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Sporadic cefiderocol resistance in *Escherichia coli* from the United Arab Emirates involves multifactorial mechanisms reversible by novel beta-lactamase inhibitors

Farah Al-Marzooq[1]✉, Hafsa Ahli[1], Rauda Aldhaheri[1], Omar Aleissaee[1], Abdulrahman Alzaabi[1], Abdullah Alsaadi[1], Hamad Almansoori[1], Akela Ghazawi[1], Lana Daoud[1], Amna Ahmad[1] & Timothy Collyns[2]

Cefiderocol (CFDC), a novel siderophore-cephalosporin, is effective against multidrug-resistant (MDR) pathogens, but the emergence of resistance threatens its future use in treating infections. This study reports the emergence of CFDC resistance in four *E. coli* strains isolated from immunocompromised and critically ill patients in the United Arab Emirates, and provides a comprehensive genomic analysis of these strains, aiming to uncover the mechanisms driving this resistance. Whole-genome sequencing with bioinformatic analysis revealed specific beta-lactamase variants (NDM-5, CMY-2/145, and OXA-181) and unique mutations in siderophore-iron transport genes (*cirA*, *fepA*, *fecA*, *fiu*, and *tonB*) and penicillin-binding proteins (PBPs) associated with resistance. Phylogenetic analysis showed that the strains were not clonally related, indicating the sporadic nature of resistance. To address this challenge, we evaluated the efficacy of several novel beta-lactamase inhibitors (BLIs) combined with CFDC. In vitro susceptibility testing demonstrated that these inhibitors restored the antibacterial activity of CFDC against resistant strains. Zidebactam, with intrinsic antibacterial activity, caused the most significant reduction in CFDC minimum inhibitory concentrations (MICs), while the activity of other inhibitors (taniborbactam and xeruborbactam) was dependent on the genetic makeup of the strains, especially mutations in the siderophore-iron uptake genes. Our findings underscore the importance of genomic surveillance in deciphering antibiotic resistance mechanisms. Novel BLIs and partner antibiotics could be added weapons in the fight against MDR bacteria; thus, we recommend using combinations with novel BLIs as innovative therapeutic options to combat the emerging threat of CFDC resistance, after proper validation of their in vivo efficacy.

Keywords Cefiderocol, *Escherichia coli*, Whole-genome sequencing, Beta-lactamase inhibitors

The emergence and spread of multidrug-resistant (MDR) bacterial pathogens is a global public health concern, posing significant challenges in the management of infectious diseases[1,2]. The arsenal of effective antibiotics is dwindling; therefore, the discovery of novel antimicrobial agents is crucial to combat these resilient MDR bacteria[3,4]. Cefiderocol (CFDC), a novel siderophore-cephalosporin, is a good addition to the therapeutic armamentarium against MDR bacteria[5]. Its unique structure incorporates a siderophore moiety, which facilitates active transport across the outer membrane of Gram-negative bacteria, by capturing iron and delivering it inside the cell through iron transporters[6]. The mechanism of action of CFDC involves inhibition of penicillin-binding proteins (PBPs), mainly PBP3, which are essential enzymes for bacterial cell wall synthesis[7]. By binding to and inactivating these targets, CFDC disrupts cell wall synthesis, ultimately leading to bacterial cell death[8]. CFDC has been approved

[1]Department of Medical Microbiology and Immunology, College of Medicine and Health Sciences, United Arab Emirates University, P.O. Box No. 15551, Al Ain, United Arab Emirates. [2]Tawam Hospital, Al Ain, United Arab Emirates. ✉email: f.almarzooq@uaeu.ac.ae

for the treatment of complicated urinary tract infections (cUTI) caused by Gram-negative pathogens in patients with limited or no alternative treatment options. Additionally, it is indicated for the treatment of hospital-acquired and ventilator-associated pneumonia caused by MDR Gram-negative pathogens in similar patient populations[9]. This underscores its importance for the management of life-threatening infections, especially where limited or no other therapeutic options are available to cure the infection[10]. Notably, CFDC demonstrates stability against most β-lactamases, including serine-β-lactamases (SBLs) and certain metallo-β-lactamases (MBLs)[7], conferring activity against otherwise resistant strains[11,12]. The rapid evolution and dissemination of β-lactamases have significantly contributed to the alarming rise of antimicrobial resistance. Traditional β-lactamase inhibitors (BLIs), such as clavulanic acid, tazobactam, and sulbactam, have been widely used in combination with β-lactam (BL) antibiotics to counteract β-lactamases[13]. However, their effectiveness is limited against emerging and more potent β-lactamases, necessitating the development of novel inhibitors with broader and more potent inhibitory activity. Currently, several promising novel BLIs are in various stages of research and development, offering potential solutions to combat resistance mediated by a wide range of β-lactamases[14]. The most promising novel agents belong to diazabicyclooctanes (DBOs) and boronic acid derivatives[15]. DBOs are a class of non-β-lactam, cyclic boronates that exhibit potent inhibitory activity against a wide range of β-lactamases, including class A, C, and a subset of class D enzymes[15]. These inhibitors bind covalently to the active site of β-lactamases, effectively inactivating them. Predecessor DBOs, such as avibactam, lacked inhibitory activity against MBLs, while newer DBOs were rationally designed to have an expanded spectrum of inhibition to cover most classes of β-lactamases. DBO inhibitors that have shown promising results in preclinical and clinical studies, particularly against carbapenem-resistant *Enterobacteriaceae* (CREs). Some DBOs, like zidebactam, have enhanced chemistries such that they can target PBPs due to their high affinity for the PBP2, in addition to their action as BLIs[16]. When these BLIs are combined with β-lactams that inhibit other PBPs, such as PBP1 and/or PBP3, they demonstrate a β-lactam-enhancing effect, in addition to β-lactamase inhibition, that is based on simultaneous inhibition of multiple PBPs[17]. Other new agents such as taniborbactam and xeruborbactam are considered pan-spectrum BLIs due to their ability to inhibit both SBLs and MBLs[18]. Taniborbactam, a bicyclic boronic acid BLI, is known as the first pan-spectrum BLI due to its capability of inhibiting almost all β-lactamases from class A to D[19]. It has a potent synergistic effect with cefepime against *Enterobacteriaceae* producing KPC, VIM, NDM, ESBLs, and AmpCs, but not on strains carrying IMP MBL. Cefepime-taniborbactam is currently in Phase 3 clinical trials for cUTI and acute pyelonephritis[20]. Xeruborbactam, an ultra-broad-spectrum cyclic boronic acid BLI, is also another pan-spectrum BLI, which can inhibit a variety of MBLs and SBLs, including KPC, AmpC, and OXA-type enzymes. Xeruborbactam in combination with meropenem is synergistically effective against multiple β-lactamase-producing *Enterobacteriaceae*[21]. These agents can possibly restore CFDC efficacy if used in combination against resistant strains, which can aid in the discovery of potent synergistic combinations for the future development of new therapeutic regimens against highly resistant bacteria.

As with any new antibiotic, bacteria possess an intrinsic ability to develop resistance mechanisms that can compromise the therapeutic effectiveness of CFDC, such as mutations in iron transport systems and/or the expression of potent degradative enzymes[22]. Recently, there have been increasing reports of CFDC resistance globally[22], with a single report documenting the emergence of resistance in *Klebsiella pneumoniae* in the United Arab Emirates (UAE)[23]. Understanding the underlying mechanisms of resistance and exploring strategies to counteract them is crucial for preserving the clinical utility of this novel antibiotic.

In this study, we report the emergence of CFDC resistance in *E. coli* strains from the UAE. We aimed to explore the genetic makeup of these strains, to reveal the resistance mechanisms, and to uncover potential strategies to overcome CFDC resistance using novel BLIs, such as zidebactam, taniborbactam, and xeruborbactam.

