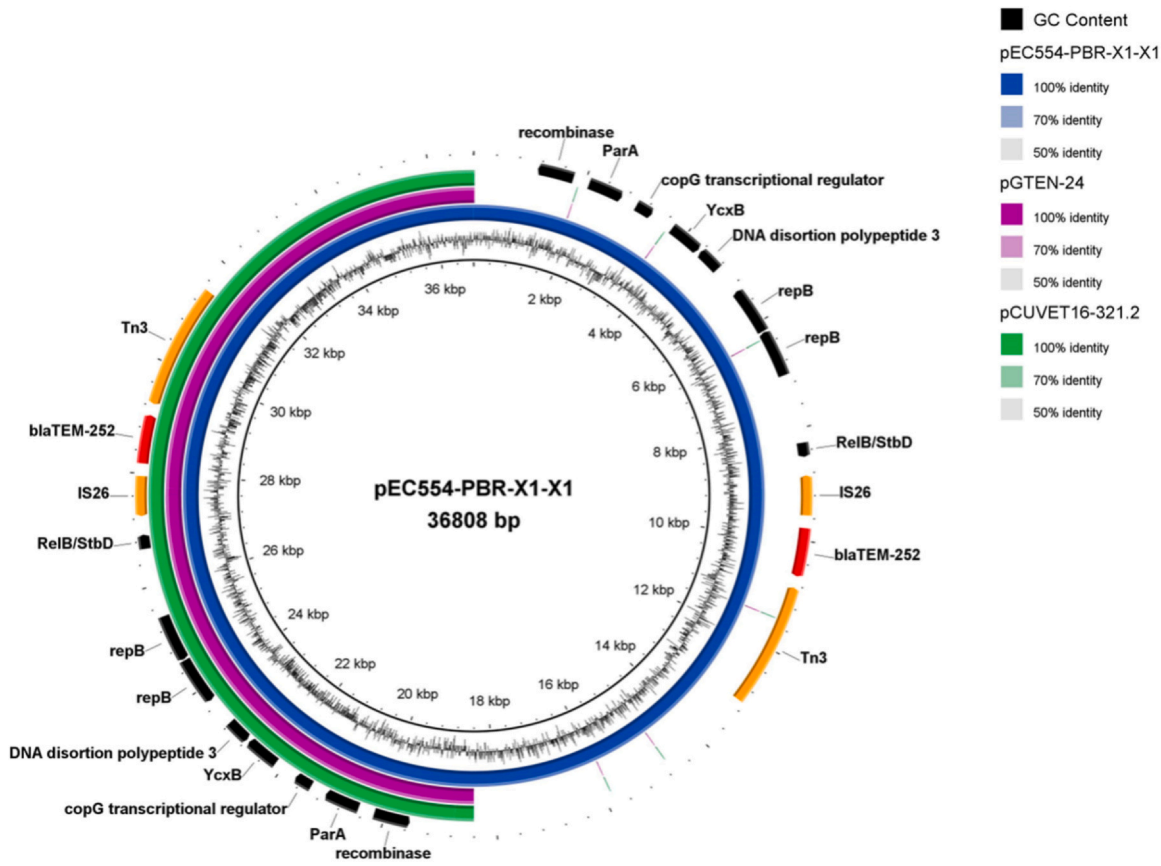


Fig. 2. The plasmid characteristics of the pEC554-PBR-X1-X1 plasmid harbouring $bla_{TEM-252}$ found in *E. coli* 554 and a comparison of the pEC554-PBR-X1-X1 plasmid harbouring $bla_{TEM-252}$ found in *E. coli* 554 with pGTEN_24 and pCUVET16–321.2 plasmids found in E. coli GTEN_24 (Massachusetts General Hospital; CP116484.1) and *E. coli* CUVET16–321.2 (Department of Veterinary Microbiology, Bangkok), respectively. The comparison was conducted using BRIG software. The red, orange, and black arrows indicate AMR genes, insertion sequences/transposes, and other proteins, respectively.

mutated. Our strains have an *ompF* gene truncated by an insertion sequence (IS26) and an *ompC* gene with a premature stop codon due to a frameshift mutation caused by a cytosine deletion in position 60 of the *ompC* gene. Regarding the TBTDs, it is well known that the primary carriers of catechol-type siderophores of the TonB system are FepA, CirA, and Fiu. [23] None of them contain a mutation leading to a truncated protein; nonetheless, they contain amino acid substitutions [FepA (K69E, S293A, T409A, and T420A), CirA (T183I), and Fiu (V77I, A283I, T493A, and M513V)]. We also found four amino acid insertions (YRIN) at position 333 of PBP3. Moreover, mutations in gyrA (S83L and D87N) and ParC (S80I) were found in EC554.

