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Letter to the Editor

Cefiderocol heteroresistance in *Klebsiella pneumoniae* is linked to mutations in the siderophore receptor *cirA* and β-lactamase activities

Editor Dr AO Olaitan

Cefiderocol is a novel siderophore-conjugated cephalosporin with potent activity against carbapenem-resistant pathogens. The siderophore molecule has greater outer membrane penetration using the active iron uptake system for cell entry. Cefiderocol is reserved for the treatment of infections in patients caused by multidrug-resistant Gram-negative bacilli with limited treatment options. However, cefiderocol-non-susceptible isolates have been reported in clinal isolates from surveillance studies. Reduced susceptibility could be associated with β-lactamases as well as other factors [1]. Deficiency in iron transporters has been reported to be associated with cefiderocol resistance in *Acinetobacter baumannii* [2], *Pseudomonas aeruginosa* and *Escherichia coli* [3].

Antimicrobial heteroresistance describes a phenomenon where subpopulations of genetically homogeneous bacteria exhibit a range of susceptibilities to a particular antibiotic. Heteroresistance has considerable clinical relevance because antibiotic treatment may select for more resistant populations. Heteroresistance is considered an important factor contributing to unexplained antibiotic treatment failure. Widespread cefiderocol heteroresistance has been reported in carbapenem-resistant Gram-negative pathogens [4]. However, there is a lack of thorough research studying the mechanisms of cefiderocol heteroresistance.

We tested the susceptibility of cefiderocol (30 μg; Hardy Diagnostics) using the disk diffusion method against 80 isolates in the Gram-negative Carbapenemase Detection panel from the CDC & FDA Antibiotic Resistance Isolate Bank. Cefiderocol exhibited potent efficacy against the majority of carbapenem-resistant strains in the panel. We identified several cefiderocol-non-susceptible *Klebsiella pneumoniae* isolates (Supplementary Table S1). Moreover, in the cefiderocol disk diffusion assay, scattered colonies appeared in the inhibition areas for *K. pneumoniae* AR 0097 (Fig. 1A), which is an indication of heteroresistance to cefiderocol. This study focused on the cefiderocol-heteroresistant strain *K. pneumoniae* AR 0097 because there is a lack of mechanistic studies regarding cefiderocol heteroresistance. *K. pneumoniae* AR 0097 belongs to sequence type ST3603 as determined by multilocus sequence typing using MLST 2.0 (https://cge.food.dtu.dk/services/MLST/). Antimicrobial resistance genes in *K. pneumoniae* AR 0097 were identified using Resistance Gene Identifier (RGI) (https://card.mcmaster.ca/analyze/rgi). The resistance genes include $bla_{\text{TEM-1}}$, $bla_{\text{OXA-9}}$, $bla_{\text{KPC-3}}$, $bla_{\text{SHV-11}}$, *sul1, sul2, dfrA12, dfrA14, aac(6′)-Ib, aadA1, aadA2, aph(6)-Id, aph(3′′)-Ib, fosA6* and several antibiotic efflux genes. We used population analysis profiling (PAP) assays to confirm cefiderocol heteroresistance in *K. pneumoniae* AR 0097. PAP assays determined the proportion of the number of resistant colonies on agar plates with cefiderocol at various concentrations against the number of colonies on agar plates without the antibiotic [5]. Cefiderocol at the susceptibility breakpoint (4 μg/mL) inactivated 3.4 log of *K. pneumoniae* AR 0097. However, a resistant subpopulation survived cefiderocol treatment at 32 μg/mL (8-fold the breakpoint). In contrast, cefiderocol at 4 μg/mL resulted in a >5 log reduction in two susceptible strains (*K. pneumoniae* AR 0039 and *K. pneumoniae* ATCC 13883) (Fig. 1B).

To investigate the heteroresistance mechanism in *K. pneumoniae* AR 0097, three colonies collected from the inhibition zone after disk diffusion assay were isolated and characterised. We first determined the cefiderocol minimum inhibitory concentration (MIC) in these three derivatives in iron-depleted cation-adjusted Mueller–Hinton broth (ID-CAMHB) according to Clinical and Laboratory Standards Institute (CLSI) procedures. ID-CAMHB is needed for accurate MIC testing because cefiderocol uses an active iron uptake system for cell entry, and iron transporters are upregulated under iron-depleted conditions. These three derivatives showed an increased cefiderocol MIC compared with the wild-type (Table 1). To identify the genetic determinants responsible for the increased cefiderocol MIC, we sequenced the whole genomes of these resistant isolates using the Illumina platform. We evaluated single nucleotide variants among the wild-type and three resistant isolates using assembly- and read-based approaches. Briefly, we used SPAdes v.3.15.3 for genome assembly, BWA v.0.7.17 for mapping reads, FreeBayes v.1.3.4 for variant calling, and IGV v.2.3 for genome browsing and visualisation. Single nucleotide polymorphism (SNP) variant analysis identified frameshift or missense mutations in the siderophore receptor *cirA* in all three resistant isolates (Table 1).

