Emergence of High-Level Cefiderocol Resistance in Carbapenem-Resistant Klebsiella pneumoniae from Bloodstream Infections in Patients with Hematologic Malignancies in China

Peng Lan,Ye Lu,Zhongju Chen,Xueqing Wu,Xiaoting Hua,Yan Jiang,Xiaoting Hua,Yan Jiang,Jiancang Zhou,Yunsong Yu

Department of Critical Care Medicine, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China
Key Laboratory of Microbial Technology and Bioinformatics of Zhejiang Province, Hangzhou, China
Regional Medical Center for National Institute of Respiratory Diseases, Sir Run Run Shaw Hospital, Zhejiang University of Medicine, Hangzhou, China
Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
Department of Infectious Diseases, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China

Peng Lan, Ye Lu, and Zhongju Chen contributed equally to this article. Author order was determined alphabetically.

ABSTRACT Cefiderocol is a novel siderophore cephalosporin exhibiting potent antimicrobial activities. Although cefiderocol has not been approved in China, resistance is emerging. A multicenter study was performed to evaluate the cefiderocol resistance in carbapenem-resistant Klebsiella pneumoniae (CRKP) strains from bloodstream infections in patients with hematologic malignancies in China. Clinical data analysis and whole-genome sequencing were conducted for collected cefiderocol-resistant CRKP strains. CRISPR-Cas9 system was employed to construct site-specific mutagenesis for gene cirA. Plasmid curing and cloning were performed to assess the effect of β-lactamases on cefiderocol resistance. Total 86 CRKP strains were collected. The MICs of cefiderocol ranged from 0.06 to 256 mg/L. Among four cefiderocol-nonsusceptible strains (4/86, 4.7%), two cefiderocol-resistant strains AR8538 (MIC = 32 mg/L) and AR8416 (MIC > 256 mg/L) were isolated from two patients with acute lymphocytic leukemia (frequency of resistance, 2/86, 2.3%). Metallo- and serine-β-lactamase inhibitors addition would decrease the MIC of cefiderocol from 32 to 1 mg/L in AR8538, which harbors blaSHV-12, blaOXA-16 and two copies of blaNDM-1 in different plasmids. Avibactam did not impact cefiderocol susceptibility of AR8416, which produces NDM-5. However, we found a deficient CirA in AR8416. Using the same K serotype strain D3, we proved CirA deficiency or carrying NDM individually reduced cefiderocol susceptibility, but their simultaneously existence rendered a high-level cefiderocol resistance. In summary, the resistance of CRKP against cefiderocol is mediated by multiple factors, including the deficiency of CirA, metallo- or serine-β-lactamases, while a high-level cefiderocol resistance could be rendered by the combined effect of NDM expression and CirA deficiency.

IMPORTANCE Cefiderocol-resistant CRKP strains are emerging in bloodstream infections in Chinese patients with hematologic malignancies, although cefiderocol has not been approved for clinical use in China. Our study proved that the resistance of CRKP against cefiderocol is mediated by multiple factors, including the deficiency of CirA, metallo- or serine-β-lactamases, while a high-level cefiderocol resistance could be rendered by the combined effect of NDM expression and CirA deficiency. As NDM production is one of the most critical mechanisms resulting in carbapenem resistance, it would pose great challenges on the clinical efficacy of cefiderocol in future.

KEYWORDS bloodstream infections, cefiderocol, CRKP, NDM, CirA
Patients with hematological malignancies have an increased risk for bloodstream infections (BSI), which are among the most common and severe infectious complications, with a prevalence of 11% to 38% and associated with a 40% rise in crude mortality (1). Particularly, those with Gram-negative pathogens, especially carbapenem-resistant *K. pneumoniae* (CRKP) has become one of the major emerging causes of fatal infections in patients with hematological malignancies (1, 2). Due to the increasing prevalence of multidrug-resistance (MDR), the treatment of infections caused by CRKP rely on tigecycline, polymyxins or new β-lactam/β-lactamase inhibitor combinations (such as ceftazidime-avibactam, CAZ/AVI) (3, 4). Unfortunately, the resistance against these antimicrobials, the last-resort therapy, is already increasingly emerging in CRKP (5). Moreover, the activity of CAZ/AVI against class B β-lactamate (eg, NDM) is limited (6). An effective antimicrobial alternative is therefore urgent.