Role of $bla_{TEM-252}$ in β-lactam resistance

The βLIM test of the EC554 isolate was positive for ceftazidime, cefepime, and cefiderocol, showing a reduced zone of inhibition in this antibiotic compared with *E. coli* ATCC 25922 (Fig. S1). However, we did not observe differences in the zone of inhibition between the strains for imipenem and meropenem, indicating that these antibiotics are not hydrolysed by TEM-252 β-lactamase (Fig. S1).

The MICs for ceftazidime, cefepime, aztreonam, and ceftolozane–tazobactam in the transformant strains (*E. coli* TOP10 + pEC554-PBR-X1-X1 and *E. coli* HB4 + pEC554-PBR-X1-X1*)* were similar to those in EC554 (Table 1). Regarding cefiderocol, the inhibition zone was wider in the transformant strains than in EC554 (18 mm versus 6 mm, respectively); however, *E. coli* TOP10 + pEC554-PBR-X1-X1 and *E. coli* HB4 + pEC554-PBR-X1-X1 remained resistant, with an inhibition halo diameter below the Area of Technical Uncertainty (ATU) according to EUCAST guidelines (Table 1).[12] Finally, the MICs for meropenem, meropenem–vaborbactam, imipenem, imipenem–relebactam, and cefepime–taniborbactam in the transformant strains showed large differences compared to those for EC554, being susceptible in the transformant strains. There was no change in their MIC value when compared with those for the *E. coli* HB4 and *E. coli* TOP10 reference strains (Table 1). These results suggest that there must be other mechanisms aside from the activity of TEM-252 that are involved in the lack of activity of these antibiotics. Moreover, in order to determine whether the TEM variant plays an important role in the resistance phenotype of EC554, an MIC assay was performed on three TEM-1 hyperproducer *E.* coli strains.[12] A lack of activity was highlighted in the results of the MIC assay, with the three *E. coli* strains hyperproducing TEM-1 without any increase in the MICs of the antibiotics tested (data not shown).

In order to explore the level of expression of this β-lactamase, we performed RT-qPCR of $bla_{TEM,}$ and compared the results with those of *E. coli* HB4 + pEC554-PBR-X1-X1 and the three TEM-1 hyperproducer *E. coli* strains. The results showed that EC554 has a significantly higher bla_{TEM} expression than the TEM-1 hyperproducer *E. coli* strains (Fig. 3).

Role of the efflux pump in β-lactam resistance

The efflux pump inhibition by PaβN caused a notable decrease in the MICs of ceftazidime–avibactam (> 256 mg/L to 16 mg/L), cefepime–taniborbactam (> 256 mg/L to 16 mg/L), meropenem (> 32 mg/L to 4 mg/L), and imipenem (> 32 mg/L to 2 mg/L). However, this decrease was moderate in meropenem–vaborbactam (16 mg/L to 4 mg/L) and imipenem–relebactam (6 mg/L to 2 mg/L) (Table 1).

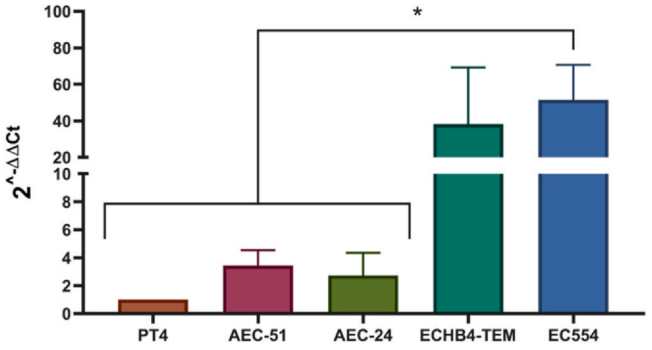


Fig. 3. bla_{TEM} gene transcription in the four *E. coli* isolates with overexpression of bla_{TEM} (ref), *E. coli* 554, and *E. coli* HB4 recombinant strain (harbouring pEC554-PBR-X1). Data were normalised to the mRNA levels of the *rpoB* gene. Three independent replicates were performed. * P < 0.05.