We further confirmed the function of *cirA* using genetic complementation by cloning the functional *cirA* from wild-type *K. pneumoniae* AR 0097 in a vector pCR™-Blunt II-TOPO™ (Invitrogen), followed by transferring into the resistant mutants by electroporation and selection with 400 μg/mL zeocin. Three resistant mutants complemented with the wild-type *cirA* showed a substantial decrease in cefiderocol MIC (Table 1). The results indicated that *cirA* mutations contributed to cefiderocol heteroresistance in *K. pneumoniae* AR 0097. CirA is a TonB-dependent outer membrane siderophore receptor protein [6]. Mutations in *cirA* may reduce the transportation of cefiderocol into *K. pneumoniae* AR 0097, resulting in the increase of cefiderocol resistance in the resistant subpopulation. A recent study showed that serial passage of an NDM-producing *K. pneumoniae* in increasing concentrations of cefiderocol resulted in functional loss of *cirA* and thus cefiderocol resistance [7]. Mutations in *cirA* may have important clinical implications in the development of cefiderocol resistance. Klein et al. reported a clinical case of the rapid development of cefiderocol resistance in a carbapenemase-producing *Enterobacter cloacae* strain in a patient within 21 days of cefiderocol therapy. Comparative

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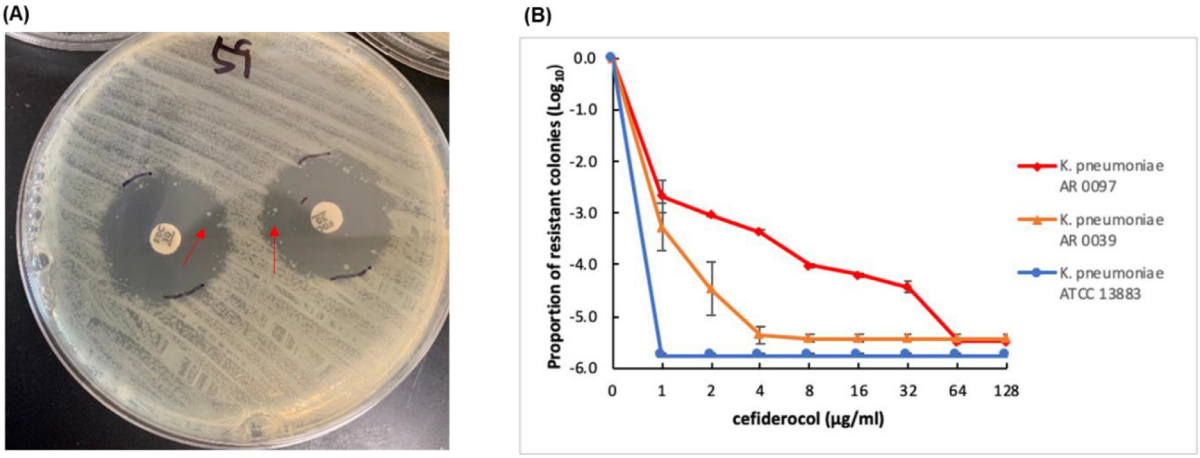

Fig. 1. Cefiderocol heteroresistance determination in *Klebsiella pneumoniae*. (A) Disk diffusion assay (30 μg; Hardy Diagnostics) for *K. pneumoniae* AR 0097. Red arrows indicate the scattered colonies in the zone of inhibition. (B) Population analysis profiling for *K. pneumoniae* AR 0097 and two susceptible *K. pneumoniae* strains.

Table 1
Mutations in the *cirA* gene contribute to cefiderocol heteroresistance in *Klebsiella pneumoniae* AR 0097

Strain	*cirA* genotype	Cefiderocol MIC (μg/mL)
AR 0097	Wild-type	4–8
AR 0097 Δ*cirA*_1	Frameshift mutation (1461delC) in *cirA*	64
AR 0097 Δ*cirA*_2	Frameshift mutation (475dupA) in *cirA*	64
AR 0097 Δ*cirA*_3	Missense mutation (1028A>C) in *cirA*	32
AR 0097 Δ*cirA*_1+pCR*cirA*	AR 0097 Δ*cirA*_1 complemented with wild-type *cirA*	2–8
AR 0097 Δ*cirA*_2+pCR*cirA*	AR 0097 Δ*cirA*_2 complemented with wild-type *cirA*	2–8
AR 0097 Δ*cirA*_3+pCR*cirA*	AR 0097 Δ*cirA*_3 complemented with wild-type *cirA*	1–4

MIC, minimum inhibitory concentration.

genomics analysis suggested that mutations in *cirA* in the *E. cloacae* strain conferred phenotypic cefiderocol resistance [8]. However, no truncation or similar mutations in the *cirA* gene were found in *K. pneumoniae* AR0047, *K. pneumoniae* AR0087, *K. pneumoniae* AR0041 and *K. pneumoniae* AR0106.