Results

Clinical data

In this study, we report the emergence of CFDC resistance in four *E. coli* strains from the UAE. Clinical presentations of the patients infected with these strains were retrieved from the medical records. None of these patients received CFDC treatment at Tawam Hospital, Al-Ain, UAE; however, all patients had prior exposure to various antibiotics, including doxycycline, fosfomycin, amoxicillin-clavulanate, piperacillin-tazobactam, meropenem, ertapenem, ceftriaxone, cefuroxime, ceftazidime-avibactam, and aztreonam. All the patients had complex medical histories and were often immunocompromised or critically ill. The first patient was a 49-year-old male who had right-sided hemiplegia due to a cerebral infarct. Strain EC647 was isolated from a rectal screening swab from this patient during hospitalization in July 2022. The second patient was a 60-year-old male who had decompensated liver cirrhosis, complicated by spontaneous bacterial peritonitis, which progressed to sepsis. Strain EC650 was isolated in July 2022 from a blood specimen collected from this patient who passed away due to sepsis. The third patient was a 67-year-old female who had B-cell lymphoma and was receiving chemotherapy for her underlying disease. Strain EC771 was isolated in February 2023 from her urinary specimen. The fourth strain (EC948) was isolated in October 2023 from a urinary specimen of a 53-year-old female with stage IV ovarian carcinoma. The patient ultimately passed away due to underlying disease progression. All the strains were highly resistant to CFDC with MICs ranging from 64 to > 256 µg/ml.

Genomic characterization of the strains

The first step in unraveling the mechanisms of CFDC resistance was to analyze the whole genomes of the strains, to detect various resistance genes and mutations linked to CFDC resistance. Genomic analyses of the strains revealed a lot of variations in the sequence types, genotypes, and different types of resistance genes in the strains, as shown in Fig. 1. Interestingly, all the strains carried bla_{NDM-5} gene, which encodes MBL enzyme. One strain (EC647) carried an additional SBL ($bla_{OXA-181}$), and an AmpC β-lactamase ($bla_{CMY-145}$). Another AmpC gene (bla_{CMY-2}) was carried by strain EC650. Two strains (EC650 and EC948) carried an ESBL gene ($bla_{CTX-M-15}$)

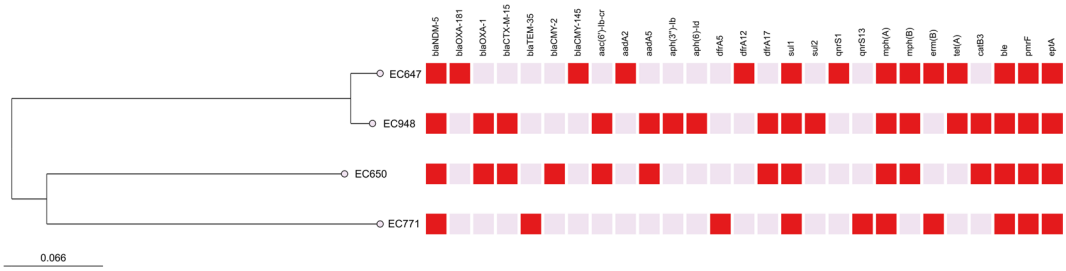


Fig. 1. Resistome analysis. Dendrogram based on the whole genome analysis, showing various resistance genes detected in each strain, whereby a red-colored square denotes the presence of the resistance gene, while a light pink colored square indicates the absence of the gene.

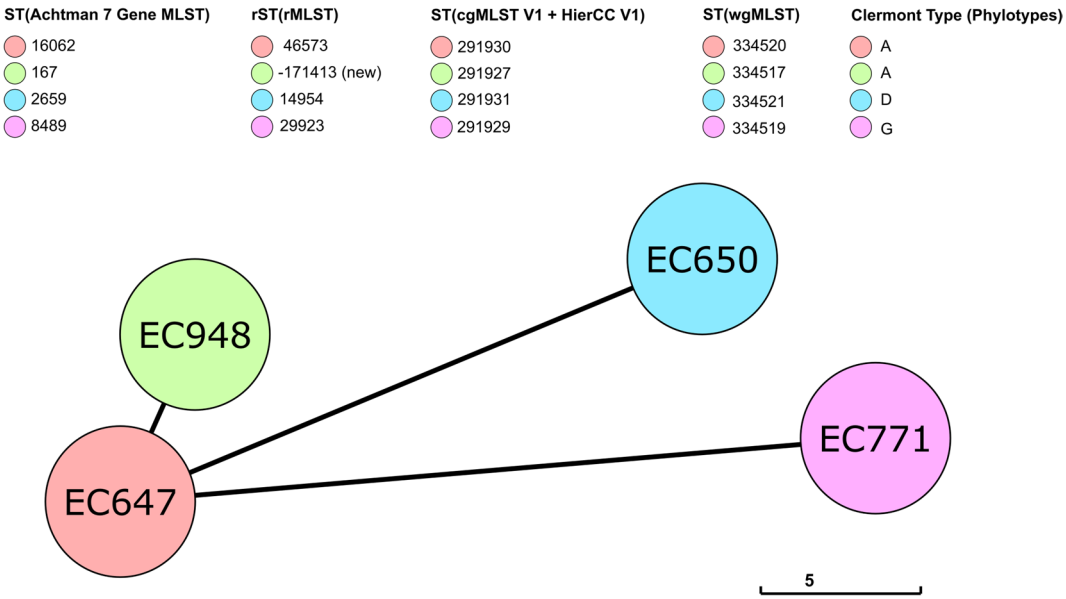


Fig. 2. Phylogenetic analyses. GrapeTree minimum-spanning tree showing ST of the isolates based on different multi-locus sequence typing (MLST) schemes [Achtman 7 gene MLST, ribosomal MLST (rMLST), core genome MLST (cgMLST), whole genome MLST (wgMLST)], in addition to the Clermont phenotyping method. The four strains were color-coded to link each to its unique sequence types and phylotype, as indicated at the top of the figure.

while another strain (EC771) carried a broad-spectrum β-lactamase (bla_{TEM-35}) without any ESBL or AmpC genes. The other genes shown in Fig. 1 encode resistance to various antibiotics, including aminoglycosides (*aac(6')-Ib-cr, aadA* and *aph*), trimethoprim (*dfr*), sulfonamide (*sul*) fluoroquinolone (*qnr*), macrolide (*mph* and *erm*), tetracycline (*tet*), chloramphenicol (*cat*), bleomycin (*ble*), cationic antimicrobial peptides, and antibiotics such as polymyxins (pmrF), and *eptA* gene encoding PmrC, which mediates the modification of lipid A and reduces polymyxin B binding.

The strains were not clonally related based on the results of various phylogenetic markers relying on the whole genome, core genome, and even phylotypes, as shown in Fig. 2**.** The four strains belonged to different sequence types (STs) [ST167 (EC948), ST2659 (EC650), ST8489 (EC771), and ST16062 (EC647)]. According to the phylogroups, EC948 and EC647 belonged to the same group (A), while the other two strains were from other groups (D and G), as shown in Fig. 2, which also demonstrates the variation in rMLST, cgMLST, and wgMLST among the strains. They also carried different plasmids based on replicon typing. Strain EC647 was a carrier of multiple plasmids (IncFIA, IncFII, and ColKP). Similarly, EC948 harbored IncFIA, IncFII, and Col(pHAD28) types, and EC650 carried three types (IncFIA, IncFIB, and IncFII), while EC771 had a single replicon, namely IncY. Variation in plasmid replicon types also reflects the diversity of CFDC-resistant strains.