Cefiderocol is a novel cephalosporin antibiotic showing a potent and broad-spectrum activity against Gram-negative MDR pathogens. Cefiderocol contains a catechol moiety, which minics catecholate-type siderophores (e.g., enterobactin) to chelate ferric iron (7). To acquire iron, bacterial cells would actively transport cefiderocol-Fe³⁺ complexes across outer membrane into the periplasmic space via iron transport systems. This “trojan horse strategy” renders increased periplasmic concentrations of cefiderocol, thus exhibiting potent activity against various carbapenemase-producing Gram-negative bacilli (8, 9). In spite of the short-term usage, strains with reduced susceptibility to cefiderocol have been reported. For example, amino acid deletions in the R2 loop of AmpC β-lactamase confered increased hydrolysis of cefiderocol, resulting in decreased susceptibility to cefiderocol but not yet reaching to resistant level (10, 11). NDM and PER were also reported contribute marginally to the decrease of the susceptibility to cefiderocol but none of them could lead to cefiderocol resistance (12). It indicated that β-lactamate production was not fully responsible for cefiderocol resistance. Meanwhile, mutations of cirA gene (encoding catecholate siderophore receptor) were observed in NDM-producing cefiderocol-resistant *Enterobacter cloacae* strains (13), but no change of cefiderocol susceptibility was observed in cirA knocked out *E. coli* (14). Therefore, the role of CirA in mediating cefiderocol resistance remains ambivalent.

The Food and Drug Administration (FDA) of USA and European Medicines Agency (EMA) have recently approved the usage of cefiderocol and it will soon be approved in China. Therefore, we collected CRKP strains isolated from bloodstream infections in patients with hematologic malignancies from 15 centers in China, to evaluate their cefiderocol susceptibility and further study the mechanisms of cefiderocol resistance, with the aim of providing guidelines for clinical practice of cefiderocol.

**RESULTS**

**Cefiderocol susceptibility.** We collected a total of 86 nonrepetitive CRKP strains from blood of hematological patients, covering 15 tertiary hospitals from January 2018 to December 2020 (Fig. S1). The MICs for cefiderocol ranged from 0.06 mg/L to greater than 256 mg/L (Fig. 1 and Table S2). The MIC⁰ and MIC₉₀ of cefiderocol against these strains was 0.5 mg/L and 4 mg/L, respectively (Fig. 1). Four nonsusceptible strains were identified, including two cefiderocol-resistant *K. pneumoniae* strains AR8538 (MIC = 32 mg/L) and AR8416 (MIC > 256 mg/L).

**Effect of β-lactamases on cefiderocol resistance.** To evaluate the effect of β-lactamate on cefiderocol resistance, cefiderocol MICs were determined in the presence or absence of β-lactamate inhibitors. Cefiderocol-nonsusceptible CRKP strains (MIC ≥ 8 mg/L) were included as shown in Table 1. All the 4 strains produced NDM-1 or NDM-5. Against 3 of 4 metallo-β-lactamate producers, decreasing 4–8-folds in MICs was observed after adding AVI except for AR8416, which still remained highly resistant in the presence of AVI. By adding DPA, a more than 8-fold decrease of cefiderocol MICs was observed and the MIC against AR8416 decreased from ≥ 256 mg/L to 1 mg/L. When adding both AVI and DPA, the MICs even decreased 16–128-folds. Combined, these results suggested both metallo- and serine-β-lactamases contributed to reduced cefiderocol susceptibility.

**Clinical and genomic characteristics of AR8416 and AR8538.** *K. pneumoniae* AR8538 and AR8416 were isolated from two young males (both 20’s) who were
diagnosed with acute lymphocytic leukemia in different hospitals. AR8538 was isolated after 1-day meropenem treatment. The patient infected by AR8416 received courses of tigecycline, polymyxin B, meropenem and cefperazone-sulbactam during the hospitalization. A diagnostic bloodstream culture grew AR8416 with cefiderocol MIC of >256 mg/L on day 3. Both patients experienced treatment failure eventually (Fig. S2).

