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Defining Baseline Mechanisms of Cefiderocol Resistance in the Enterobacterales

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The objective of this study was to identify putative mechanisms contributing to baseline cefiderocol resistance among carbapenem-resistant Enterobacterales (CRE). We evaluated 56 clinical CRE isolates with no previous exposure to cefiderocol. Cefiderocol and comparator agent minimum inhibitory concentrations (MICs) were determined by broth microdilution. Short-read and/or long-read whole genome sequencing was pursued. Cefiderocol nonwild type (NWT; *i.e.,* MICs ≥4 mg/L) CRE were compared with species-specific reference genomes and with cefiderocol wild type (WT) CRE isolates to identify genes or missense mutations, potentially contributing to elevated cefiderocol MICs. A total of 14 (25%) CRE isolates met cefiderocol NWT criteria. Of the 14 NWT isolates, various β-lactamases (*e.g.,* carbapenemases in *Klebsiella pneumoniae* and AmpC β-lactamases in *Enterobacter cloacae* complex) in combination with permeability defects were associated with a ≥ 80% positive predictive value in identifying NWT isolates. Unique mutations in the sensor kinase gene *baeS* were identified among NWT isolates. Cefiderocol NWT isolates were more likely to be resistant to colistin than WT isolates (29% vs. 0%). Our findings suggest that no consistent antimicrobial resistance markers contribute to baseline cefiderocol resistance in CRE isolates and, rather, cefiderocol resistance results from a combination of heterogeneous mechanisms.

Keywords: carbapenem-resistant Enterobacterales, cefiderocol, mechanisms of resistance

Introduction

The magnitude of carbapenem resistance in gram-negative bacteria has reached an alarming level.[1] In recent years, several new antibiotics have become available for clinical use; however, these new agents have a limited spectrum of activity compared with cefiderocol.[2]

Cefiderocol is a synthetic conjugate composed of a cephalosporin moiety to inhibit cell wall synthesis and a catechol-type siderophore moiety that gains entry into bacterial cells using active iron transporters, independent of porin channels, efflux pumps, or hydrolysis by carbapenemases.[3,4] Cefiderocol is the first siderophore-conjugated antibiotic to progress beyond phase 1 human trials. Commercial development of several previous siderophore antibiotic candidates was terminated early, primarily owing to adaptive resistance, presumably a consequence of downregulation of iron transport receptors because of competition with native siderophore production.[5] Available data suggest that adaptive resistance may be less of a concern for cefiderocol.[6–9]

Our insights into mechanisms of resistance to cefiderocol remain incomplete.[10] Although a number of mutations resulting in cefiderocol inactivity have been described in *Pseudomonas aeruginosa*, limited preclinical and even more limited clinical data suggest mutations specific to the Enterobacterales generally involve TonB-dependent receptors[11–17] or amino acid changes in the R2 loop of AmpC β-lactamases.[18,19] Furthermore, Enterobacterales producing New Delhi metallo-β-lactamase (NDM), generally have elevated cefiderocol minimum inhibitory concentrations (MICs), compared with Enterobacterales producing other carbapenemases.[5,20,21]

The objective of this study was to identify putative mechanisms contributing to baseline cefiderocol resistance among carbapenem-resistant Enterobacterales (CRE) isolates. Cefiderocol nonwild type (NWT) CRE isolates were compared with species-specific reference genomes and with cefiderocol wild type (WT) CRE isolates to identify genes or missense mutations leading to changes in amino acid composition, and potentially contributing to cefiderocol resistance.

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Methods

Isolate selection

Fifty-six consecutive clinical ertapenem-resistant Enterobacterales isolates obtained from unique patients hospitalized at The Johns Hopkins Hospital in 2017 were evaluated for *in vitro* activity against cefiderocol and comparator agents. None of the patients contributing isolates had previous exposure to cefiderocol. Isolates consisted of the following: *Citrobacter freundii* (2), *Enterobacter cloacae* complex (15), *Escherichia coli* (15), *Klebsiella aerogenes* (2), *Klebsiella oxytoca* (4), *Klebsiella pneumoniae* (15), and *Serratia marcescens* (3). Isolates were stored at −80°C in glycerol until further testing was performed. This study was approved by the Johns Hopkins University institutional review board.