Detection of mutations in the siderophore-iron uptake genes and PBPs

We also checked the genomes for mutations associated with CFDC resistance (shown in Fig. 3), including PBPs-encoding genes, and siderophore-iron uptake genes, namely *cirA*, which encodes a receptor for colicins that transports catecholate-type siderophores, *fecA,* which encodes a transporter mediating ferric citrate uptake, *fepA,* which encodes a receptor for enterobactin siderophore, and *fiu,* which encodes a receptor for catecholate

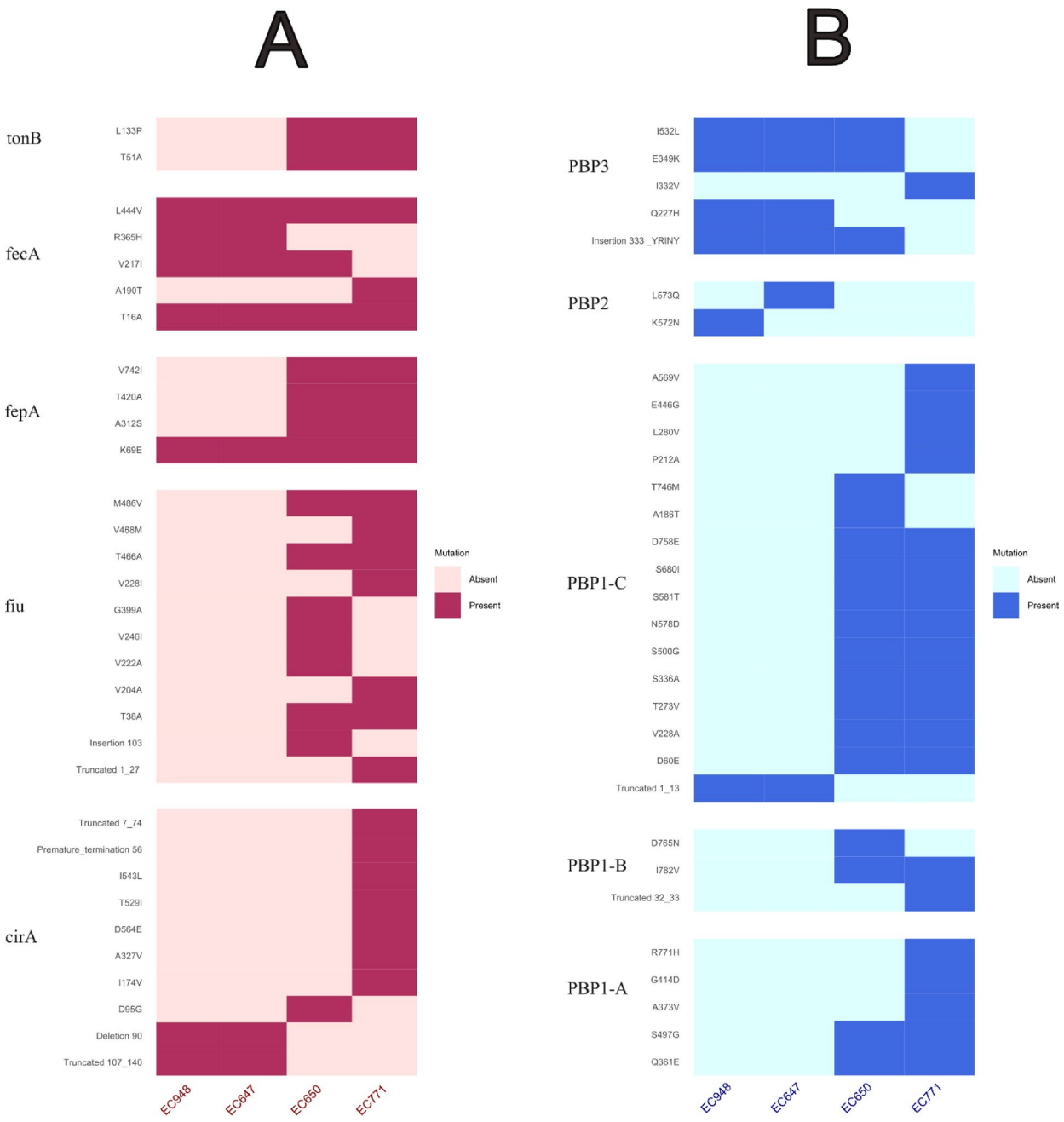


Fig. 3. Heatmaps listing all the mutations in the siderophore-iron uptake genes (**A**), and PBP encoding genes (**B**) associated with CFDC resistance. The letters denote amino acids, and the numbers represent the location of specific amino acid(s) with mutations or truncation.

siderophores. Furthermore, we investigated *tonB* gene, which encodes an energy transduction system that facilitates the movement of iron-siderophore complexes across the outer membrane by interacting with outer membrane receptors, like FecA, FepA, Fiu, and CirA, to facilitate the movement of these complexes into the periplasmic space[24,25]. Gene alignment data is shown in the supplementary file.

Interestingly, all the strains had various mutations in several genes. The Venn diagram (Fig. 4A) illustrates the similarities in mutations found in siderophore-iron uptake genes and PBPs of various types among the four strains. It is obvious that EC711 had the maximum number of mutations (n = 43), 20 of which were shared with EC650. The latter strain also had 4 mutations in PBP3 and *fecA*, which are shared with both EC948 and EC647. These two strains were similar for four mutations in *cirA*, *fecA,* PBP3, and PBP1, and each had a single unique mutation in PBP2.

For *cirA,* EC650 had a single mutation (D95G), whereas the other three strains exhibited frameshift mutations resulting in truncation. For EC771, premature termination of the protein was noted with truncation, in addition to many mutations noted in the second fragment of the gene. As for EC647 and EC948, both had missing aa (S) at

position 90 due to deletion mutation causing a frameshift with altered amino acids distal to the mutation leading to premature termination of the first fragment of the protein. Both had a truncated first segment, followed by a second fragment of the protein starting at amino acid 141, without any observed mutations.

All four strains shared the same three mutations in *fepA* (K69E) and *fecA* (T16A and L444V), as shown in the Venn diagram (Fig. 4A-number surrounded by a star), indicating the number of shared and unique mutations. Multiple mutations in *fepA* and *fecA* were shared between EC948 and EC647, whereas EC771 and EC650 shared multiple mutations in *fepA* and *tonB,* and harbored several mutations in *fiu* gene, which exhibited a wild type in both EC948 and EC647.

For PBP3, we found several mutations in the *ftsI* gene, which encodes this protein. Most of the variations were found in EC647 and EC948, which carried identical mutations (Q227H, aa insertion at 333rd position: YRINY, E349K, I532L), while EC650 had the same mutations, except Q227H. EC771 had a single aa substitution (I332V).

As for PBP2, MrdB subunit did not show any mutation, while MrdA had mutations in two strains, namely EC647 (L573Q) and EC948 (K572N). Finally, we examined PBP1, with its three subunits (A, B, and C), and identified several mutations in strain EC771, followed by EC650. PBP1-C had the most variations, compared to the other subunits. Both EC650 and EC771 shared several mutations, while some were found exclusively in each of them. Both EC948 and EC647 had one alteration, which included a missing 1–13 aa. Multiple sequence alignments of the genes with mutations at nucleotide and amino acid levels are shown in the supplementary file (Supplementary Fig. 1).

Moreover, we explored the association of various resistance determinants, including β-lactamase genes (carbapenemases, ESBLs, AmpC), iron acquisition genes, and target modification through altered PBPs involved in peptidoglycan synthesis and β-lactam resistance mechanisms. As shown in Fig. 4B, the chord network revealed interconnected resistance strategies, with complex patterns of co-occurrence of resistance genes and mutations within the strains. Chord thickness reflects the association strength, revealing the coordinated selection of β-lactamases, iron acquisition, and cell wall synthesis determinants. It is worth mentioning that catecholate and colicin transporter genes (i.e., *fiu* and *cirA*) exhibited the strongest associations with various genetic modifications detected in the strains, highlighting their key role in CFDC resistance.

Antibiotic susceptibility profiles and response to the novel BLIs

The next step was to check the antibiotic susceptibility profiles of the strains. Figure 5 shows the MICs for various antibiotics with the results of testing for the novel BLIs alone [BLIs with intrinsic antibacterial activities (i.e. xeruborbactam, and zidebactam)], and in combination with BLs. All the strains were highly resistant to multiple antibiotics from various classes, i.e., they are all MDR strains. The strains were highly susceptible to colistin and tigecycline, which are considered as last resort drugs. Among BL/BLI combinations, meropenem-xeruborbactam was the most effective (MICs: < 0.031–0.5 µg/ml), followed by cefepime-zidebactam (MIC: 0.062–0.5 µg/ml). On the other hand, cefepime-taniborbactam did not exhibit a potent effect, except for one strain (EC771; MIC: 0.5 µg/ml). Interestingly, zidebactam had a potent antibacterial effect, even when tested alone (MIC: 0.125–0.5 µg/ml), compared to xeruborbactam, which did not show any sole effect (MIC > 4 µg/ml).