According to whole-genome sequencing data, strain AR8538 belonged to a novel sequence type, ST5820, which was a single-locus variant of ST3520 and was identified as *K. quasipneumoniae* by Kleborate. AR8538 harbored 4 plasmids with IncX3-type plasmid pAR8538_3 carrying *bla*$_{NDM-1}$ and *bla*$_{SHV-12}$ and IncFIA (HI1)-type plasmid pAR8538_4 carrying *bla*$_{NDM-1}$ and *bla*$_{DHA-1}$ (Fig. 2A and B). pAR8538_3 had 32% query coverage and 99.99% nucleotide identity with pNDM_IncX3 (CP050161), which also carried *bla*$_{NDM-1}$ and *bla*$_{SHV-12}$ (Fig. 2A). Another plasmid pAR8538_4 showed 82% coverage and 100% identity with pFDAARGOS_I (CP069967), which lack genetic contexts of *bla*$_{NDM-1}$, while pNDM-XZA88 (CP076461) aligned well to genetic contexts of *bla*$_{NDM-1}$ in pAR8538_4 (Fig. 2B). The cirA gene was intact in AR8538.

Confirmed by whole-genome sequencing, AR8416 belonged to ST 5214 and K locus (KL) 1. Colicin I receptor (CirA) of *K. pneumoniae* consists of a transmembrane β-barrel with a luminal ‘plug’ domain positioned inside (15). Chromosomal cirA gene of AR8416 had a deletion of guanine base at position 1300 (Fig. 3A), rendered an early stop codon at position 444 of CirA, leading to the deficiency of β-barrel structure of CirA (Fig. S3). Among the 86 CRKP strains, the cirA mutation was only detected in AR8416. The strain

| TABLE 1 | In vitro activity of cefiderocol with or without β-lactamase inhibitors$^a$ |
|----------|-----------------------------------------------|
| Strains  | β-lactamase        | Cefiderocol MIC (mg/liter) | AVI | DPA | AVI+DPA |
| AR8416   | NDM-5             | >256                        | >256 | 1   | 1        |
| AR8538   | NDM-1 × 2, DHA-1, SHV-12 | 32                        | 8    | 2   | 1        |
| AR8334   | NDM-5, SHV-208    | 8                          | 2    | 1   | 0.5      |
| AR8335   | NDM-1, SHV-12, IMP-4 | 8                          | 1    | 1   | 0.06     |

$^a$AVI, avibactam; DPA, dipicolinic acid; MIC, minimum inhibitory concentration.

$^b$No β-lactamase inhibitor was added.
harbored plasmid-mediated \( \text{bla}_{\text{NDM-5}} \), \( \text{qnrS1} \), \( \text{sul2} \), \( \text{floR} \), tet(A) and chromosomal \( \text{bla}_{\text{SHV-1}} \). AR8416 carried 4 plasmids, with \( \text{bla}_{\text{NDM-5}} \) carried by a 45-kb IncX3-type plasmid, designated pAR8416-NDM5 (Fig. 3B). Insertion sequence (IS) IS\( \text{swi1} \), IS300 and IS5 were upstream of \( \text{bla}_{\text{NDM-5}} \) while \( \text{ble}_{\text{MBL}} \) (mediating bleomycin resistance), \( \text{trpF} \) (encoding a phosphoribosylanthranilate isomerase), \( \text{dsbD} \) (encoding a twin-arginine translocation pathway signal sequence domain protein) were downstream, which were common genetic contexts of \( \text{bla}_{\text{NDM}} \) (16). A copy of IS26 was also involved in downstream \( \text{bla}_{\text{NDM-5}} \). BLASTN analysis revealed that pAR8416-NDM5 displayed 100% query coverage and 100% nucleotide identity with plasmid pABC369-NDM-5 (MK372393) carried by a \textit{K. pneumoniae} strain in the United Arab Emirates, and plasmid pNDM5-SCNJ1 (MK715437) carried by a \textit{K. pneumoniae} strain isolated in China.