Laboratory methods

Frozen isolates were subcultured twice to tryptic soy agar with 5% sheep blood. AST was determined using custom, lyophilized Sensititer broth microdilution (BMD) panels (Thermo Fisher Scientific, Waltham, MA), as previously described.[22] The BMD panel contained cefiderocol concentrations ranging from 0.03 to 64 mg/L and a proprietary chelator in the wells removing the requirement for iron-depleted cation-adjusted Mueller–Hinton broth.[23] Quality control organisms were performed each day of testing, including *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603, and *K. pneumoniae* ATCC 2814.

Because of differences in breakpoint interpretations between the various standards development organizations (*e.g.,* Clinical and Laboratory Standards Institute [CLSI], European Committee on Antimicrobial Susceptibility Testing [EUCAST], and United States Food and Drug Association [FDA]), the Enterobacterales epidemiologic cutoff value (ECV) was used to define isolates as wild type (WT; ≤2 mg/L) versus nonwild type (NWT; ≥4 mg/L).[24] The ECV divides microbial populations into those with (NWT) and without (WT) phenotypically detectable mechanisms of resistance. CLSI interpretive criteria were applied to most comparator agents (*i.e.,* ceftazidime-avibactam, meropenem-vaborbactam, imipenem-cilastatin-relebactam, colistin).[25] For agents where CLSI criteria were not available for the Enterobacterales (*i.e.,* tigecycline, eravacycline), FDA susceptibility criteria were applied.[26]

Resistance mechanism identification

Genomic DNA was extracted from the 56 CRE isolates using the DNeasy PowerBiofilm Kit (Qiagen, Inc., Valencia, CA). Whole genome sequencing (WGS) was pursued to identify cefiderocol resistance markers for the 56 isolates. WGS was conducted using Illumina MiSeq/HiSeq short-read sequencing (Illumina, San Diego, CA) and/or long-read Nanopore sequencing as previously described.[27] Whole genome assemblies were deposited to the SRA under bioproject PRJNA686978.

Fourteen of the 56 CRE isolates (25%) had cefiderocol MICs of ≥4 mg/L (*i.e.,* NWT). To characterize potential resistance mechanisms contributing to cefiderocol NWT results, the 14 cefiderocol NWT isolates were compared with their respective reference genomes (*e.g.,* a cefiderocol NWT *K. pneumoniae* genome was compared with *K. pneumoniae* ATCC 13883 reference genome) to identify genes and/or missense mutations resulting in changes to amino acid composition, with a focus on previously proposed cefiderocol resistance targets defined in the Enterobacterales (Table 1).

Sequenced isolates were evaluated using FASTQC v0.11.6 and MultiQC v1.6. Trimmomatic v0.39 removed adapters and trimmed low-quality paired-end reads. Trimmed and deduplicated reads (FastUniq v1.1) were *de novo* assembled with SPAdes v3.12.0 and annotated with Prokka v1.13. Quast v4.6.3 confirmed assembly quality. Genomic distances for cluster analysis were calculated with SourMash 2.0.0a. MUMmer3 v3.23 was used for pairwise differential genome analysis. Genes present in the clinical isolate but absent in the reference genome, or vice versa, as well as genes with point mutations were extracted for manual inspection. Gene annotations were determined with nucleotide BLAST v2.9.0+ against the reference genome for each species. Resistance genes were identified using ARESdb.[28] Isolate variant analysis was carried out with Snippy 4.6.0 against the reference genome for each species using default parameters. Intergenic and synonymous variants were removed. All bioinformatics analyses were performed by Ares Genetics.

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value of antimicrobial resistance markers was investigated by comparing cefiderocol NWT with WT isolates. The role of markers with a PPV of 80% or greater for identifying cefiderocol NWT isolates were investigated as putative mechanisms of cefiderocol resistance. Susceptibility profiles of antimicrobial agents and the presence of markers (genes and genetic variants) were compared among cefiderocol NWT and WT isolates using chi-square testing. Analyses were performed using GraphPad QuickCalcs. p-Values of ≤0.05 were considered significant.