Besides, each novel BLI was tested in combination with CFDC using the checkerboard assay. Treatment with CFDC + BLIs caused a significant reduction in CFDC MICs, as shown in Fig. 6**.** Both taniborbactam and xeruborbactam were effective in reducing CFDC MICs significantly for all the tested strains (Fig. 6A,B); however, the combinations were more effective on strains EC647 and EC948 compared to the other two strains (EC771 and EC650), for which CFDC was either dropped to borderline susceptible range (MIC: 4 µg/ml) or remained in the resistance range (MIC ≥ 16 µg/ml for EC650 treated with CFDC + taniborbactam). The same strain did not show a response to the combination with zidebactam, although it was the most effective combination, causing the maximum drop in CFDC MIC (0.015 and 0.125 µg/ml for strains EC948 and EC771, respectively; Fig. 6C). Nevertheless, EC647 showed the least response to the combinations with zidebactam, which failed to drop CFDC MIC to the susceptible range (MIC: ≥ 8 µg/ml).

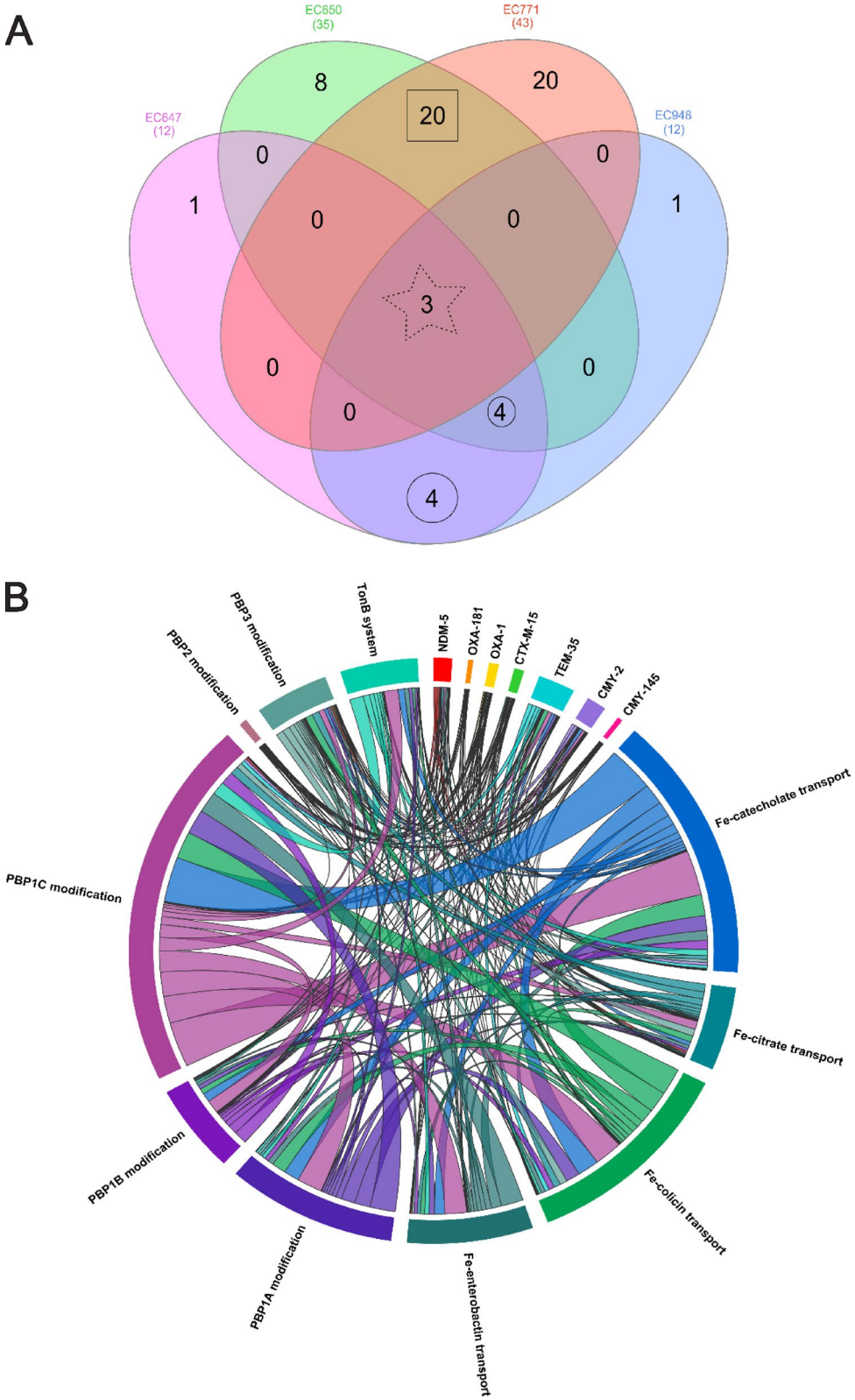
For taniborbactam, the standard combination of cefepime-taniborbactam was less effective than combining the same BLI with CFDC for strains EC647 and EC948 (MICs: 1 µg/ml for the latter compared to > 16 µg/ml for the former combination).

For xeruborbactam, the standard combination of meropenem-xeruborbactam was more effective than combining the same BLI with CFDC for all strains, as MIC did not drop below 1 µg/ml using the latter combination.

For zidebactam, the standard combination of cefepime-zidebactam was less effective than combining the same BLI with CFDC for all the strains except EC650. Combining CFDC with ½ MIC of zidebactam showed a more potent effect than using the same drug at its MIC, with a significant reduction of CFDC MICs.

Genotype–phenotype correlation to explain response to the combination therapy

When the genotypes and phenotypes were linked, we found that strain EC650 with the least response to the combination therapy with CFDC carried an AmpC gene (bla_{CMY-2}), and many mutations in all types of PBPs (except PBP2) and mutations in all iron transport genes. A total of 20 mutations in EC650 were shared with strain EC771, which harbored the highest number of mutations (n = 43) in all types of PBPs (except PBP2) and mutations in all iron transport genes. Surprisingly, it exhibited the least MIC to CFDC when used solely (MIC: 64 µg/ml), although in the resistance range, and was the only strain responding to cefepime-taniborbactam (MIC: 0.5 µg/ml). This strain had a single mutation in PBP3, compared to the other three strains which harbored more mutations in this gene, including YRIN insertion. Interestingly, both strains (EC771 and EC650) exhibited the best response to meropenem-xeruborbactam (MICs: < 0.031 µg/ml), while cefepime-zidebactam was most effective on EC771 (MICs: < 0.062 µg/ml).



Another strain (EC647) harbored an AmpC gene ($bla_{CMY-145}$) and SBL (bla_{OXA181}), in addition to bla_{NDM-5}. It exhibited weak response, especially to the combination consisting of zidebactam, although it was shown to be the most potent BLI with the best activity and maximum reduction of CFDC MIC on other responding strains. EC647 harbored a mutation in PBP2, which was unique compared to EC948, which had another type of mutation in the same gene. Both strains (EC647 and EC948) had higher MICs to zidebactam (0.5 µg/ml), but it was not significantly different from the other two strains without PBP2 mutations (0.25–0.125 µg/ml).

Overall, two strains (EC647 and EC948) can be considered the best responders to the combinations of CFDC with BLIs compared to the other strains (EC771 and EC650), as the latter two strains had exclusive mutations in

◀**Fig. 4**. Co-occurrence of mutations. Venn diagram (**A**) of mutations in the four strains. The diagram summarizes the similarities and variations in these mutations, whereby the numbers between parentheses represent the total number of mutations per strain. The black square indicates the number of shared mutations between EC771 and EC650, while the circles were used to show the number of shared mutations between EC948 and EC647 (large circle) and those shared between the latter two strains with EC650 (small circle). The star refers to the mutations shared among the four strains. Other numbers represent unique mutations in each strain. A chord diagram (**B**) visualizes the co-occurrence relationship of various CFDC resistance mechanisms, including beta-lactamase resistance genes, alterations of PBPs, and iron transport systems for colicins (*cirA*), catecholate (*fiu)*, ferric citrate (*fecA*), enterobactin siderophore (*fepA),* and *tonB* energy transduction system. Chord thickness reflects association strength based on Jaccard similarity coefficients (≥0.5), whereby thicker chords indicate stronger co-occurrence relationships.

