International Journal of Antimicrobial Agents 61 (2023) 106698

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

In Vitro Selection of *Enterobacter cloacae* with Cefepime, Meropenem, and Ceftazidime-Avibactam Generates Diverse Resistance Mechanisms

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ARTICLE INFO

Article history:
Received 17 January 2022
Accepted 26 November 2022

Editor: Professor A Tsakris

Keywords:
β-lactam/β-lactamase inhibitor combinations
carbapenem
in vitro resistance
E. cloacae

ABSTRACT

Five *Enterobacter cloacae* isolates were subjected to 10-day serial passage in broth microdilution with cefepime, meropenem, or ceftazidime-avibactam to evaluate increases in minimum inhibitory concentration (MIC) and resistance mechanisms after exposure. Post-exposure isolates displaying >2-fold changes from the parent isolate were analysed alongside the parent isolate. Increases in MIC were 4- to 256-fold (median: 16-fold) after cefepime exposure, 16- to 128-fold (64-fold) after meropenem, and 2- to 32-fold (8-fold) after ceftazidime-avibactam. Post-exposure isolates had diverse mechanisms, identified using a combination of short and long whole-genome sequencing. All agents selected for AmpC alterations in one isolate set. OmpC and TetA/AcrR regulator alterations were noted in meropenem and ceftazidime-avibactam post-exposure isolates of the same set. Other mutations in AmpC were noted when isolates were exposed to cefepime or ceftazidime-avibactam. A premature stop codon in the cell division inhibitor protein, MioC was observed when one parent isolate was exposed to any of the agents, indicating a cell persistence mechanism. Mutations in less common transporter systems and protein synthesis components were also noted. All agents showed cross-resistance to other β-lactams and resistance mechanisms were diverse, with some not usually associated with β-lactam resistance in Enterobacterales. This initial evaluation indicates that cefepime and meropenem select for isolates with higher MIC values compared to ceftazidime-avibactam. Further studies evaluating these findings should be performed for other species for which the primary β-lactam resistance mechanism is not gene acquisition. These studies should evaluate these observations in vivo to assess their translation into patient treatment policies.

1. Introduction

Among the different species in the *Enterobacter cloacae* complex, *E. cloacae sensu stricto* is the most common in human infections [1]. This organism was included in the ESKAPE pathogen list because it causes serious nosocomial infections with elevated morbidity and mortality rates and develops resistance during antimicrobial treatment [2,3].

Despite the numerous descriptions of extended-spectrum β-lactamases (ESBLs) and carbapenemase genes encoding β-lactam resistance among *E. cloacae* isolates, acquired β-lactamases are not the most common β-lactam resistance mechanism in this species [4,5]. The development of β-lactam resistance in *E. cloacae* is usually attributed to changes in global regulatory genes that alter the expression of different proteins [1]. Overexpression of the constitutive AmpC is probably the most important contributor to β-lactam resistance in *E. cloacae* [6]. This mechanism alone can elevate the MIC values for ceftazidime and other cephalosporins. Cefepime is stable against *Enterobacter* AmpC hydrolysis; however, resistance can develop during treatment when outer membrane protein (OMP) mutations combine with decreased β-lactam permeability and/or increased efflux of the agent by the efflux system, such as in the case of AcrAB-TolC [1,7]. Combination of these mechanisms and AmpC overproduction also increases the carbapenem MIC values in *E. cloacae* isolates [8].

In the context of resistance to many β-lactam agents and the potential for developing resistance during therapy, cefepime and the carbapenems have been considered the preferred treatments for *E. cloacae* infections [9,10], but the increased use of carbapenems to treat infections caused by *E. cloacae* may generate higher resistance levels in this species [1,11]. *E. cloacae* is the second most common carbapenem-resistant Enterobacterales (CRE; data from the SENTRY Program). Many CR-*E. cloacae* isolates do not produce carbapenemases. Instead, these isolates have a combination

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https://doi.org/10.1016/j.ijantimicag.2022.106698

of resistance mechanisms that include de-repression of AmpC, alterations in OMPs, and overexpression of efflux systems [1].