Results

Description of cefiderocol wild type CRE isolates

Overall, cefiderocol MICs were ≤2 mg/L for 42 of the 56 isolates (75%). Eighteen of the 42 isolates (43%) were carbapenemase producing, most commonly because of the presence of a bla_{KPC} gene, except for two *E. coli* with $bla_{OXA\text{-}181}$, and a *S. marcescens* with a $bla_{SME\text{-}2}$. Susceptibility across the 42 cefiderocol WT isolates to other commercially available antimicrobial agents with anticipated activity against CRE were as follows: ceftazidime-avibactam (100%), meropenem-vaborbactam (98%), imipenem-relebactam (64%), tigecycline (100%), and eravacycline (86%). Although a CLSI susceptible category no longer exists for colistin, 100% of 39 cefiderocol WT isolates (excluding three *S. marcescens* owing to intrinsic resistance to the polymyxins) had colistin MICs ≤2 mg/L.

Description of cefiderocol nonwild type CRE isolates

The 14 cefiderocol NWT CRE isolates included the following: *K. pneumoniae* (7), *E. cloacae* complex (6) [including: *Enterobacter asburiae* (4), *Enterobacter hormaechei* (1), *Enterobacter kobei* (1)], and *C. freundii* (1) (Table 2).

Table 1. Previously Identified Mutations Contributing to Elevated Minimum Inhibitory Concentrations in the Enterobacterales to Cefiderocol and Other Siderophore-Conjugated Antibiotic Candidates

Target	*Organism(s)*	*Function*	*Description of findings*
tonB	*Escherichia coli*	Component of inner membrane protein complex providing energy to TonB-dependent transporters	*tonB* mutants had significantly decreased susceptibility to the siderophore-conjugated antibiotics KP-736,[16] BMS-180680,[11] E-0702,[17] pirazmonam,[15] and U-78,608[15]
cirA	*E. coli*, *Enterobacter cloacae*	Encodes receptor which preferentially transports catecholate siderophores	Double knockout of both *cirA* and *fiu* resulted in a 16-fold increase in cefiderocol MICs[12]; double mutants of *cirA* and *fiu* had decreased susceptibility to the siderophore-conjugated antibiotics KP-736,[16] BMS-180680,[11] pirazmonam,[15] and U-78,608[15]; heterogeneous mutations in the *cirA* gene conferred resistance to cefiderocol[13]
fiu	*E. coli*	Encodes receptor that preferentially transports catecholate siderophores	Double knockout of both *cirA* and *fiu* resulted in a 16-fold increase in cefiderocol MICs[12]; double mutants of *cirA* and *fiu* had decreased susceptibility to the siderophore-conjugated antibiotics KP-736,[16] BMS-180680,[11] pirazmonam,[15] and U-78,608[15]
baeS	*Klebsiella pneumoniae*	Encodes a sensor kinase protein of the two-component BaeSR signal transduction system reported to affect a variety of envelope stress response pathways.	Mutations in *baeS* increased cefiderocol MICs 32-fold.[14] Val295Gly, Thr279Pro and Thr200Pro associated with elevated cefiderocol MICs in mutants[44]
exbD	*K. pneumoniae*	TonB-dependent energy transduction system reported to affect the function of iron transporters	A Leu49frame shift mutation led to elevated cefiderocol MICs[44]
envZ	*K. pneumoniae*	Two-component transcriptional regulator reported to affect the expression of iron transporters	Val124Gly, Val147Gly, Ile152Asp, Leu18frame shift and Val54Gly mutations led to elevated cefiderocol MICs in mutants[44]
ompR	*K. pneumoniae*	Two-component transcriptional regulator reported to affect the expression of iron transporters	Met62Arg mutation led to elevated cefiderocol MICs in mutants[44]
yicM	*K. pneumoniae*	Unknown function	Mutations in Gly32ASP in two separate mutants led to elevated cefiderocol MICs[44]
ampC	*E. cloacae* complex	Chromosomal β-lactamase gene	Two amino acid deletion in the R2 loop of AmpC beta-lactamase (*i.e.,* alanine and leucine at positions 292 and 293, respectively) led to resistance to cefiderocol in two separate clinical *E. hormaechei* isolates[19]; alanine-proline deletion at positions 294 and 295 and leucine-to-valine substitution in position 296 increased cefiderocol MICs in an *E. cloacae* complex isolate[18]
bla_{NDM}	*K. pneumoniae* and *E. cloacae*	Carbapenemase enzyme	Resistance among *E. cloacae* and *K. pneumoniae* associated with the metallo-β-lactamase, NDM. When testing these isolates in combination with the beta-lactamase inhibitor dipicolonic acid, the cefiderocol MICs decreased[5]

MIC, minimum inhibitory concentration; NDM, New Delhi metallo-β-lactamase.