There are four β-lactamases genes ($bla_{\text{TEM-1}}$, $bla_{\text{OXA-9}}$, $bla_{\text{KPC-3}}$ and $bla_{\text{SHV-11}}$) in the genome of *K. pneumoniae* AR 0097 (accession no. **GCF_003571655.1**). Each of the four identified β-lactamase gene in *K. pneumoniae* AR 0097 was cloned into a pCR™-Blunt II-TOPO™ vector, followed by transformation into *E. coli* cells. The *E. coli* cells carrying $bla_{\text{KPC-3}}$ and $bla_{\text{SHV-11}}$ showed an increase in cefiderocol MIC compared with the control strain *E. coli* TOP10 (Supplementary Table S2). The results suggested that the combination of two β-lactamases may contribute to the baseline cefiderocol non-susceptibility in *K. pneumoniae* AR 0097. To further confirm the impact of β-lactamases on cefiderocol susceptibility in *K. pneumoniae* AR 0097, we determined the bactericidal activity of cefiderocol in vitro with or without the β-lactamase inhibitor avibactam at a fixed concentration of 4 μg/mL. When used alone, cefiderocol or avibactam did not effectively inhibit the growth of *K. pneumoniae* AR 0097. In the presence of avibactam at 4 μg/mL, cefiderocol at $\geq$8 μg/mL eradicated almost all bacterial cells (Supplementary Fig. S1). The results suggested that β-lactamases contributed to cefiderocol non-susceptibility in *K. pneumoniae* AR 0097.

In summary, cefiderocol heteroresistance in *K. pneumoniae* AR 0097 could be multifactorial and is linked to mutations in the catecholate siderophore receptor *cirA* and β-lactamase activities. Cefiderocol heteroresistance may present a challenge for the treatment of infections because these isolates are already resistant to most available antibiotics. The combination of cefiderocol with a β-lactamase inhibitor or other antibiotics may enhance the treatment outcome.

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Competing interests

None.

Ethical approval

Not required.

Sequence information

The whole-genome shotgun project has been deposited at GenBank under the accession no. **PRJNA851446**.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2022.106635.

References

[1] Kohira N, Hackel MA, Ishioka Y, Kuroiwa M, Sahm DF, Sato T, et al. Reduced susceptibility mechanism to cefiderocol, a siderophore cephalosporin, among clinical isolates from a global surveillance programme (SIDERO-WT-2014). J Glob Antimicrob Resist 2020;22:738–41. doi:10.1016/j.jgar.2020.07.009.

[2] Malik S, Kaminski M, Landman D, Quale J. Cefiderocol resistance in *Acinetobacter baumannii*: roles of β-lactamases, siderophore receptors, and penicillin binding protein 3. Antimicrob Agents Chemother 2020;64 e01221-20. doi:10.1128/AAC.01221-20.

[3] Ito A, Sato T, Ota M, Takemura M, Nishikawa T, Toba S, et al. In vitro antibacterial properties of cefiderocol, a novel siderophore cephalosporin, against Gram-negative bacteria. Antimicrob Agents Chemother 2018;62 e01454-17. doi:10.1128/AAC.01454-17.

[4] Choby JE, Ozturk T, Satola SW, Jacob JT, Weiss DS. Widespread cefiderocol heteroresistance in carbapenem-resistant Gram-negative pathogens. Lancet Infect Dis 2021;21:597–8. doi:10.1016/S1473-3099(21)00194-8.

[5] Sherman EX, Wozniak JE, Weiss DS. Methods to evaluate colistin heteroresistance in *Acinetobacter baumannii*. Methods Mol Biol 2019;1946:39–50. doi:10.1007/978-1-4939-9118-1_4.

[6] Noinaj N, Guillier M, Barnard TJ, Buchanan SK. TonB-dependent transporters: regulation, structure, and function. Annu Rev Microbiol 2010;64:43–60. doi:10.1146/annurev.micro.112408.134247.

[7] McElheny CL, Fowler EL, Iovleva A, Shields RK, Doi Y. In vitro evolution of cefiderocol resistance in an NDM-producing *Klebsiella pneumoniae* due to functional loss of CirA. Microbiol Spectr 2021;9:e0177921. doi:10.1128/spectrum.01779-21.

[8] Klein S, Boutin S, Kocer K, Fiedler MO, Störzinger D, Weigand MA, et al. Rapid development of cefiderocol resistance in carbapenem-resistant *Enterobacter cloacae* during therapy is associated with heterogeneous mutations in the catecholate siderophore receptor *cirA*. Clin Infect Dis 2022;74:95–8. doi:10.1093/cid/ciab511.

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