Ceftazidime-avibactam is approved by the United States Food and Drug Administration (US FDA) and the European Medicine Agency (EMA). Avibactam restores the activity of ceftazidime in the presence of Ambler class A (ESBLs and KPC), class C (AmpC), and some class D (OXAs) enzymes [12]. Ceftazidime-avibactam has inhibitory activity against the *E. cloacae* AmpC [13] and is active against isolates belonging to this Enterobacterales species [14]. However, there are few data on the potential development of resistance in *E. cloacae* isolates against ceftazidime-avibactam.

Five *E. cloacae sensu stricto* isolates were subjected to 10-day serial passage experiments at progressively increasing concentrations of cefepime, meropenem, or ceftazidime-avibactam to determine how rapidly resistant mutants appear and how high the MIC results would become post-exposure. Whole-genome sequencing using a combination of short- and long-read methods was conducted to evaluate the single nucleotide polymorphisms (SNPs) between the post-exposure and parental isolates to understand the resistance mechanisms that would emerge.

2. Materials and Methods

2.1. Isolates, antimicrobial exposure, and susceptibility testing

Five *E. cloacae sensu stricto* clinical isolates collected from US hospitals in 2016 and 2017 were selected from the INFORM Program for this study. Isolates exhibited low MIC values for β-lactam agents active against this species, including ceftazidime, cefepime, meropenem, and ceftazidime-avibactam.

Broth microdilution panels, prepared and inoculated according to the CLSI guidelines [15], were used for the passaging experiments. Panels contained increasing concentrations of cefepime, meropenem, and ceftazidime-avibactam. After overnight incubation, the entire content of the well displaying visible growth in the presence of the highest concentration of each antimicrobial was used to prepare a new 0.5 McFarland standard. This standard was diluted to a final concentration of 5×10^5 CFU/mL in a new panel with the same antimicrobial agent that the suspension was then grown into. This process was repeated for an additional 9 days. Isolates were subcultured twice before further testing and storage.

Baseline and post-exposure *E. cloacae* isolates were susceptibility tested by the reference broth microdilution method [15] against cefepime, meropenem, ceftazidime-avibactam (inhibitor at 4 mg/L), and comparator agents.

2.2. Whole-genome sequencing

Final mutants that displayed >2-fold changes from the baseline and baseline isolates were sequenced using a combination of short- and long-read sequencing methods. Total genomic DNA (1 ng) was used as input for DNA library construction using the Nextera XT^{TM} library construction protocol and index kits (Illumina, San Diego, California, USA) following the manufacturer's instructions. Libraries were sequenced to achieve a 100X coverage on a MiSeq Sequencer (Illumina) using the MiSeq Reagent Kit v3 (600cycle).

Long-stranded DNA was obtained using the Qiagen Genomic Tip 100/G (Qiagen, Germantown, Maryland, USA) according to manufacturer's instructions. Sequencing library preparation was carried out with Nanopore Rapid Barcode Sequencing Kit using 400 ng input DNA on the MinION sequencer controlled by MinKNOW version 19.06.8 (Oxford Nanopore Technologies Ltd, Oxford, UK).

2.3. Bioinformatic Analysis

FASTQ format files generated in the MiSeq for each sample set were assembled independently using de novo assembler SPAdes 3.11.1 [16]. A FASTA format file of contiguous sequences with the best N50 value was generated. FASTQ files were used as input for guided assemblies on the Lasergene SeqMan NGen software (DNASTAR, Madison, WI) for analysis of the genes encoding AmpC, AmpR, AmpD, OmpC, OmpF, AcrA, AcrB, TolC, MarA, MarB, MarR, RamA, RamR, CsrA, RobA, and SoxS. Multilocus sequence typing (MLST) used the PubMLST database available at https://pubmlst.org.