Table 2. Summary of Antimicrobial Resistance Genes and Chromosomal Mutations in 14 Enterobacterales Cefiderocol Nonwild Type Isolates

Organism	*FDC MIC (mg/L)*	*ST*	*Source*	*mCIM result*	*β-lactamase genes*	*Acquired resistance genes*	*Mutations in genes previously associated with cefiderocol resistance*	*Chromosomal mutations leading to amino acid changes*
K. pneumoniae	32	258	Skin and soft tissue	Negative	$bla_{CTX-M-2}$, bla_{OXA-2}, bla_{SHV-11}	*aac, ant, aph, dfrA12, cat1, sul1, sul2, chrA*	BaeS (A103S, P376A)	OmpK36 (E232R, F207Y, T222L, L59V, L229A, F198Y, N304E, A217S, D223G, G189T), OmpK37 (I70M, I128M, N230G, M233Q, R239K, E244D, N274S, V277I), PmrB (R256G), GyrA (F83I), GyrB (E466D), ParC (S80I, T57S), AcrR (R173G, F197I, P161R, F172S, L195V, K201M, G164A), CycA (S263A), GlpT (G206A), UhpT (E350Q)
K. pneumoniae	32	258	Stool	Positive	bla_{KPC-2}, bla_{OXA-9}, bla_{SHV-40}, bla_{TEM-1}	*aac, aad, aph, cat1, dfrA12, oqxA/B, sul1, chrA*	BaeS (A103S, P376A)	OmpK36 (E232R, F207Y, T222L, L59V, L229A, F198Y, N304E, A217S, D223G, G189T), OmpK37 (I70M, I128M, N230G, M233Q, R239K, E244D, N274S, V277I), PmrB (R256G), GyrA (F83I), GyrB (E466D), ParC (S80I, T57S), AcrR (R173G, F197I, P161R, F172S, L195V, K201M, G164A), CycA (S263A), GlpT (G206A), UhpT (E350Q), BamB (A176T)
K. pneumoniae	16	258	Urine	Positive	bla_{KPC-3}, bla_{SHV-12}, bla_{TEM-1}	*aac, cat1, dfrA14, mphA, qnRS7, oqxA/B, chrA*	BaeS (A103S, P376A)	OmpK36 (D223G, G189Y, E232R, T222L, L59V, L229A, F198Y, N304E, F207Y, A217S), OmpK37 (I70M, I128M, N230G, M233Q, R239K, E244D, N274S, V277I), PmrB (R256G), GyrA (F83I), ParC (S80I, T57S), AcrR (R173G, F197I, P161R, F172S, L195V, K201M, G164A), CycA (S263A), GlpT (G206A), UhpT (E350Q), BamB (A176T)

(*continued*)

TABLE 2. (CONTINUED)