Role of OmpC/OmpF in β-lactam resistance

As described above, we found two loss-of-function mutations in the main porins associated with antimicrobial resistance in EC554 (OmpC and OmpF). These results were confirmed via SDS-PAGE analysis, and it was found that EC554 lacks the OmpC/OmpF band (Fig. S2). In order to better understand the impact of porin loss on the susceptibility profile of EC554, we compared the β-lactams' MICs in EC554, *E. coli* HB4 (the porin-deficient strain), *E. coli* TOP10 + pEC554-PBR-X1-X1, *E. coli* HB4 + pEC554-PBR-X1-X1, and *E. coli* TOP-10. We found that porin deficiency, on its own, led to a slight increase in the MIC for all the β-lactams tested, with these increases being more notable for cefepime, cefepime–taniborbactam, and meropenem (see *E. coli* HB4 versus *E. coli* TOP-10; Table 1). In any case, this increase indicates resistance. The increases in MICs observed for cefepime–taniborbactam and meropenem are more evident when comparing the MIC values of *E. coli* TOP10 + pEC554-PBR-X1-X1 and *E. coli* HB4 + pEC554-PBR-X1-X1; this is not true for cefepime due to the high level of resistance presented by both transformant strains. The cefepime–taniborbactam and meropenem MICs observed via broth microdilution for *E. coli* HB4 + pEC554-PBR-X1-X1 were 32-fold and 8-fold higher than the MIC for *E. coli* TOP10 + pEC554-PBR-X1-X1 (Table 1).

Discussion

In this study, we report a pan-β-lactam-resistant, non-carbapenemase-producing *E. coli*. We found that the extremely resistant phenotype of this isolate is due to the combination of several resistance mechanisms working in perfect synchrony.

First, we found that $bla_{TEM-252}$ and its level of expression in the pEC554-PBR-X1-X1 plasmid were directly responsible for the resistance to ceftazidime, cefepime, aztreonam, ceftolozane–tazobactam, and cefiderocol. We know that TEM-10 is an ESBL that confers resistance to ceftazidime, cefepime, and aztreonam, but it is inhibited by classic inhibitors such as tazobactam.[24] Therefore, it is not expected to confer resistance to ceftolozane–tazobactam. S268G substitution in the TEM protein has been reported to be a neutral replacement by itself and only leads to an increase in the MIC when it is combined with other mutations.[25] Moreover, the latter substitution does not affect the susceptibility to the inhibitor.[25] Thus, the level of expression could be the main factor responsible for tazobactam's incapacity to reduce the MIC of ceftolozane. This hypothesis is supported by a previous study, wherein the hyperproduction of TEM-1 was found to be involved in the acquisition of piperacillin–tazobactam resistance.[12]

Resistance to ceftazidime–avibactam was also mediated by the $bla_{TEM-252}$ β-lactamase in EC554. However, the MICs of the transformant strains (8 mg/L) differ in three dilutions from the MIC in the EC554 strain (64 mg/L) (Table 1). This suggests that another additional non-β-lactamase mechanism must be involved in this phenotype. When we inhibited the efflux pumps, the MIC of EC554 decreased to 16 mg/L. Thus, it seems that the combination of TEM-252 and efflux pump activity underlies the ceftazidime–avibactam resistance in EC554. In Enterobacterales, the role of the efflux pumps in ceftazidime–avibactam resistance is unclear,[26,27] although the active expulsion of avibactam by efflux pumps has been previously reported in *P.* aeruginosa.[27] Our results suggest that avibactam can also be actively expulsed in *E. coli*. Resistance to cefepime–taniborbactam was also mediated by $bla_{TEM-252}$ β-lactamase and efflux pumps with similar behaviour when the efflux pumps were inhibited. By contrast, the MICs of the transformant strains were 4 and 0·125 mg/L for *E. coli* HB4 + pEC554-PBR-X1-X1 and *E. coli* TOP10 + pEC554-PBR-X1-X1, respectively. The MIC values were probably reduced due to taniborbactam, which could be more active against TEM β-lactamase than avibactam. Moreover, the difference in the MIC values between both transformant strains confirmed the contribution of the impermeability to the cefepime–taniborbactam resistance. Some studies have reported that taniborbactam crosses the outer membrane via the porin pathway, suggesting that permeability defects could contribute to the cefepime-–taniborbactam resistance.[28,29] Thus, our results indicate that a combination comprising TEM-252 plus porin deficiency and efflux pump activity is responsible for the cefepime–taniborbactam resistance in EC554.