PBP1A, PBP1B, *tonB*, and *fiu*, and had more mutations in PBP1C, *cirA,* and *fepA,* when compared to the former strains, which had no or less mutations in these genes (Fig. 4).

Discussion

Resistance to CFDC has emerged globally[23]. Here, we report discovering CFDC resistance in four clinical isolates of *E. coli* from the UAE. All the strains were isolated from critically ill patients with a history of chronic or immunosuppressive illness, repeated hospitalization, and frequent exposure to antibiotics. All these are risk factors for increased susceptibility to MDR bacterial infections[26]. None of the patients were exposed to the drug or treated with it during the course of illness. It is unclear whether these bacteria were initially carried by the patients and underwent a series of mutations due to repeated exposure to antibiotics, leading to CFDC resistance impacted by the selective pressure imposed by other BL antibiotics[27]. Another possible scenario is that these bacteria were present in the hospital, that has a high prevalence of persistent MDR bacteria, and were acquired during hospitalization[28]. We can consider this study as the first report of CFDC resistance in *E. coli* within the UAE. However, a previous study reported detecting CFDC resistance in a patient with leukemia originating from the UAE and hospitalized in the US. The patient initially had *E. coli* with NDM-5 gene which was susceptible to CFDC but acquired resistance after repeated exposure to the drug during hospitalization in the US[29]. There was a gradual increase in CFDC MIC which was attributed to the increased expression of NDM-5 gene during the course of treatment.

We delved into the whole genomes of our strains to uncover the mechanisms of CFDC resistance. Noteworthy, all the strains carried bla_{NDM-5} gene which is an MBL associated with CFDC resistance according to multiple reports worldwide[29–31]. NDM-5 is a variant of the New Delhi MBL, which is becoming prevalent in the UAE[32]. It can efficiently hydrolyze CFDC, undermining its effectiveness, as demonstrated experimentally using *E. coli* DH10B cells expressing various MBLs, whereby NDM-1 and NDM-5 efficiently hydrolyze CFDC, while IMP-1 and VIM-2 showed poor activity, based on steady-state kinetic measurements[33]. Two broad-spectrum AmpC β-lactamases, namely bla_{CMY-2} and $bla_{CMY-145}$ (a derivative of bla_{CMY-2}), were detected in two strains. This concurs with other reports documenting the presence of CMY genes in CFDC-resistant *E. coli*, particularly those co-harboring NDM gene[34]. A recent study linked the expression of chromosomally encoded AmpC to high levels of CFDC resistance in *E. coli*[35], and several studies collectively support the notion that carriage of bla_{CMY} plays a critical role in the development of resistance to CFDC, especially when present alongside other resistance mechanisms like NDM-5 or specific mutations in PBP3 and iron transport genes[36,37]. One of our strains carried an SBL (OXA-181), an OXA-48-like carbapenemase, which is not reported in any CFDC-resistant strains. To the best of our knowledge, this is the first report of co-production of NDM-5, CMY-145, and OXA-181 in a CFDC-resistant strain. Indeed, this is the first report of such gene combinations in the UAE due to the lack of comprehensive genomic studies. Nosocomial outbreaks by strains co-producing NDM-5 and OXA-181 genes were reported in other parts of the world, and some of these strains belonged to ST410, which is a high-risk clone [38–41].

Interestingly, our strains were highly diverse in terms of their genotypes and belonged to various STs, which contradicts most studies reporting the emergence of CFDC resistance, typically linking it to specific dominant clones. For instance, a recent French study reported CFDC resistance in *E. coli* strains carrying NDM-5, with mutations in siderophore-iron uptake genes and PBP3, from four main clones (ST410, ST167, ST361, and ST405)[30]. The same notion on the association of NDM-5, *cirA* and PBP3 mutations with high levels of CFDC resistance was reported in a Chinese study, which found that 76.9% of the resistant strains belonged to ST167, while a few were from ST410, ST746, and ST11738 [42]. One of our strains (EC948) belonged to ST167, which is classified as a high-risk clone, and a source of several nosocomial outbreaks[43]. The other strains belonged to other STs, particularly, strain EC647 belonged to the new ST16062. Both ST167 and 16,062 belong to the ST10 clonal complex, which is known as lineage A. On the other hand, strain EC650 belonged to ST2659, which is a part of clonal complex ST38, but EC771 belonged to ST8489, which does not belong to any known clonal complex. To the best of our knowledge, ST16062 and ST8489 were not reported before in the UAE; thus, they are considered new types identified in this part of the world[32,44,45]. The strains were also unique using other typing schemes based on the core genome and the whole genome, and they carried different plasmid types. Based on replicon typing, each strain carried a unique set of plasmids, with the IncF type being the dominant one. This is in line with recent reports highlighting the association of IncF plasmids with carriage of NDM-5 gene in high-risk *E. coli* clones[46,47]. These plasmids, along with other mobile genetic elements, play a crucial role in the horizontal gene transfer (HGT) of resistance determinants[48]. Antibiotic pressure in hospitals accelerates HGT, and bacterial diversification, which is also a major driver of resistance evolution[48–50]. The variations in the genetic makeup

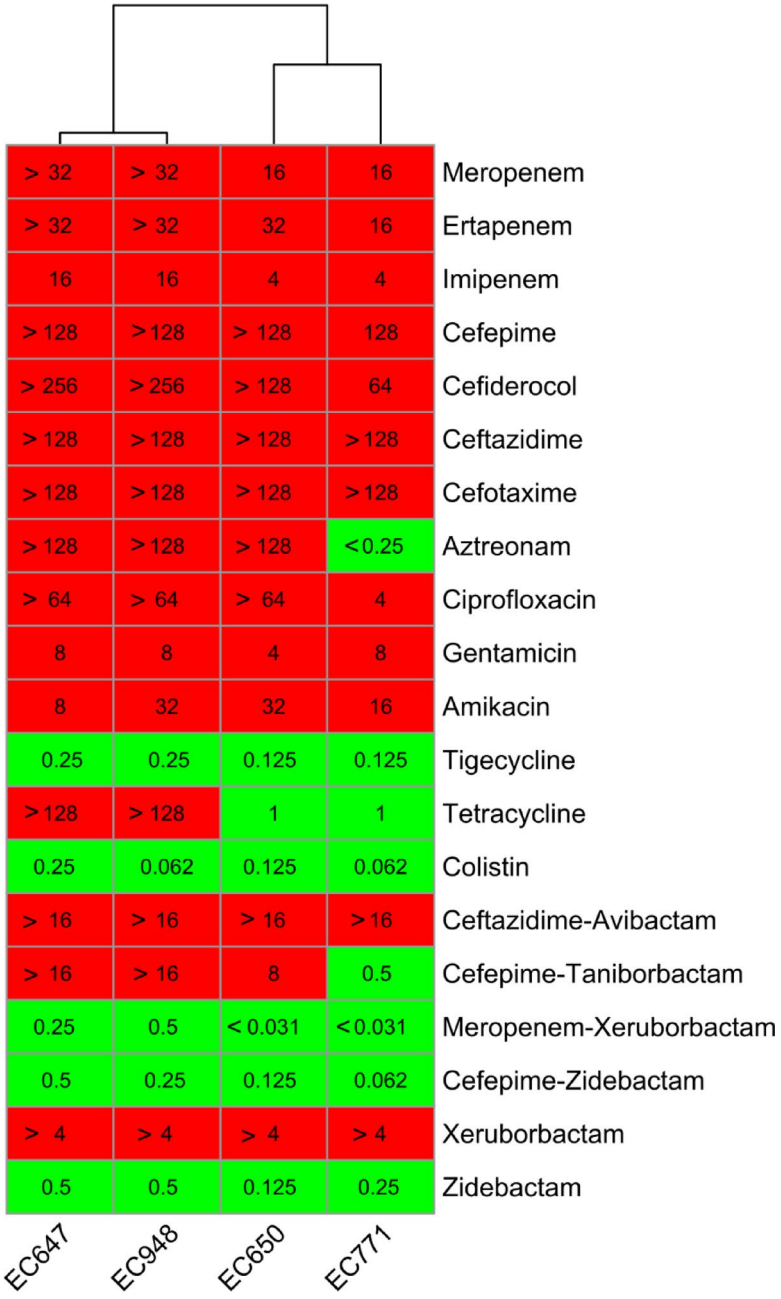


Fig. 5. Antibiogram for the four *E. coli* strains tested for susceptibility to various antibiotics, including novel BLIs. BLIs with intrinsic antibacterial activities (i.e. xeruborbactam and zidebactam) were tested individually and in combinations. The numbers shown in the graph represent the MICs (μg/ml), whereby red color represents non-susceptibility and green color represents susceptibility to the indicated antibiotic. The dendrogram was generated based on the antibiogram profile of the four strains.

of the strains reflect the non-clonal nature of CFDC resistance, demonstrating that the strains did not originate from a resident clone acquired during the hospital stay. The accumulation of multiple mutations elucidates the evolving nature of resistance as an adaptive strategy to counter antibiotic selective pressure.