Organism	FDC MIC (mg/L)	ST	Source	mCIM result	β-lactamase genes	Acquired resistance genes	Mutations in genes previously associated with cefiderocol resistance	Chromosomal mutations leading to amino acid changes
K. pneumoniae	4	231	Blood	Positive	bla_{CMY-59}, $bla_{CTX-M-15}$, bla_{NDM-1}, $bla_{OXA-181}$, bla_{SHV-1}, bla_{TEM-1}	*rmtF, aac, aad, aph, arb, brp(MBL), catB, dfrA12, ermB, mphA, qnrB1, sul1, sul2, sugE, chrA*	BaeS (A419V), YicM (I262V, T299M)	OmpK36 (E232R, L59V, A217S, D223G, N304E, F207Y, N218H), OmpK37 (I70M, I128M, N230G, M233Q, R239K, E244D, N274S, V277I), GyrA (F83I), ParC (S80I, T57S), AcrR (R173G, F197I, P161R, F172S, L195V, K201M, G164A), CycA (S263A), GlpT (G206A), UhpT (E350Q), BamB (A176T)
K. pneumoniae	4	231	Urine	Positive	bla_{CMY-59}, $bla_{CTX-M-15}$, bla_{NDM-1}, $bla_{OXA-181}$, bla_{SHV-1}, bla_{TEM-1}	*rmtF, aac, aad, aph, arb, brp(MBL), catB, dfrA12, ermD, mphA, qnrB1, sul2, sugE*	BaeS (A419V), YicM (I262V, T299M)	OmpK36 (E232R, L59V, A217S, D223G, N304E, F207Y, N218H), OmpK37 (I70M, I128M, N230G, M233Q, R239K, E244D, N274S, V277I), GyrA (F83I), ParC (S80I, T57S), AcrR (R173G, F197I, P161R, F172S, L195V, K201M, G164A), CycA (S263A), GlpT (G206A), UhpT (E350Q), BamB (A176T)
K. pneumoniae	4	307	Respiratory	Positive	$bla_{CTX-M-15}$, bla_{OXA-1}, bla_{SHV-28}, bla_{TEM-1}	*aac, aph, catB3, dfrA14, tetA, qnrB1, sul2*	BaeS (F3L, A103T, D106N, S192N)	OmpK37 (I70M, I128M, N230G, M233Q, R239K, E244D, N274S, V277I), GyrA (F83I), ParC (S80I, T57S), AcrR (R173G, F197I, P161R, F172S, L195V, K201M, G164A), CycA (S263A), GlpT (G206A), UhpT (E350Q), BamB (A176T)
K. pneumoniae	4	258	Stool	Positive	bla_{SHV-11}, bla_{TEM-1}, bla_{OXA-9}, bla_{KPC-2}	*aac, aad, aph, dfrA12, cat1, sul1, oqxA/B, chrA*	BaeS (A103S, P376A)	OmpK36 (E232R, F207Y, T222L, L59V, L229A, F198Y, N304E, A217S, D223G, G189T), OmpK37 (I70M, I128M, N230G, M233Q, R239K, E244D, N274S, V277I), PmrB (R256G), GyrA (F83I), GyrB (E466D), ParC (S80I, T57S), AcrR (R173G, F197I, P161R, F172S, L195V, K201M, G164A), CycA (S263A), GlpT (G206A), UhpT (E350Q), BamB (A176T)

(*continued*)

Table 2. (Continued)

Organism	*FDC MIC (mg/L)*	*ST*	*Source*	*mCIM result*	*β-lactamase genes*	*Acquired resistance genes*	*Mutations in genes previously associated with cefiderocol resistance*	*Chromosomal mutations leading to amino acid changes*
Enterobacter hormaechei	64	1296	Blood	Negative	bla_{TEM-1}, bla_{ACT-70}	*dfrA12, oqxA/B, sul1, sul2, romA*		ParC (S57T), ParE (I355T), CycA (263A), GlpT (G206A, Q444E), UhpT (E350Q), SetB (I307V), 5' OmpC truncation
Enterobacter asburiae	16	24	Urine	Negative	bla_{ACT-1}	*mcr-10, oqxA/B*		GyrA (S83T), ParC (S57T), ParE (I355T), CycA (263A), GlpT (Q444E), UhpT (E350Q), SetB (I307V), OmpC Δ AA 30–75
E. asburiae	8	1056	Stool	Negative	bla_{MIR-8}	*oqxA/B, romA*		ParC (S57T), ParE (I355T), CycA (263A), GlpT (G206A, Q444E), UhpT (E350Q), SetB (I307V)
E. asburiae	4	807	Intra-abdominal	Negative	bla_{ACT-4}	*mcr-10, oqxA/B, romA*		ParC (S57T), ParE (I355T), CycA (263A), GlpT (Q444E), UhpT (E350Q), SetB (I307V), OmpC Δ AA 35–75
E. asburiae	4	Novel	Urine	Negative	bla_{ACT-4}, bla_{RomA}	*oqxA/B*		GyrA (S83T), ParC (S57T), ParE (I355T), CycA (263A), GlpT (Q444E), UhpT (E350Q), SetB (I307V)
Enterobacter kobei	4	125	Urine	Negative	bla_{ACT-28}	*mcr-10, oqxA/B, romA*		ParC (S57T), ParE (I355T), CycA (263A), GlpT (Q444E), UhpT (E350Q), SetB (I307V)
Citrobacter freundii	8	Novel	Intra-abdominal	Negative	bla_{CMY-59}	*qnrB38*		OmpC 5′ truncation, GyrA (S83T), ParC (S57T), SoxR (R4K, T38S), cycA (S263A), GlpT (G206A, E448K), UhpT (E350Q)

FDC, cefiderocol; mCIM, modified carbapenem inactivation method; ST, sequence type.