pAR8416-NDM5 is not the only factor resulting in high-level ceferocol resistance in AR8416. Knocking out of \( \text{bla}_{\text{NDM-5}} \) failed in AR8416. To explore the influence of pAR8416-NDM5 on the high-level resistance of AR8416, we obtained isolate AR8416-1 with only plasmid pAR8416-NDM5 cured. As expected, curing of plasmid pAR8416-NDM5 resulted in a sharp decrease in ceferocol resistance (MIC, from >256 to 0.25 mg/L) (Table 2). The resistance was then reverted with the complement of pAR8416-NDM5. It was indicative that pAR8416-NDM5 might be a decisive factor for ceferocol resistance in AR8416. However, when pAR8416-NDM5 was introduced into D3 (a ST23 and K1 serotype clinical \textit{K. pneumoniae} strain isolated from liver abscess, GenBank accession number: ERS3900049) and \textit{E. coli} DH5\( \alpha \), the ceferocol MIC increased 8-fold (MIC, from 0.25 to 4 mg/L) and 16-fold (MIC, from 0.125 to 2 mg/L), respectively, yet still remaining susceptible. Hence, pAR8416-NDM5 reduces ceferocol susceptibility but was not the determining factor resulting in ceferocol resistance.

\textbf{NDM expression and CirA deficiency jointly confer ceferocol resistance.} While CirA is involved in the permeation of ceferocol into bacterial cells (14), we speculated that the deficiency of CirA contributed to ceferocol resistance in AR8416, in combination with pAR8416-NDM5. We then constructed a cirA mutation in D3, which was the same as that in AR8416. The CirA deficiency rendered the ceferocol MIC against D3
increased 2-fold (MIC from 0.5 to 1 mg/L) (Table 3). While pAR8416-NDM5 was transformed into D3-cirA mutant, cefiderocol MIC reached greater than 256 mg/L, suggesting that the presence of pAR8416-NDM5 and the deficiency of CirA jointly conferred high-level cefiderocol resistance of AR841, but an individual factor could not have this effect. To determine whether blaNDM-5 or other genetic contexts in pAR8416-NDM5 were responsible for cefiderocol resistance, the blaNDM-5 gene derived from pAR8416-NDM5 was inserted into E. coli DH5a to confirm the effect of the blaNDM-5 gene derived from pAR8416-NDM5 on cefiderocol susceptibility.

### TABLE 2  Effect of plasmid pAR8416-NDM5 on cefiderocol susceptibility in *K. pneumoniae* AR8416, D3 and *E. coli* DH5α

| Strain          | Description                      | MIC (mg/L) |
|-----------------|----------------------------------|------------|
| AR8416          | Wild type                        | >256       |
| AR8416-1        | pAR8416-NDM5 cure strain of AR8416 | 0.25       |
| AR8416-2        | AR8416-1 complemented with pAR8416-NDM5 | >256      |
| D3              | Wild type                        | 0.5        |
| D3 + pAR8416-NDM5 | D3 transformed with pAR8416-NDM5 | 4          |
| DH5α            | Wild type                        | 0.125      |
| DH5α + pAR8416-NDM5 | DH5α transformed with pAR8416-NDM5 | 2          |

*MIC, minimum inhibitory concentration.*
NDM5 was cloned in pCR2.1 vector. As shown in Table 3, both AR8416-1 and D3 strains with cirA mutation became highly resistant to cefiderocol after acquiring pCR2.1-NDM5, while wild-type D3 and DH5α still remained susceptible with pCR2.1-NDM5 transformed, though the MICs enhanced marginally. However, other types of carbapenemases, including KPC-2, IMP-4, OXA-48 and OXA-232 did not show the similar synergistic effect with the deficiency of CirA (Table 4).

**DISCUSSION**

Cefiderocol is a promising novel cephalosporin antibiotic showing expanded activity against Gram-negative bacteria producing all four Ambler classes of β-lactamases, including extended spectrum β-lactamases (ESBL) and carbapenemases. In meropenem-nonsusceptible strains in SIDERO-WT study, more than 99% Enterobacterales and *Pseudomonas aeruginosa* were susceptible to cefiderocol (17). In SIDERO-CR study, the susceptibility rate for meropenem-nonsusceptible is 97% (18). Cefiderocol-resistant strains are uncommon, and the underlying mechanisms have been little elucidated. In this study, we reported for the first time that NDM and the deficiency of catecholate siderophore receptor CirA individually contributed to reduced cefiderocol susceptibility, while combining these two factors resulted in a high-level cefiderocol resistance in *K. pneumoniae*.