We deeply explored mutations in both PBPs and iron transport systems, that can be implicated in CFDC resistance and found an array of genotypic variations. In most CFDC-resistant isolates (3/4), a unique frameshift mutation was identified with new amino acids (YRINY) inserted at the 333rd position of PBP3. Notably, the same mutations were reported repeatedly in CFDC-resistant *E. coli* [30,34,42]. Although many reports support the association between CFDC resistance and PBP3 mutations, a recent study indicated that such a mutation had no impact on CFDC MICs in *E. coli* strain MG1655 upon modifications of the PBP3 sequence by inserting YRIK or YRIN within PBP3, compared to the wild-type strain, which is devoid of mutations[35]. It is worth mentioning that the recombinant strains lacked any β-lactamases that may degrade CFDC in the latter study, which highlights the important role of these enzymes in conferring a high level of resistance and the demand for using potent BLIs

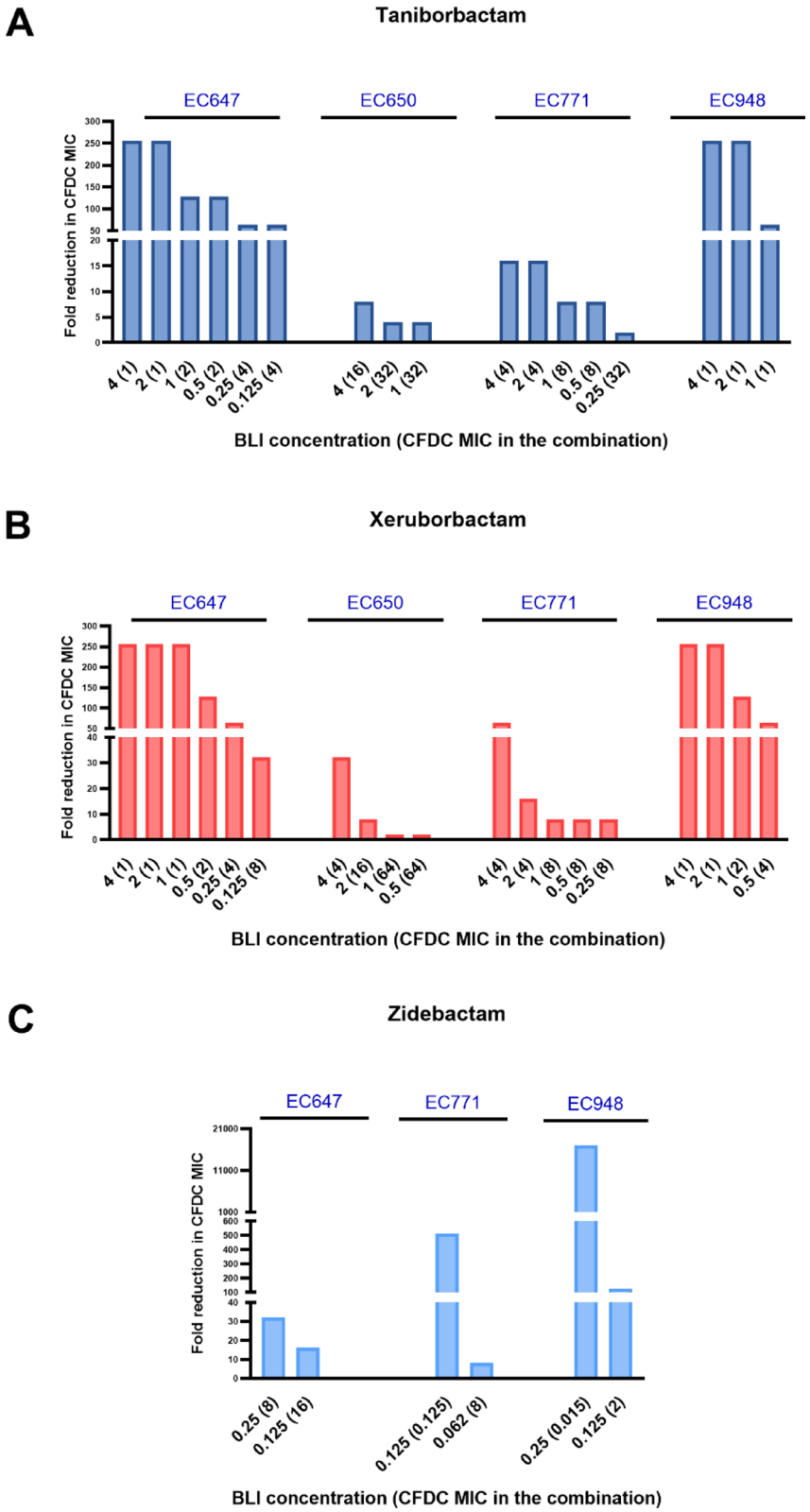


Fig. 6. Fold reduction in CFDC MIC when used in combination with different concentrations of BLIs (taniborbactam, xeruborbactam, and zidebactam). For zidebactam, only strains against which the combinations were effective are shown, with the best two concentrations that exhibited a significant reduction in CFDC MICs. For taniborbactam and xeruborbactam, serial concentrations associated with a significant reduction in MICs are shown, with 4 μg/ml being the highest BLI concentration used. Inhibitory CFDC concentration when used in combination with the indicated concentration of the BLIs are shown between the parentheses.

to counteract their hydrolytic action. Zhang et al. 2017 studied the impact of mutations in various PBPs in *E. coli* and highlighted that PBP3 modifications demonstrate the highest impact on susceptibility to BL drugs. The same study performed molecular modeling of the *E. coli* PBP3 native enzyme, shedding light on the location of mutations relative to the transpeptidase active site of PBP, as one of the factors determining whether a mutation can impact the bacterial response to the BL alone or coupled with BLIs, such as ceftazidime-avibactam[51]. The transpeptidase activity of PBP3 relies on eight residues (Ser307, Lys310, Ser359, Asn361, Lys494, Thr495, Gly496, and Thr497), forming three conserved sequence motifs which are responsible for the binding of BL to the active site of PBPs[52]. Notably, none of our four strains had a mutation in these specific residues, but indeed, they exhibited multiple other mutations, not only in PBP3 but also in other types of PBP. Several mutations were found in PBP1C in line with a previous study, showing mutations in this subunit of PBP1 among CFDC-resistant *E. coli* strains [42]. Noteworthy, two of our strains (EC650 and EC771) with poor response to the combination therapy had multiple mutations in all three subunits of PBP1. As CFDC targets PBP3, it is not clear if mutations in other types of PBPs will affect the drug activity or the process of cell wall synthesis in the mutant strains. As for PBP2, which is the molecular target for zidebactam, two strains (EC647 and EC948) exhibited single mutations in one of the subunits (MrdA). Both strains had slightly higher MICs to the drug compared to strains without PBP2 mutations. Their response to the combination therapy was variable, as strain EC647 did not show a reduction of CFDC MIC to the susceptible range, which is most likely linked to its genomic content as it harbors both AmpC gene ($bla_{CMY-145}$) and SBL (bla_{OXA181}), in addition to NDM-5. The cumulative degradative effect of these enzymes is the most likely cause of poor response to zidebactam, but it did not affect the response to other BLIs which significantly lowered the MICs in these strains due to their pan-BL inhibitory effect, preserving the activity of CFDC by protecting it from degradation.