Six of the NWT CRE isolates harbored carbapenemases genes, including bla_{NDM} and $bla_{OXA\text{-}181}$ [3] and bla_{KPC} [3]. All carbapenemase-producing genes in NWT CRE were identified in *K. pneumoniae*.

Susceptibility across the 14 isolates to other CRE-active antibiotics were as follows: ceftazidime-avibactam (86%), meropenem-vaborbactam (71%), imipenem-relebactam (50%), tigecycline (93%), and eravacycline (79%). Seventy-one percent of cefiderocol NWT isolates had colistin MICs ≤2 mg/L.

Chromosomal mutations and acquired resistance genes potentially contributing to cefiderocol resistance

Initially, antimicrobial resistance genes known to contribute to cefiderocol resistance were explored among NWT isolates (Tables 1 and 2). Mutations leading to amino acid changes in BaeS were present in 7 of 7 (100%) NWT *K. pneumoniae* isolates and only 1 of 8 (13%) WT *K. pneumoniae* isolates, as detailed further in Table 2. Mutations in *yicM* leading to amino acid substitutions (I262V and T299M) were present in 2 (29%) NWT isolates; however, substitutions in YicM were also present in 6 (25%) WT isolates. No mutations in *envZ* leading to amino acid substitutions were observed among NWT isolates, whereas two WT *K. pneumoniae* had mutations in *envZ* resulting in amino acid substitutions at D188Y and A443T. *ompR* and *exbD* were 100% conserved among all *K. pneumoniae* isolates. All three *K. pneumoniae* isolates harboring bla_{NDM} and $bla_{OXA\text{-}181}$ had elevated cefiderocol MICs, ranging from 4 to 16 μg/mL. Mutations within the chromosomal *ampC* genes of *E. cloacae* complex isolates were not observed, as previously described (Table 1).

Mutations and/or antimicrobial resistance genes identified among cefiderocol NWT isolates that may contribute to cefiderocol resistance in comparison with a respective reference genome were then explored. As all included isolates were multidrug resistant, an extensive list of antimicrobial resistance genes were identified, including porin mutations, efflux pumps, and β-lactamase genes (Table 2). Cefiderocol NWT to WT isolates were compared to identify resistance markers with at least 80% PPV in identifying NWT *K. pneumoniae* isolates (Table 3). There were seven NWT and eight WT *K. pneumoniae* isolates. β-lactamases were associated with a high PPV in identifying NWT *K. pneumoniae* including AmpC β-lactamases (*e.g., $bla_{CMY\text{-}59}$*) and carbapenemases (*e.g., $bla_{NDM\text{-}1}$* and $bla_{OXA\text{-}181}$), all in conjunction with permeability defects (*e.g.,* mutations associated with *ompK36* and *ompK37*). Each NWT isolate harbored between 3 and 6 β-lactamase genes; 6 (86%) of 7 NWT *K. pneumoniae* were carbapenemase producers (including bla_{KPC} and the combination of $bla_{NDM\text{-}1}$ and $bla_{OXA\text{-}181}$). This is in contrast to WT isolates that harbored between 1 and 4 β-lactamase genes and only 4 (50%) WT *K. pneumoniae* were carbapenemase producers (limited to KPC production). Two efflux transporters (*i.e., sugE* and *chrA*) also had high PPVs in identifying cefiderocol NWT isolates in *K. pneumoniae*.

The *chrA* gene encoding a chromate ion transporter was identified among 5 NWT (71%) and 1 WT (13%) isolate. On further inspection of 4 NWT isolates harboring *chrA,* where hybrid short- and long-read assemblies were available, we found the gene was harbored on a IncFIB/IncFII plasmid within an iron operon (*fec*IRABCDE) for three isolates and on an IncM1 plasmid not associated with the iron operon in a single isolate. Various other genes linked with resistance to non-β-lactam agents frequently carried on plasmids

Table 3. The Association of Antimicrobial Resistance Markers Identified in Carbapenem-Resistant *Klebsiella pneumoniae* Isolates with Cefiderocol Minimum Inhibitory Concentrations of 4 mg/L or Higher, Only Markers with at Least an 80% Positive Predictive Value for Identifying Cefiderocol Nonwild Type Isolates Are Shown