For the SNP analysis, short- and long-reads were assembled using Unicycler v0.4.8-beta, which builds an initial assembly graph from short-reads using the de novo assembler SPAdes and then simplifies the graph using information from short- and long-reads. SNPs were called between the baseline and follow-up pairs of isolates using MAUVE V2.4.0. All SNPs determined by MAUVE were confirmed by mapping quality trimmed reads independently to the baseline assembly. Reads were mapped using BWA v0.7.12-r1039. Insertion/deletion (INDEL) sites were realigned using IndelRealigner from the GATK toolbox v3.8-1-0gf15c13ef. High confidence variants were called by samtools v1.8 and filtered by bcftools v1.8. Filtering criteria for the variant call format (VCF) file was a minimum read depth of 4 ($\geq$2 reads per strand), >30 map quality, >50 average base quality, no significant strand bias, and >75% of mutations within reads to support the presence of any given alteration. Repeat regions of >50 bp were removed from VCF using MUMmer v3.0. Baseline assembly was annotated using Prokka v1.14.0. SNPs were annotated using Snpeff 4.3t. Short reads were subjected to quality trimming using a sliding window threshold of Q18. The reference sequence was built using the default parameters of the unicycler. SNP quality metrics were then applied [17].

All potential SNPs were confirmed using reference-guided alignments on short-read data with a minimum depth of 4X, >30 map quality, >50 average base quality, no significant strand bias, and >75% of reads supporting the variant calling. Additional analysis was performed by nucDiff (https://omictools.com/nucdiff-tool) to capture all INDELS and uncovered regions. Uncovered regions were identified as stretches of nucleotide sequences missing from the comparator when matched with the reference sequence.

Sequences analysed in this study were submitted to the Sequence Read Archive under the BioProject ID PRJNA774541.

3. Results

The initial MIC results and genetic characteristics of the *E. cloacae* isolates used for the passaging experiments are summarized in Table 1. Isolates were selected to display low MIC values for cefepime, meropenem and ceftazidime-avibactam. The baseline isolates carried different AmpC genes from the ACT family, but no other β-lactamase-encoding genes.

After 10-day serial passaging in subinhibitory concentrations of meropenem, the meropenem MIC values for the 5 isolates increased from 8- to 128-fold compared to the initial results (Table 2). Exposure to subinhibitory concentrations of cefepime resulted in higher MIC increases than meropenem, despite the same overall median fold increase (16-fold). The median MIC increase for ceftazidime-avibactam after exposure to subinhibitory concentrations of this combination was 8-fold for all five isolates. Two isolates had increases of only 2-fold after 10-day passaging, two isolates had final MIC values 8-fold greater than the initial MIC, and one isolate displayed a 32-fold increase. Notably, one isolate set (#1) had a 32-fold increase in MIC values for all three agents.

Resistant MIC results were only observed for one passaged isolate for meropenem and one for ceftazidime-avibactam despite the

Table 1
Basal MIC values and resistance genes detected among *E. cloacae* isolates.

	MIC (mg/L)			
E. cloacae isolate	Meropenem	Ceftazidime-avibactam	Cefepime	Resistance genes
ECL #1	0.03	0.5	0.5	*act-18, aph(6)-Ia*
ECL #2	0.06	0.5	1	*act-17, fosA*
ECL #3	0.03	0.25	0.5	*act-41-like, aadA2, sul1*
ECL #4	0.03	0.5	0.25	*act-15-like, aph(6)-Ia*
ECL #5	0.06	0.5	0.25	*act-12-like, fosA*

Table 2
Overall fold changes in MIC by day of passaging.

	MIC Fold change for *E. cloacae* isolates during serial passaging of antimicrobial agent:								
	Meropenem			Ceftazidime-avibactam			Cefepime		
Serial Passage Day	Median	Mode	Geo Mean	Median	Mode	Geo Mean	Median	Mode	Geo Mean
1	1	1	0.9	1	1	1.0	1	1	1.5
2	1	1	1.5	2	2	1.7	4	No mode	4.0
3	2	2	2.6	2	2	1.7	4	4	5.3
4	4	No mode	4.0	2	2	1.7	8	No mode	9.2
5	4	No mode	4.6	2	No mode	2.3	8	No mode	10.6
6	4	4	7.0	4	4	3.0	16	No mode	13.9
7	8	4	10.6	4	No mode	3.5	16	16	24.3
8	16	No mode	13.9	4	No mode	4.0	16	16	24.3
9	16	No mode	18.4	8	2	7.0	16	16	24.3
10	16	8	21.1	8	No mode	6.1	16	16	24.3

increase in MIC values. In contrast, two passaged isolates for cefepime displayed resistant MIC results (Figure 1).