In the same way, cefiderocol resistance seems to be mediated by a combination of mechanisms in the EC554 strain. On one hand, we found that the TEM-252 β-lactamase was able to hydrolyse cefiderocol, although the disc diameter in the transformant strains was wider than that in the EC554 strains (18 mm (R) vs. 6 mm (R)) (Table 1), which indicates another complementary resistance mechanism is required to achieve such resistance. However, contrary to the findings for ceftazidime–avibactam, efflux pump inhibition did not alter the diameter for cefiderocol, so we can exclude this mechanism from consideration. Resistance to cefiderocol not mediated by β-lactamases has been reported in Enterobacterales due to different mechanisms, such as mutations in iron-transport-related proteins (e.g., *tonB, cirA,* and *fiu*) [4,30] and PBP3 modifications.[23] However, as far as we know, resistance to cefiderocol mediated by TEM β-lactamases has not been reported previously. Focusing on the PBPs, a four-fold increase in the MIC of cefiderocol has been reported in strains with mutations in PBP3,[23] including a four-amino acid insertion (YRIN) or the same duplication with a single mismatch (YRIK/YRIP) after position 333. These changes modify the active site of PBP3, affecting its accessibility to cefiderocol.[23] Our genome analysis showed a YRIN motif insertion in EC554, which could explain the increase in cefiderocol's MIC. The combination of both mechanisms (TEM-252 and PBP3 motif insertion) could be responsible for the level of cefiderocol resistance found in EC554.

Contrary to what has been reported in cephalosporins, resistance to carbapenems and new drug combinations (meropenem–vaborbactam and imipenem–relebactam) does not appear to be affected by TEM-252 β-lactamase in EC554. Our results show that the efflux pump system plays a major role in the resistance to these compounds, as the MICs of meropenem and imipenem decreased from > 32 mg/L to 4 and 2 mg/L, respectively, when the efflux pumps were inhibited by PAβN (Table 1). In the same manner, the MICs of meropenem–vaborbactam and imipenem–relebactam also exhibited a decrease (Table 1). The porin-deficient strain (HB4) has an meropenem MIC that is slightly higher than that of the TOP-10 strain (Table 1), though this was not found to be the case for imipenem. However, in the EC554 strain, the MIC of carbapenems does not decrease to values similar to HB4 (0·5 mg/L) when the efflux pumps are inhibited. We hypothesise that this could be attributed to two factors: 1) a complementary mechanism different to those characterised herein and 2) the combination of porin loss plus the activity of the TEM-252, which produces a synergistic effect against carbapenems. More in-depth studies are needed to explore this issue further.

Conclusions

In this study, we have documented the emergence of a pan-β-lactam-resistant, non-carbapenemase-producing *E. coli* clinical isolate (EC554) from a patient with extensive prior exposure to ceftolozane–tazobactam and cefiderocol. The resistance phenotype of EC554 is attributed to a synergistic combination of multiple mechanisms, including the novel $bla_{\text{TEM-252}}$ variant, overexpression of $bla_{\text{TEM-252}}$ β-lactamase, efflux pump activity, and significant porin loss (OmpC and OmpF). As far as we know, this study is the first comprehensive report of a non-carbapenemase-producing, pan-β-lactam-resistant *E. coli* isolate, emphasising the necessity for robust antimicrobial stewardship and continuous surveillance to mitigate the spread of such highly resistant pathogens. This research contributes significantly to the growing body of knowledge on antibiotic resistance, providing a foundation for future studies aimed at combating the dissemination of resistance genes in clinical settings.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2024.106268.

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