Antimicrobial resistance marker	*Definition*	*TN* (n)	*FP* (n)	*FN* (n)	*TP* (n)	*Sensitivity* (%)	*Specificity* (%)	*PPV* (%)	*NPV* (%)
K. pneumoniae									
AadA2	Aminoglycoside adenyltransferase	8	0	3	4	57.1	100	100	72.7
CatI	Chloramphenicol acetyltransferases	8	0	3	4	57.1	100	100	72.7
DfrA12	Dihydrofolate reductase	8	0	3	4	57.1	100	100	72.7
DNA_topoisomerase_subunit_gyrB|E466D	DNA gyrase	8	0	4	3	42.9	100	100	66.7
ChrA	Heavy metal ion transporter	7	1	2	5	71.4	87.5	83.3	77.8
RmtF	16S rRNA methyltransferase	8	0	5	2	28.6	100	100	61.5
AAC(6′)-Ib-Hangzhou	Aminoglycoside acetyltransferase	8	0	5	2	28.6	100	100	61.5
APH(3′)-VI	Aminoglycoside 3′-O-phosphotransferase	8	0	5	2	28.6	100	100	61.5
CMY-59	AmpC β-lactamase	8	0	5	2	28.6	100	100	61.5
NDM-1	NDM metallo-β-lactamase	8	0	5	2	28.6	100	100	61.5
OXA-181	OXA carbapenemase	8	0	5	2	28.6	100	100	61.5
BRP(MBL)	Bleomycin-resistant protein	8	0	5	2	28.6	100	100	61.5
CatB	Chloramphenicol acetyltransferases	8	0	5	2	28.6	100	100	61.5
SugE	Putative efflux transport	8	0	5	2	28.6	100	100	61.5

FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive.

carrying β-lactamase genes (*e.g., aad2, cat1, dfrA12, rmtF, aac, aph, brp,* and *catB*) as well as chromosomal mutations commonly associated with successful multidrug-resistant clones (*e.g., gyrB* mutations among ST258)[29,30] also had a high PPV of being associated with NWT isolates. Comparisons between NWT and WT isolates were not performed between *C. freundii* and *E. cloacae* complex isolates because of the small number of comparators at the species level.

Resistance genes associated with colistin resistance among cefiderocol NWT isolates

As colistin resistance (*i.e.,* MICs ≥4 μg/mL) was more commonly identified with cefiderocol NWT versus WT isolates at 29% versus 0%, mechanisms of colistin resistance were further evaluated (Table 2). An R256G substitution in the sensor kinase, PmrB (also known as BasS[31]), was identified in 4 (57%) *K. pneumoniae* NWT isolates, whereas 3 (38%) *K. pneumoniae* WT isolates harbored this mutation.

Discussion

Cefiderocol remains an intriguing compound with great promise for clinical efficacy against a broad range of aerobic, gram-negative pathogens. Surveillance studies of isolates with no preceding cefiderocol exposure indicate cefiderocol activity against the Enterobacterales approaches 100%.[32–38] Surveillance studies specifically evaluating cefiderocol activity against CRE are limited but estimate between 74% and 100% of isolates have cefiderocol MICs ≤4 mg/L, in the absence of preceding cefiderocol exposure.[32,38,39] Large surveillance studies of cefiderocol activity against CRE isolates since the clinical introduction of this agent are not yet available. Using a cohort of clinical CRE isolates from patients without prior cefiderocol exposure, we sought to identify potential mechanisms contributing to elevated cefiderocol MICs. In our cohort of 56 CRE isolates, 25% met criteria as cefiderocol NWT (≥4 mg/L).

We were unable to identify consistent mechanisms leading to elevated cefiderocol MICs among NWT CRE isolates. However, a combination of β-lactamase production and permeability defects were present in all NWT CRE isolates. This is somewhat analogous to noncarbapenemase-producing CRE where the combination of ESBL/AmpC β-lactamase production with reduced membrane permeability leads to carbapenem resistance.[40]