The SNP analysis of the parent and post-exposure isolates revealed multiple changes in two isolate sets (#1 and #2) and discrete alterations in the other three sets (Figure 2). The #1 set (average nucleotide length for set: 491524 bp; >99.9% parent coverage) of post-exposure isolates had AmpC alterations either in position 61 (Glu→Val) or 213 (Gly→Asp; 2 isolates and 1 isolate, respectively). The isolate exposed to meropenem in this set also displayed a missense alteration in position 100 (Gly→Asp) of the OmpC-encoding gene, a premature stop in position 356 of the gene encoding the glycosyltransferase family 4 protein, and a missense alteration in the β-galactosidase gene (Ser1003Leu). Notably, the meropenem-exposed isolate displayed an elevated MIC (≥8-fold) for imipenem, doripenem, and cefepime in addition to meropenem. This isolate also displayed a 4-fold increase for ceftazidime-avibactam . The ceftazidime-avibactam post-exposure isolate in this set exhibited alterations in a TetR/AcrR family transcriptional regulator (Arg107His) and in the multidrug efflux resistance nodulation family (RND) transporter permease subunit (Leu527Arg). In addition, this isolate had a premature stop (position 126) on the gene encoding an EAL domain-containing protein [18] and missense alterations in a chitoporin (His434Tyr) and in NlpE, an envelope stress response activation lipoprotein (Gly19Cys). The cefepime-exposed isolate from set #1 only displayed changes in AmpC (Glu61Val). A 4-fold increase in meropenem MIC was noted for this isolate.

All post-exposure isolates from set #2 (average nucleotide length for set: 4870236 bp; >99.9% parent coverage) exhibited missense mutations in the ATP-dependent RNA helicase RhlE (Asn215Thr) and in the UrtB, a urea ATP-Binding Cassette (ABC) transporter permease subunit, compared to the parent isolate (Ala468Gly). Furthermore, all three isolates had a premature stop in the MioC cell division inhibition protein (position 136) and an alteration upstream of the CorC, a CBS-pair domain divalent metal cation transport mediators (CNNMs) family that is responsible for magnesium/cobalt homeostasis. The isolate exposed to ceftazidime-avibactam also displayed upstream alterations in the peptide chain release factor 1 gene and missense mutations in the translation initiation factor, IF-2, and in EnvZ, a two-component system sensor histidine kinase. A premature stop codon was noted in the GalU gene encoding for UTP-glucose-1-phosphate uridylyltransferase in the cefepime post-exposure isolates from this set. The cefepime post-exposure isolates in this set had elevated carbapenem, cefepime, and ceftazidime-avibactam MIC results.

The meropenem post-exposure isolate from set #3 (average nucleotide length for set: 4788139 bp; >99.9% parent coverage) had an alteration in the UDP-glucose 6-dehydrogenase (Thr202Ile). This isolate displayed 8- to 16-fold elevated MIC values for meropenem and doripenem only. No SNPs were detected in the cefepime post-exposure isolate from set #3, but this isolate had only a 4-fold increase in the cefepime MIC. The ceftazidime-avibactam post-exposure isolate was not sequenced because its MIC values after 10-day serial passaging were the same as, or only 2-fold different from, the parent isolate.

Missense alterations were detected for one gene per post-exposure isolate from set #4 (average nucleotide length for set: 4749791 bp; >99.9% parent coverage). The isolate passaged into meropenem had an alteration in the transcription regulator, SlyA (Pro61Leu). The ceftazidime-avibactam post-exposure isolates had an alteration in the AmpC gene (Val303Glu). The cefepime post-exposure isolate in this set had an alteration in the glycosyltransferase family 4 protein (His113Arg). All these terminal isolates had ≥4-fold higher MIC values for meropenem, doripenem, ertapenem and cefepime compared to the parent strain.