Until now, a variety of factors have been demonstrated to be associated with reduced cefiderocol susceptibility but limited data presents cefiderocol resistance mechanism. Among which, β-lactamase production is considered to be an important reason for cefiderocol resistance. For NDM-producing *K. pneumoniae* strains, individual serine-β-lactamase inhibitor (AVI) or metallo-β-lactamase inhibitor (DPA) exhibits little effect on cefiderocol

**TABLE 3** Combined effect of deficiency of CirA and NDM-5 on cefiderocol resistance

| Strain                  | Description                  | MIC (mg/L)*                  |
|------------------------|------------------------------|------------------------------|
| D3                     | Wild type                    | 0.5                          |
| D3-cirA-mutant         | D3 with cirA mutation        | 1                            |
| D3-cirA-mutant+pAR8416-NDM5 | D3-cirA-mutant transformed with pAR8416-NDM5 | >256                         |
| D3+pCR2.1-NDM5         | D3 transformed with pCR2.1-NDM5 | 4                            |
| D3-cirA-mutant+pCR2.1-NDM5 | D3-cirA-mutant transformed with pCR2.1-NDM5 | >256                         |
| AR8416-1+pCR2.1-NDM5   | AR8416-1 transformed with pCR2.1-NDM5 | >256                         |
| D3+pCR2.1 vector       | D3 transformed with pCR2.1 vector | 0.5                          |
| AR8416-1+pCR2.1 vector | AR8416-1 transformed with pCR2.1 vector | 0.25                         |
| DH5α + pCR2.1 vector  | DH5α transformed with pCR2.1 vector | 0.125                        |
| DH5α + pCR2.1-NDM5     | DH5α transformed with pCR2.1-NDM5 | 2                            |

*aMIC, minimum inhibitory concentration.*

**TABLE 4** Combined effect of deficiency of CirA and other types of carbapenemases on cefiderocol resistance

| Strain                  | Description                  | MIC (mg/L)*                  |
|------------------------|------------------------------|------------------------------|
| D3+pCR2.1_NDM1         | D3 transformed with pCR2.1_NDM1 | 4                            |
| D3+pCR2.1_KPC2         | D3 transformed with pCR2.1_KPC2 | 2                            |
| D3+pCR2.1_IMP4         | D3 transformed with pCR2.1_IMP4 | 0.5                          |
| D3+pCR2.1_OXA48        | D3 transformed with pCR2.1_OXA48 | 0.5                          |
| D3+pCR2.1_OXA232       | D3 transformed with pCR2.1_OXA232 | 0.5                          |
| D3-cirA-mutant+pCR2.1_NDM1 | D3-cirA-mutant transformed with pCR2.1_NDM1 | >256                         |
| D3-cirA-mutant+pCR2.1_KPC2 | D3-cirA-mutant transformed with pCR2.1_KPC2 | 4                            |
| D3-cirA-mutant+pCR2.1_IMP4 | D3-cirA-mutant transformed with pCR2.1_IMP4 | 1                            |
| D3-cirA-mutant+pCR2.1_OXA48 | D3-cirA-mutant transformed with pCR2.1_OXA48 | 1                            |
| D3-cirA-mutant+pCR2.1_OXA232 | D3-cirA-mutant transformed with pCR2.1_OXA232 | 1                            |
| AR8416-1+pCR2.1_NDM1   | AR8416-1 transformed with pCR2.1_NDM1 | >256                         |
| AR8416-1+pCR2.1_KPC2   | AR8416-1 transformed with pCR2.1_KPC2 | 1                            |
| AR8416-1+pCR2.1_IMP4   | AR8416-1 transformed with pCR2.1_IMP4 | 0.25                         |
| AR8416-1+pCR2.1_OXA48  | AR8416-1 transformed with pCR2.1_OXA48 | 0.5                          |
| AR8416-1+pCR2.1_OXA232 | AR8416-1 transformed with pCR2.1_OXA232 | 0.25                         |