All three *K. pneumoniae* isolates producing NDMs had cefiderocol MICs in the NWT range. This is in agreement with other studies indicating an association of NDM carbapenemases with elevated MICs.[5,20,21] All NWT isolates had outer membrane porin mutations. Similar findings were identified in a study by Rolston *et al.* where 20% of CRE isolates had cefiderocol MICs of 4 mg/L or higher.[38] These investigators were also unable to identify a clear mechanism of resistance contributing to elevated cefiderocol MICs, but observed a predominance of outer membrane porin disruption in combination with various β-lactamases.[38] We also identified two efflux transporters to be associated with 80% PPV among cefiderocol NWT *K. pneumoniae.* One of these was a plasmid-mediated heavy metal ion transporter (ChrA; chromate ion transporter) was associated with cefiderocol NWT isolates. Combining these findings, membrane permeability defects in the presence of β-lactamase production appear to be necessary, although not always sufficient, in contributing to elevated cefiderocol MICs. It has been hypothesized that the bulky chlorocatechol side chain at position C3 of cefiderocol provides steric hindrance and reduces its capacity to enter efflux pumps.[12,41] However, our findings suggest that upregulation of efflux pumps may contribute to cefiderocol resistance in *K. pneumoniae*.

We also found that cefiderocol NWT isolates were more likely to have elevated polymyxin and meropenem-vaborbactam MICs compared with WT isolates. Meropenem-vaborbactam cross-resistance is not surprising, as the three NDM-producing isolates would not be expected to be inactivated by this agent. Polymyxin resistance, however, is more perplexing. Polymyxin resistance most commonly occurs because of a reduction in the net negative charge of the gram-negative cell wall.[42] We observed PmrB mutations among 57% of NWT *K. pneumoniae* isolates compared with 38% of WT *K. pneumoniae* isolates. Similarly, we identified the plasmid-mediated *mcr*-10 gene among half of the NWT *E. cloacae* complex isolates and in no WT isolates. Although cefiderocol has a net −1 physiologic charge, its antibacterial activity is enhanced by a positively charged cyclic quaternary ammonium moiety on the C3 side chain allowing better orientation toward the negatively charged inner membrane of the gram-negative cell wall.[43] It is possible that a further reduced negative charge associated with colistin resistance may impact cefiderocol activity.[43] Future studies are required to evaluate this hypothetical mechanism of cross-resistance.

By evaluating previously described genes contributing to cefiderocol resistance, we identified unique mutations in the *baeS* gene among the cefiderocol NWT isolates in our cohort. Kohira *et al.* described 32-fold increases in cefiderocol MICs against *K. pneumoniae* owing to mutations in the *baeS* gene, responsible for encoding a sensor kinase protein of the two-component BaeSR signal transduction system.[14] BaeSR may affect envelope stress response pathways; however, the particular genes impacting cefiderocol activity have not been identified.[44] Furthermore, we were unable to identify mutations in *tonB*, *exbD*, envZ, or *ompR* associated with elevated cefiderocol MICs among our NWT CRE isolates—underscoring the heterogeneity in antimicrobial resistance markers that may contribute to elevated cefiderocol MICs. Of importance, these mutations may be more common after exposure to cefiderocol, and uncommon in isolates naive to cefiderocol exposure, as in our study.

There are a number of limitations to this study. This is hypothesis-generating work and our goal was to explore mutational patterns among cefiderocol-resistant CRE. Future molecular studies are needed to confirm the significance of specific mutations we identified in contributing to cefiderocol resistance. Second, an understanding of all the necessary components of the cefiderocol ferric iron complex transport system is incomplete and other cefiderocol-specific transport determinants likely exist—particularly for Enterobacterales other than *E. coli*. In addition, our cohort was small and from one geographic region. Larger studies with isolates from more diverse regions are needed to evaluate the generalizability of our findings. Finally, WGS is unable to detect mechanisms of resistance associated with changes in expression or other post-transcriptional alterations in

structure, dynamics, and substrate specificity of proteins, enzymes, and cell wall components.[45] These limitations notwithstanding, this is the largest study to date exploring potential baseline mechanisms of resistance to cefiderocol among the Enterobacterales.

In summary, we found that 25% of CRE isolates had cefiderocol MICs ≥4 mg/L without previous exposure to this agent. Evaluating the 14 isolates exhibiting cefiderocol NWT MICs further, we identified heterogeneous mechanisms that include the combination of β-lactamase production and permeability defects contributing to elevated cefiderocol MICs. As cefiderocol is prescribed with increasing frequency, an understanding of the true risks of emergence of resistance will become more apparent.

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