For *E. cloacae* set #5 (average nucleotide length for set: 4730007 bp; >99.9% parent coverage), only the meropenem and cefepime post-exposure isolates were sequenced because there was only a 2-fold change in MIC for the terminal ceftazidime-avibactam isolate after 10-days of passaging. The meropenem post-exposure isolate displayed 16- to 32-fold elevated MIC values for all carbapenems except ertapenem. Cefepime was elevated 8-fold from baseline in this isolate, which had an alteration in the DNA-3-methyladenine glycosylase. An alteration in the ABC transporter substrate-binding protein and the upstream region of the hypothetical protein M942_13365 were noted in the cefepime post-

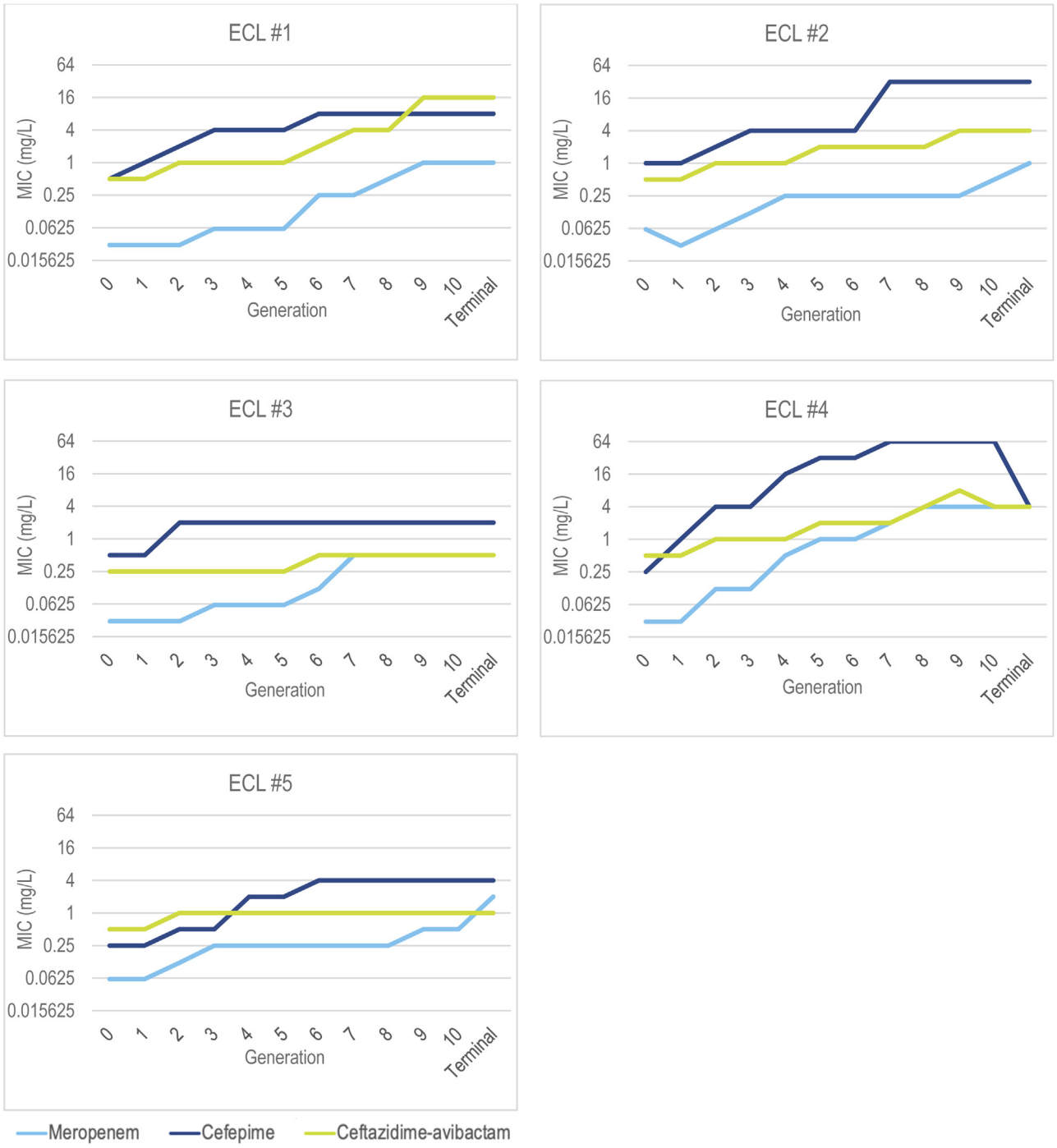

Figure 1. MIC variation for the 5 *E. cloacae* isolates during the 10-day serial passage in the presence of meropenem, ceftazidime-avibactam, and cefepime.

exposure isolate. This isolate displayed higher MIC values for all carbapenems apart from imipenem, cefepime, and piperacillin-tazobactam.