*aMIC, minimum inhibitory concentration.*

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susceptibility (12). However, when both AVI and DPA were added for one strain, the MIC decreased 16-fold (from 4 to 0.25 μg/mL) (12). These results suggest that both metallo- and serine-β-lactamases contribute to ceferodolc resistance. Even so, different types of β-lactamases present versatile hydrolysis ability against ceferodolc. In present study, IMP4, OXA-48 and OXA-232 had no effect on ceferodolc susceptibility while ceferodolc MIC against D3 increased from 0.5 to 2 mg/L after acquiring KPC-2. It suggested KPC-2 might possess a higher hydrolysis ability against ceferodolc, though KPC-2 is not sufficient to result in ceferodolc resistance, even plus CirA deficiency. In contrast, NDM-5, as well as NDM-1, is the most effective β-lactamase contributing to ceferodolc resistance in our tested lactamases. Our results support previous findings that NDM confers greater MIC50 values (2 μg/mL) than KPC, GES, IMP, VIM and OXA (0.12 - 1 μg/mL) (8) and NDM-producers showed an MIC distribution of ceferodolc with a higher concentration range compared with other carbapenemase producers (19). Interestingly, a recent study demonstrated that NDM but not KPC-2 and OXA-48 facilitate the acquisition of CirA mutation (20). Further kinetic parameters are required to explain the superiority of NDM. Worryingly, recent measurements from Poirel L et al. showed that the specific activities against ceferodolc of β-lactamase PER-1 is greater than NDM-1 (2.14 versus 0.28 μmol/min·mg) (21). It is noteworthy that PER might cause threat for future ceferodolc resistance.

**blaNDM**, in the two ceferodolc-resistant strains were all located on plasmids. Different from pAR8416-NDM5, the **blaNDM**-bearing plasmids in AR8538 were less common. Plasmid pAR8538_3 is a large recombinant while the **blaNDM** of pAR8538_4 might be acquired via IS26-mediated translocatable unit mobilization. Interestingly, AR8538 harbored two copies of **blaNDM** located on different plasmids, which was seldom reported. In addition to NDM-1, DHA-1 and SHV-12 also contributed to ceferodolc resistance of AR8538. It suggested that the effect of additive of β-lactamases could also result in resistance, despite of the low-level resistance (32 mg/L).

As reported before, CirA was the gateway for ceferodolc entry into bacterial cells (22), hence its deficiency must impede ceferodolc penetration. In this study, we showed the contribution of CirA deficiency on ceferodolc resistance. A base deletion rendered a frameshift mutation, which caused a shortened CirA length (from 657 aa to 443 aa). Actually, this shortened CirA was also observed in ceferodolc-resistant *E. cloacae*, due to base deletions or insertions (13). Coincidentally, the *E. cloacae* strain was also NDM-5 positive. Further *in vitro* evolution experiments revealed that NDM-5 facilitates the emergence of CirA mutation, resulting in ceferodolc resistance in *Enterobacter cloacae* (20). The similar findings were also reported in *K. pneumoniae* recently (23). Together with our molecular validation in this study, the high-level resistance to ceferodolc was the result of both NDM production and CirA deficiency.

Actually, CirA is not the unique receptor for ceferodolc entry. The uptake of siderophore (and ceferodolc) into the periplasm requires energy that generated from TonB-ExbB-ExbD system (24). When ferri-siderophores or ceferodolc are bound to outer membrane receptors, TonB system transfer energy to the receptors and induce a conformational change, resulting in internalization of ceferodolc and siderophores (25–27). This active transportation renders a high ceferodolc concentration in periplasm (Fig. 4). In addition to CirA, these TonB-dependent outer membrane transporters (TBDT) include FepA, IroN, FecA, FhuE, IutA, Fiu, Iha, FyuA and FitA in *Enterobacteriales*, with FepA, Fiu and CirA as the common catechol receptors. On the other hand, ceferodolc molecules can also cross the outer membrane of by passive diffusion through porin channels (28). Therefore, the transportation of ceferodolc is redundant and not surprisingly when only *cirA* was knocked out in *E. coli*, ceferodolc MIC was unchanged, whereas the MIC increased 16-fold by the double knockout of *cirA* and *fiu*, though it still remained susceptible (1 mg/L) (14). Consistently, in our test strain D3, mutation of *cirA* had little influence on ceferodolc susceptibility. However, for those NDM-producers (such as AR8416), when CirA is inactivated, NDM is sufficient to hydrolyze decreased ceferodolc in periplasmic space (Fig. 4). These highly resistant strains could not be inhibited even by the combination of ceferodolc and avibactam, a potential
therapeutic alternative recently proposed to against carbapenem- and ceferocol-resistant strains (21).