Iron transporters are involved in the permeation of CFDC into bacterial cells. The siderophore moiety of CFDC leverages the active transport mechanisms of siderophores to enhance the penetration of the drug into the bacteria. By utilizing iron transport channels for cell entry via this Trojan horse strategy, CFDC can circumvent resistance mediated by efflux pumps and membrane permeability barriers, which often limit the effectiveness of other β-lactam antibiotics[22]. As expected, all our CFDC-resistant strains carried mutations in siderophore-iron transport genes. Catecholate siderophore receptor (CirA) has a major effect on siderophores uptake, then entry across the bacterial outer membrane; thus, it has been linked to CFDC resistance in multiple bacterial species[23,53]. Complementation studies of *cirA* into deficient strains proved that adding a functional transporter was able to fully restore susceptibility to CFDC[54]. A previous study reported the presence of a premature stop codon in the siderophore receptor *cirA* among several resistant strains[42], which is in line with our findings, as two of our strains had the same type of mutation. All our strains had additional mutations in enterobactin receptor (*fepA*) and ferric iron citrate receptors (*fecA*) genes, which are critical for siderophore-iron uptake and transport across the bacterial outer membrane and are thought to be implicated in CFDC resistance, as we reported in our prior work[55]. As seen in our study, mutations in *cirA, fepA,* and *fecA* were reported in multiple CFDC-resistant strains from China[42], while a single CFDC-resistant strain carried *fiu* mutations.

The same two strains (EC771 and EC650) had both *fiu* mutations and similar mutations in *tonB*, suggesting that these strains have a defective siderophore-iron transport system, contributing to their poor response to the combination therapy. This limited response can be attributed to the impact of the *fiu* gene encoding ferric iron uptake (Fiu) transporter, which mediates the movement of iron-catecholate complexes across the bacterial outer membrane[25]. Being a distinct clade of iron-catecholate transporters, which can bind to monomeric catecholate compounds, either alone or in complex with iron[25], Fiu can impact CFDC activity, as a catechol siderophore mimetic drug, using this iron transport pathway to access bacterial cells. This was supported by a previous report showing a twofold increase in CFDC MIC against *E. coli* by knocking out either *cirA* or *fiu*, whereas the MIC of CFDC increased 16-fold by the double knockout of both genes[6]. Thus, the effect of mutations in these receptors seems to be cumulative. TonB-dependent transporters are able to selectively capture iron-siderophore complexes through interaction with the energy-transducing protein TonB[56]. Thus, mutations in *tonB* can significantly impact CFDC activity as noted in poorly responding strains to combination therapy, due to the fact that TonB system interacts with various types of outer membrane transporters that recognize iron-siderophore complexes, which enter the periplasm in a process dependent on the inner-membrane protein TonB[24]. This has been confirmed in a previous study showing that CFDC was inactive against a ΔtonB strain[57]. The same study has shown that double and triple knockout of *cirA,* and *fiu* with or without *fepA* led to CFDC resistance. Multiple siderophore receptors are implicated in drug uptake; thus, resistance can arise when multiple pathways of entry are blocked via concurrent mutations in iron uptake genes. This is further supported by our co-occurrence analysis, which revealed unique patterns of association among various resistance determinants and mutations, suggesting biologically significant relationships. Co-occurrence patterns suggest that these mechanisms are coordinately selected, likely due to their co-location on mobile genetic elements (as with β-lactamase genes) and shared regulatory networks that respond to antibiotic stress, with coordinated selection applicable to chromosomal mutations in PBPs and iron transport systems.

Despite all the mutations, combinations with novel BLIs were effective in significantly lowering CFDC MICs, which holds great promise for the effective eradication of highly resistant bacteria. The best BLI with the most potent effect, even when used alone, was zidebactam owing to its dual activity as both a PBP2 inhibitor and a β-lactamase inhibitor[16]. This is in line with previous reports demonstrating low MICs for zidebactam alone (MIC_{50}/MIC_{90}: 0.5/1 µg/ml) on *E. coli* strains with insertion mutations in PBP3, coupled with NDM and CMY co-production[58]. The latter study reported that modifications in PBP3 did not impact the MICs for zidebactam and its combination with cefepime, suggesting that their activity is attributed to the antibacterial potential of zidebactam. On the contrary, amino acid insertion into the PBP3 sequences significantly impacted the MICs of other BL, including cefepime-taniborbactam. Our results concur with these findings, as one of our strains (EC771) showed the best response to this drug, compared to the other three strains having YRIN insertion

mutations in PBP3, which seems to affect the response to the combinations. The effect of taniborbactam combined with CFDC was also dependent on the presence of mutations in the iron transport systems. The same was noted for xeruborbactam with regard to its effect on CFDC. Only a single study reported the combined effect of CFDC and taniborbactam, as well as the ability of the latter to protect CFDC from hydrolysis by potent β-lactamases such as PER, and other types of MBLs in recombinant *E. coli* MG1655 strains[59]. Alterations in PBP3 in these strains had an impact on CFDC used alone and in the combination, especially on strains with certain MBL types. Compared to zidebactam, xeruborbactam has less potent intrinsic antibacterial activity against some Gram-negative bacteria, including CREs (MIC_{50}/MIC_{90}:16/32 µg/ml)[21]. It can bind to different PBP types, including PBP1a/PBP1b, PBP2, and PBP3. Consistent with inhibition of PBPs, xeruborbactam can enhance the potencies of β-lactam antibiotics even against strains that lacked β-lactamases. Studies have shown that strains with substitutions in PBP2 had increased MICs of the highly selective PBP2 inhibitors like zidebactam, without any change in xeruborbactam MICs. Similarly, strains with mutations in PBP3 did not exhibit any increase in xeruborbactam MICs[21]. However, the impact of dual mutation in both PBP2 and PBP3 was not reported in the latter study. As for our strains, we believe that the cumulative effect of multiple mutations in different types of PBPs with other defects in the iron transport system were all determinants for the response to xeruborbactam with CFDC.

A recent study reported a superior effect of BL/BLI combinations with xeruborbactam than those with tanibornactam, on *E. coli* strains with specific types of NDM, including NDM-5, which was detected in all our strains[60]. A recent study reported excellent response to novel BL/BLI agents in a pan-BL-resistant isolate, with the best activity reported for combinations of meropenem-xeruborbactam and cefepime-zidebactam (MIC for both ≤ 0.25 µg/ml) being superior to cefepime-taniborbactam (MIC: 16 µg/ml)[36]. The same response was noted in our strains, which responded to both BL/BLI agents, although they harbored multiple mutations in iron transport genes and PBP3.

Indeed, all the studies that were conducted on these novel BLIs, including this study, were limited by the sample size. Further studies, including bigger cohorts of strains with diverse genomic contents, must be conducted in the future to prove the efficacy of the novel synergistic combinations. This should be supplemented by in vivo pre-clinical and clinical studies to examine the pharmacokinetics and pharmacodynamics of these combinations of CFDC and novel BLIs. Furthermore, functional studies are necessary to investigate the impact of each mutation on drug activity, as some of the mutations reported in the study are novel.

Conclusions

This study reports the emergence of CFDC resistance in *E. coli*, identifying several new genes and sequence types in the UAE for the first time. Our results confirm the multifactorial nature of CFDC resistance, attributed to mechanisms including the expression of potent beta-lactamases (co-production of MBL, SBL, and/or AmpC), defects in PBPs, and siderophore iron transport systems.

These findings highlight the importance of ongoing surveillance and innovative approaches to preserve the efficacy of last-resort antibiotics against evolving bacterial resistance.

Our study did not only elucidate the genomic basis of emerging CFDC resistance in *E. coli* but also present promising avenues for developing new therapeutic strategies. Our study highlighted the efficacy of novel BL/BLI combinations with BL enhancer effect (zidebactam) and pan-beta lactamase inhibitory activity (tanibornactam, and xeruborbactam). However, their efficacy was impacted by the type of mutations affecting CFDC entry through iron receptors, transporters, and its target on PBP3.

The emergence of CFDC resistance is an anticipated challenge that could limit its long-term efficacy. Ongoing research, clinical evaluation, and the judicious use of novel agents are crucial to ensure their long-term effectiveness and mitigate the emergence of resistance. By extending the lifespan of these valuable therapeutic options, we can contribute to the ongoing efforts to combat multidrug-resistant bacterial infections.