Beyond these, synonymous mutations were observed in redox-regulated ATPase YchF (2 isolates, set #5), cation-transporting P-type ATPase (2 isolates; set #2), and the MerR family transcriptional regulator (1 isolate; set #2).

4. Discussion

E. cloacae is naturally resistant to ampicillin, amoxicillin-clavulanate, cephalothin, and cefoxitin because of its basal production of AmpC. In addition, approximately 50% of *E. cloacae* isolates are expected to have higher levels of AmpC expression, which would confer resistance to third-generation cephalosporins [1]. Considering these characteristics, the preferred antimicrobial treatment option for *E. cloacae* infections is either cefepime or the carbapenems. Tamma et al. [9] expanded upon the list of therapeutic agents to other classes, including trimethoprim-sulfamethoxazole and fluoroquinolones. A subanalysis of a study published by our group [14] demonstrated that these agents had >89.9% coverage for 314 *E. cloacae* isolates recovered from patients with pneumonia in US ICUs from 2015 to 2017. Despite the elevated susceptibility rates for the overall population, when only the 97 (30.9%) isolates

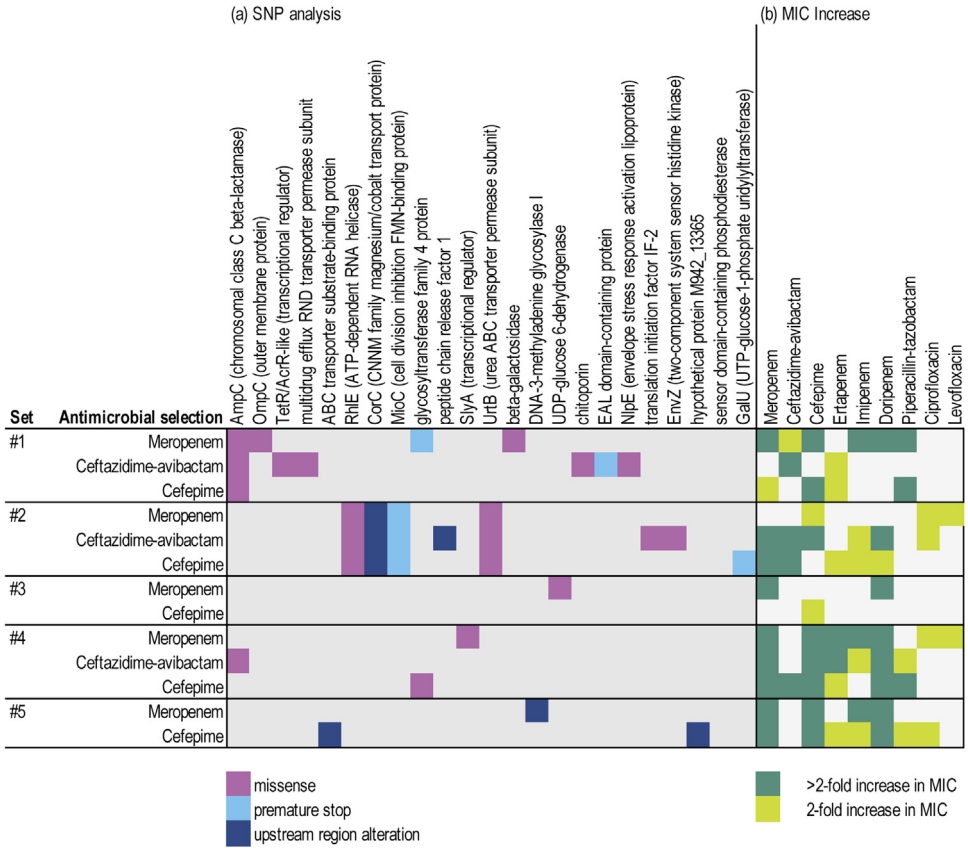

Figure 2. Genetic alterations detected in the single nucleotide polymorphism analysis and the MIC increase for β-lactams and comparator agents.