Clinical analysis of the patients indicate that induced resistance is of concern for ceferocol. In the phase 3 clinical trial CREDIBLE-CR (29), 12 (15%) patients with a carbapenem-resistant pathogen (including *A. baumannii*, *S. maltophilia*, *K. pneumoniae*, and *P. aeruginosa*) at baseline treated with ceferocol had at least a 4-fold increase in ceferocol MICs. Recently, highly ceferocol-resistant *Enterobacter cloacae* was identified after ceferocol treatment and *cirA* mutations were discovered in resistant isolates (13). It indicated the emergence of ceferocol resistance after its clinical use. In our study, no ceferocol or its analogue (ceftazidime and cefepime) was used to treat infections caused by AR8416 and AR8538. Of note, the patients infected by AR8416, which exhibited high-level ceferocol resistance, experienced extensive exposure to multiple antimicrobial classes, including carbapenems, third-generation cephalosporins, tigecycline, polymyxin B and β-lactamase inhibitors, raising the concerns about the impact of multiple antimicrobial exposure on ceferocol resistance. It requires further investigation to explore the factors driving the emergence of ceferocol-resistant strains.

In conclusion, ceferocol-resistant CRKP strains are emerging from bloodstream infections in patients with hematologic malignancies prior to the approval of ceferocol clinical use in China. Cefiderocol resistance is mediated by multiple factors, including the deficiency of CirA, metallo- and serine-β-lactamases. High-level ceferocol resistance could be rendered by the combined effect of NDM expression and CirA deficiency.

**MATERIALS AND METHODS**

**Bacterial isolates.** A retrospective multicenter cohort study was performed to evaluate the cefiderocol resistance of CRKP strains isolated from bloodstream infections in patients with hematological malignancies from January 2018 to December 2020. This study covered 15 tertiary referral hospitals from 10 provinces (or cities) in China. *K. pneumoniae* strains were identified via matrix-assisted laser desorption/
ionization mass spectrometry. This study was approved by the ethics committees of Sir Run Run Shaw Hospital (20201231-57).

Antimicrobial susceptibility testing. Carbenepenem resistance was determined by broth microdilution method with meropenem, imipenem and ertapenem. MICs of cefiderocol were determined using broth microdilution method in iron-depleted cation-adjusted Mueller-Hinton broth (ID-CAMHB) (30). All tests were repeated in triplicates and interpreted following the Clinical and Laboratory Standards Institute 2019 guidelines (cefiderocol MIC: ≤4 mg/L, susceptible; 8 mg/L, intermediate; and ≥16 mg/L, resistant). E. coli ATCC 25922 was served as a quality control strain. To examine the effect of β-lactamase inhibitors. We used 100 μg/ml of dipicolinic acid (DPA) to inhibit metallo-β-lactamase and 4 μg/ml of AVI to inhibit serine-β-lactamase.

WGS and sequence analysis. Genomic DNA was extracted using a Qiagen minikit (Qiagen, Hilden, Germany). We used the TruePrep DNA Library Prep kit v2 (Vazyme Biotech, Nanjing, China) to prepare DNA from AR8416 and AR8538 for next-generation sequencing on the Illumina HiSeq X 10 platform (Illumina, San Diego, CA) with 100-bp paired-end reads and using HiSeq SBS kit v4 (Illumina, San Diego, CA). The quality of the reads was checked using FastQC v.0.4.5. The two isolates were also sequenced using the MinION platform (Nanopore, Oxford, UK). To assemble whole-genome sequences, the long-read sequencing data from Nanopore sequencing were hybrid assembled with the Illumina sequencing data using Unicycler v.0.4.8. Unicycler uses miniasm to construct a string graph assembly using both the short read contigs and the long reads. Complete and circular chromosome and plasmids were finally obtained. Unicycler uses the SPAdes read error correction module to reduce the number of errors in the short read before SPAdes assembly. Parameter “--only-error-correction” was used in SPAdes assembly.