Methods

Ethics statement

This study was approved by Tawam Human Research Ethics Committee, Al-Ain, UAE (approval number: MF2058-2021-761). The study was conducted in compliance with national and international standards, including the Declaration of Helsinki. All methods and procedures were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained for the use of the clinical data presented in the study.

Bacterial strains

While screening a group of 80 clinical strains of *E. coli* isolated from patients attending Tawam Hospital, Al-Alin, UAE, during 2022–2023, four strains were identified as resistant to CFDC (MIC ≥ 64 µg/ml). The strain's identity was confirmed as *E. coli* using the VITEK 2 system (BioMérieux, Craponne, France). All isolates were preserved in brain heart infusion broth (MAST, United Kingdom) containing 20% glycerol and stored in − 80°C freezer. Before each experiment, the strains were checked for purity[61].

Whole genome sequencing and bioinformatic analyses

The genomic DNA of the strains was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. DNA quality and quantity were explored by a nanodrop (Thermo Scientific, USA). Fluorometric quantification was also done by Qubit 2 (Thermo Scientific, USA). Whole genome sequencing (WGS) was performed using DNBSEQ-G400RS (DNA nanoball sequencing platform by MGI-Tech, Hong Kong) at a coverage of > 200X. Before analysis, sequences were checked for quality by assessing

FASTQ files of individual samples with FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Several quality metrics (sequencing depth, read length distribution, read quality distribution, mean read quality along the read, base frequency at each read position) were compared across samples. De novo assembly was accomplished for the paired-end sequence reads using Unicycler (https://github.com/rrwick/Unicycler), followed by quality assessment using QUAST v5.0.2 (http://bioinf.spbau.ru/en/quast)[62,63] and annotation using the rapid prokaryotic genome annotation tool (PROKKA)[64,65].

The resultant assembled genomes were analyzed and screened for sequence type, plasmid replicon types, resistance genes, and any mutations known to be associated with CFDC resistance. The Comprehensive Antibiotic Resistance Database (CARD) was used for the screening of antibiotic resistance genes (https://card.mcmaster.ca/)[66]. A heatmap depicting the presence–absence status of antimicrobial resistance genes was plotted using the Microreact tool provided by the Centre for Genomic Pathogen Surveillance (CGPS)[67]. Plasmid incompatibility types were obtained by the plasmid finder provided by the Center for Genomic Epidemiology (https://cge.food.dtu.dk/services/PlasmidFinder/). To explore the evolutionary relationships among the strains, phylogenetic trees were reconstructed using GrapeTree tool via MSTree v2 (https://github.com/achtman-lab/GrapeTree)[63,68] based on Achtman 7 gene MLST[69], Ribosomal MLST (rMLST)[70], Core Genome MLST (cgMLST), and Whole Genome MLST (wgMLST)[71] derived from the genome assembly of sequenced reads for each strain, which were analyzed using EnteroBase analysis tools[72]. Additionally, the Clermont typing method was used for in silico phylotyping of the strains from the genomic data[73,74]. The latter is a special epidemiological typing method for *Escherichia* species into eight main phylogroups (A, B1, B2, C, D, E, F, and G), derived from Clermont and colleagues' methods developed in 2013 and 2019, linking phylogenetic groups to the lifestyle of different strains and adaptation for various niches in human, animal or environment [75,76].

For mutation analysis, pairwise alignment of the genes linked to CFDC resistance (siderophore-iron uptake genes, including *cirA*, *fecA, fepA, tonB,* and *fiu* [30] and genes encoding PBP type 1,2, and 3) was carried out using ClustalW for multiple sequence alignments. Wild-type *E. coli* strain K-12 MG1655 (GenBank accession no. NC_000913) was used as a reference, as recommended by other investigators[30,34,42]. For mutations in PBP3, a reference sequence was obtained from publications reporting mutations in this gene[42]. To check for the presence of similar mutations among the strains, InteractiVenn tool was used to generate a Venn diagram for unique and shared mutations[77]. Furthermore, a heatmap was generated using ggplot2 package in R (version 4.1.2) software to provide a list of the mutations detected in each strain.

Gene presence/absence data were converted to binary matrices for the assessment of co-occurrence relationships between pairs of genes (beta-lactamases) and mutations (PBPs and iron transporters), using the Jaccard similarity coefficient[78]. A threshold of 0.5 was used to define substantial associations for co-occurrences between pairs of resistance genes and mutations, which is more than what is expected by random chance[79]. The gene co-occurrence network was visualized using a chord diagram implemented in the circlize package in R (version 4.1.2). Connection strength was represented by chord thickness, depending on the Jaccard similarity coefficient[78].

Antibiotic susceptibility testing

Antibiotics were purchased from Sigma-Aldrich (Saint-Louis, USA), except for CFDC, taniborbactam, xeruborbactam, and zidebactam, which were procured from MedChemExpress, USA. Antibiotic susceptibility testing was conducted according to the latest Clinical Laboratory Standards Institute (CLSI) guidelines using *E. coli* ATCC 25,922 as a quality control[80]. Broth microdilution test was used to estimate the minimum inhibitory concentration (MIC) for 14 antibiotics covering different antimicrobial classes, including meropenem, ertapenem, imipenem, cefepime, ceftazidime, cefotaxime, aztreonam, ciprofloxacin, gentamicin, amikacin, tigecycline, tetracycline, colistin, and CFDC. Besides, BLIs with expected intrinsic antibacterial activities (i.e. xeruborbactam, and zidebactam) were tested alone before testing them in combination with other BLs. BL/BLI combinations (ceftazidime-avibactam, cefepime-taniborbactam, meropenem-xeruborbactam, and cefepime-zidebactam) were tested for use as controls. For the latter combination, both drugs were used at 1:1 ratio (i.e. equal concentrations of cefepime and zidebactam) while the other BLIs were used at a fixed concentration of 4 µg/ml as per the recommendations in previous research work[44,81]. For all the antibiotics except CFDC, Mueller–Hinton broth obtained from Oxoid, United Kingdom, was used for the assessment of MICs in accordance with the latest CLSI guidelines[80]. Tigecycline and colistin broth microdilution results were interpreted based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines[82]. CFDC MIC was performed in iron-depleted cation-adjusted BBL™ Mueller Hinton II Broth (Becton Dickinson, USA), prepared as per the CLSI guidelines, as described in our previous work[55]. Based on antibiotic susceptibility profiles, the strains were classified as multidrug-resistant (MDR) if they were resistant to at least one antibiotic in three or more antimicrobial categories. Antibiogram data were depicted using pheatmap package in R version 4.1.2 software.

Testing CFDC in combination with novel BLIs using the checkerboard synergy assay

A checkerboard test was performed on the four CFDC-resistant strains to determine the lowest concentration of BLIs that causes a significant reduction in the MIC of CFDC. Three BLIs with different mechanisms of action were used, namely zidebactam, tanibornactam, and xeruborbactam. Synergy testing was carried out in 96-well microtiter plates with an initial inoculum of 5×10^5 CFU/ml with a final volume of 100 µl as described in our previous work[61,83]. CFDC was serially diluted starting from a concentration of 64 down to 0.0625 µg/ml. Zidebactam was tested starting with the MIC of each isolate, while tanibornactam, and xeruborbactam were tested starting from 4 down to 0.25 µg/ml. MICs of the drugs alone and in combination were determined as the lowest concentrations inhibiting bacterial growth following an overnight incubation at 37°C. All the experiments were performed in duplicate. Fold reduction of CFDC MIC was calculated by subtracting MIC

of the combination from the MIC of CFDC alone and plotted using GraphPad Prism® Version 10.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Data availability

Data was deposited in the Sequence Read Archive (NCBI) under BioProject accession number PRJNA1131975, with the following bio-samples: SAMN42328620 (EC647), SAMN42328621 (EC650), SAMN42328622 (EC771), and SAMN42328623 (EC948).

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Author contributions

FA: conceptualization, study design, funding acquisition, investigation, methodology, data curation, formal analysis, visualization, software, writing—review and editing, supervision, HA, RA, OA, AA, HA, and AAL: investigation and formal analysis; AG, LD and AA: methodology, and formal analysis; TC: investigation, clinical data, and review.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to F.A.-M.

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