that were ceftazidime-nonsusceptible (a phenotypic marker for AmpC overexpression) were analysed, the susceptibility rates for trimethoprim-sulfamethoxazole and fluoroquinolones ranged from 69.2% to 77.3% (data not published). Furthermore, the susceptibility rates for these agents were only 6.9% to 21.1% when multidrug-resistant (MDR; $n = 29$; 9.2%) isolates were evaluated. Cefepime was active against 24.1% of the MDR isolates whereas meropenem was only active against 55.2% of these isolates. In this prior study [14] only ceftazidime-avibactam inhibited all Enterobacterales isolates, including the 314 *E. cloacae*.

Antimicrobial therapies that prevent the emergence of resistance should be included in stewardship efforts to reduce the burden of antimicrobial resistance. In a study in which ESKAPE pathogens were exposed to increasing concentrations of various antimicrobial agents, the *E. cloacae* strain grew in cefepime concentrations 1000-fold higher than the MIC of the tested baseline isolate [19]. A similar observation was made for exposure to ciprofloxacin, indicating that it is not only AmpC expression that can be modulated in this species. The same authors noted that all isolates resistant to meropenem also displayed resistance to cefepime [19]; therefore, cefepime may not be considered an option for therapy once meropenem resistance occurs.

In the current study, five genetically distinct *E. cloacae* clinical isolates were exposed to cefepime, meropenem and ceftazidime-avibactam to assess how exposure to these agents impacts the MIC values for β-lactam and the resistance mechanisms that would emerge post-exposure. The isolates selected were susceptible to all three agents. The results indicate that exposure to cefepime or meropenem may generate isolates with higher MIC values when compared to ceftazidime-avibactam. Despite the differences noted in the level of resistance, cross-resistance to other β-lactams was observed with all three agents. Furthermore, cross-resistance with other antimicrobial classes occurred in one ceftazidime-avibactam post-exposure isolate due to alterations in efflux regulation.

Changes in the AmpC, OMP amino acid alterations, and efflux regulation were expected in most of the post-exposure isolates. These changes occurred in four isolates, three of which were from a single parent. The alterations were noted in two amino acid residues from the AmpC gene, in OmpC, and in the TetR/AcrR transcriptional regulator. In the remaining isolates, changes were observed in genes that have not been commonly associated with β-lactam resistance. Among these changes, some could be compensatory or spontaneous, but others could be involved with β-lactam resistance.

Changes in transcriptional regulator SlyA were noted in one isolate that was exposed to meropenem. This conferred higher MIC values for β-lactams and other antimicrobial classes. Alterations in genes involved in less common membrane transporting systems, including systems of the RND, ABC and CNNMs family transporters, were observed in multiple isolates. However, post-exposure isolates with alterations in these less common transporter genes only displayed elevated MIC results against the β-lactams.

One isolate displayed a premature stop in MioC, a gene involved in cell division [20], when exposed to the three agents. As replication and division are tightly coordinated in bacteria, cells with stalled division could became persistent in the presence of antimicrobial agents [21]. Alterations in genes that could stall other vital processes in the cell, such as protein synthesis, were also observed. There is limited evidence in the literature that these genes have an impact on antimicrobial resistance and further studies are needed to identify their role in β-lactam resistance.

The results from the current study indicate that the use of cefepime and meropenem to treat infections caused by *E. cloacae* could select for a population that has higher MIC results for

these agents. Moreover, cefepime, meropenem, and ceftazidime-avibactam selected isolates displaying cross-resistance to other β-lactams and, in some cases, cross-resistance to other antimicrobial classes due to the overexpression of efflux systems. These study findings are limited by the number of isolates tested and the differences that can be observed between in vitro assays and in vivo treatment. Further studies, including in vivo animal and clinical studies, need to be conducted to fully understand how these observations translate into clinical practice.

Acknowledgments

The authors would like to thank Brieanna Roth for performing the passaging experiments.