MLST, serotype, antimicrobial resistance and virulence genes were identified using Kleborate (31). Plasmid type was identified by PlasmidFinder-2.0. Synteny plots of plasmid alignment were generated by R v.3.6.2 after pairwise BLASTN comparisons. Complete sequences of the chromosome and plasmids from K. pneumoniae AR8538 and AR8416 have been deposited in GenBank under accession numbers CP081827–CP081831 and CP081815–CP081819, respectively.

Plasmid curing experiment was performed using sublethal concentration of sodium dodecyl sulfate (SDS) as previously described (32), to confirm whether the blaOXA-232-bearing plasmid pAR8416-NDM5 has contributed to cefiderocol resistance. Curing of pAR8416-NDM5 was verified by PCR and colonies with only pAR8416-NDM5 cured were collected. All the primers used in this study are shown in Table S1.

Cloning and sequencing. The complete sequences of blaOXA-232, blaNDM5, blaPER-1, blaIMP-4, bladoxa-48 and bladoxa-222 containing native promoters were identified and amplified by PCR. Purified PCR products were cloned into pCR2.1 vector and transformed into E. coli DH5α. Spread 40 μL of 40 mg/mL X-gal and 40 μL of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) on LB agar plates, which contained 50 mg/L kanamycin. The transformants were then plated on LB agar plates and incubated at 37°C overnight. Pick 10 white or light blue colonies rather than dark blue ones. The recombinant plasmids were verified by PCR using the M13 primers (Table S1) and sequenced on both strands by Sanger sequencing. All operation steps were followed by the instruction of TOPO TA Cloning kit.

Site-specific mutagenesis for cirA. To explore the impact of termination of cirA coding, we performed single-base deletion mutation as present in AR8416 for cirA via a CRISPR-Cas9-mediated genome-editing method (33). Generally, the single guide RNA (sgRNA) directs the Cas9 protein to a target sequence in the presence of a 5′-NGG-3′ protospacer adjacent motif (PAM). Cas9 nuclease then cleaves the target sequence to cause a double-strand break. To repair the break, homologous recombination occurs with the presence of exogenously supplied donor DNA repair templates.

Specifically, a 20 bp-spacer sequence before a PAM site (5′-NGG-3′) in the target sequence was ligated into plasmid pSGKP, which expressed the sgRNA. Two reversed Bsal sites were inserted between the promoter and the sgRNA scaffold for assembly of spacers (33). Cotransform the spacer introduced plasmid pSGKP and the donor DNA (containing deletion mutation) into the L-arabinose induced recipient cells harboring the pCasKP plasmid, which expressed the Cas9 protein and lambda Red recombination system. The MH agar plate containing 50 μg/mL apramycin and 100 μg/mL hygromycin was used to screen the positive transformants at 30°C overnight. The successful mutant was verified by PCR and sanger sequencing. Of note, a silent mutation should be introduced in the PAM site on donor DNA to prevent the cutting of Cas9 when performing complementation. Primers used in this study are shown in Table S1. Plasmid pCasKP and pSGKP contained the temperature-sensitive replicon repA101(Ts) and the sucrose-sensitive gene sacB, respectively (33). To cure these two plasmids after successful editing, bacterial cells were streaked onto an MH agar plate containing 5% sucrose and incubated at 37°C overnight. Survival colonies were plated onto MH plates with or without the supplementation of apramycin or hygromycin, respectively. The colonies with the successful curing of both plasmids could only grow on the plate without antibiotic.

Data availability. Complete sequences of the chromosome and plasmids from K. pneumoniae AR8538 and AR8416 have been deposited in GenBank under accession numbers CP081827–CP081831 and CP081815–CP081819, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.
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We have no conflicts of interest to disclose.

This study was approved by the ethics committees of Sir Run Run Shaw Hospital (20201231-57).

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