Declarations

Funding: This study was supported by AbbVie. AbbVie was involved in the design and decision to present these results and JMI Laboratories received compensation fees for services in relation to preparing the manuscript. Allergan (now AbbVie) was not involved in the collection, analysis, and interpretation of data.

Competing Interests: JMI Laboratories contracted to perform services in 2018-2020 for Achaogen, Inc., Affinity Biosensors, Albany College of Pharmacy and Health Sciences, Allecra Therapeutics, Allergan, Amicrobe Advanced Biomaterials, Inc., American Proficiency Institute, AmpliPhi Biosciences Corp., Amplyx Pharma, Antabio, Arietis Corp., Arixa Pharmaceuticals, Inc., Artugen Therapeutics USA, Inc., Astellas Pharma Inc., Athelas, Becton, Basilea Pharmaceutica Ltd., Bayer AG, Becton, Beth Israel Deaconess Medical Center, BIDMC, bioMerieux, Inc., bioMerieux SA, BioVersys Ag, Boston Pharmaceuticals, Bugworks Research Inc., CEM-102 Pharmaceuticals, Cepheid, Cidara Therapeutics, Inc., Cipla, Contrafect, Cormedix Inc., Crestone, Inc., Curza, CXC7, DePuy Synthes, Destiny Pharma, Dickinson and Company, Discuva Ltd., Dr. Falk Pharma GmbH, Emery Pharma, Entasis Therapeutics, Eurofarma Laboratorios SA, Fedora Pharmaceutical, F. Hoffmann-La Roche Ltd., Fimbrion Therapeutics, US Food and Drug Administration, Fox Chase Chemical Diversity Center, Inc., Gateway Pharmaceutical LLC, GenePOC Inc., Geom Therapeutics, Inc., GlaxoSmithKline plc, Guardian Therapeutics, Hardy Diagnostics, Harvard University, Helperby, HiMedia Laboratories, ICON plc, Idorsia Pharmaceuticals Ltd., IHMA, Iterum Therapeutics plc, Janssen Research & Development, Johnson & Johnson, Kaleido Biosciences, KBP Biosciences, Laboratory Specialists, Inc., Luminex, Matrivax, Mayo Clinic, Medpace, Meiji Seika Pharma Co., Ltd., Melinta Therapeutics, Inc., Menarini, Merck & Co., Inc., Meridian Bioscience Inc., Micromyx, Microchem Laboratory, MicuRx Pharmaceutics, Inc., Mutabilis Co., N8 Medical, Nabriva Therapeutics plc, National Institutes of Health, NAEJA-RGM, National University of Singapore, North Bristol NHS Trust, Novartis AG, Novome Biotechnologies, Oxoid Ltd., Paratek Pharmaceuticals, Inc., Pfizer, Inc., Pharmaceutical Product Development, LLC, Polyphor Ltd., Prokaryotics Inc., QPEX Biopharma, Inc., Ra Pharmaceuticals, Inc., Rhode Island Hospital, RIHML, Roche, Roivant Sciences, Ltd., Safeguard Biosystems, Salvat, Scynexis, Inc., SeLux Diagnostics, Inc., Shionogi and Co., Ltd., SinSa Labs, Specific Diagnostics, Spero Therapeutics, Summit Pharmaceuticals International Corp., SuperTrans Medical LT, Synlogic, T2 Biosystems, Taisho Pharmaceutical Co., Ltd., TenNor Therapeutics Ltd., Tetraphase Pharmaceuticals, The Medicines Company, The University of Queensland, Theravance Biopharma, Thermo Fisher Scientific, Tufts Medical Center, Universite de Sherbrooke, University of Colorado, University of Southern California-San Diego, University of Iowa, University of Iowa Hospitals and Clinics, University of North Texas Health Science Center, University of Wisconsin, UNT System College of Pharmacy, URMC, UT Southwestern, VenatoRx, Viosera Therapeutics, Vyome Therapeutics Inc., Wayne State University, Wockhardt, Yukon Pharmaceuticals, Inc., Zai Lab, and Zavante Therapeutics, Inc. There are no speakers' bureaus or stock options to declare.

Ethical Approval: Not required.

Sequence Information: Not applicable.

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