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Abstract:	Purpose: To assess the in vitro activity of cefiderocol (CFDC) against a collection of both ceftazidime-avibactam (CZA) susceptible and resistant KPC-producing Enterobacterales (KPC-EB) isolates. Secondly, to assess its synergistic activity in combination with different antibiotics. Methods: One hundred KPC-EB isolates were tested: 60 CZA susceptible and 40 CZA resistant. Among them, 17 pairs of CZA susceptible and resistant KPC-producing Klebsiella pneumoniae (KPC-Kp) isolates were collected from 17 distinct patients before and after CZA treatment, respectively. Results: CFDC susceptibility was evaluated by both broth microdilution (lyophilized panels; Sensititre; Thermo Fisher) and disk diffusion testing. Results were interpreted using EUCAST breakpoints. Synergistic activity of CFDC in combination with CZA, meropenem-vaborbactam, imipenem, and amikacin against six characterized KPC-Kp strains, before and after acquisition of CZA resistance, was evaluated using gradient diffusion strip crossing method. CFDC resistance rate was significantly higher in CZA resistant EB subset than in the susceptible one ($p<0.001$): 82.5% vs 6.7%. MIC 50 and MIC90 values were 0.25 and 2 mg/L, 8 and 64 mg/L in CZA-susceptible and CZA-resistant subset, respectively. KPC-Kp isolates harboring KPC-D179Y or KPC-Δ242-GT-243 variants showed CFDC MICs ranging from 4 to 64 mg/L. CFDC showed in vitro synergistic effect mostly with

	CZA, against both CZA susceptible and resistant isolates, resulting in a synergy rate of 66.7%. Conclusions: CZA resistance mechanisms in KPC-EB impair the in vitro activity of CFDC, often leading to co-resistance. CFDC in combination with the new β-lactamases inhibitors might represent a strategy to enhance its activity.
Response to Reviewers:	We would like to thank the Editorial Team for his helpful suggestions, which in our view have greatly enhanced the quality and strength of our study. We hope that in this revised version the manuscript is now suitable for publication in European Journal of Clinical Microbiology and Infectious Disease. Please, note that the changes to the original manuscript have been highlighted in the text. The response to the Editor's comments and ensuing modifications in the manuscript are also clearly indicated in the rebuttal. Comments from Reviewers and point-by-point answers Reviewer #3: I'm afraid that the authors did not address my most important comment, which would not be difficult, at least for the 17 isolates' pairs that were submitted to WGS. These isolates could be analyzed by core-genome MLST or SNP analysis and phylogenetic trees could be constructed. I also suggest that the authors provide some data on the geographical and temporal distribution of the isolates and the varying or not resistotypes (although these are not very meaningful). If, as I feel, the isolates were clonal, this has to be mentioned as a limitation, which hampers the generalizability but does not eliminate the validity of the results. We thank the Reviewer for this comment. Five pairs of the susceptible and resistant KPC-producing K. pneumoniae isolates were previously characterized by WGS. Pairwise comparisons in the phylogenetic analysis showed tight clustering of isolates (0-1 single nucleotide polymorphism, SNP) in 4 of the 5 patients. We added these data in the revised manuscript (see lines 178-181). Unfortunately, we did not analyze by WGS all the other pairs of isolates. In the revised version of the manuscript, we added TABLE S1 as Supplementary material (see line 103) to provide some data on the geographical, temporal distribution and antimicrobial susceptibility patterns of the isolates selected in our study. The lack of a full molecular typing of all isolates represents the main limitation of our study and we added it in the text (see lines 240-241). Editor-in-Chief: As for the comments of the other referees, I see that the Introduction and Discussion sections were not shortened at all (Referee 2, comment 1), a comment should be added in the text that "The clinical relevance of synergistic testing remains controversial at best" (Referee 2, comment 3). We thank you for this appraisal. Accordingly, we shortened the Introduction and Discussion sections. We added in the revised manuscript a comment regarding the controversial clinical relevance of synergistic testing (see lines 246-247).

In vitro activity of cefiderocol against ceftazidime-avibactam susceptible and resistant KPC-producing Enterobacterales: cross-resistance and synergistic effects

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Abstract

Purpose: To assess the *in vitro* activity of cefiderocol (CFDC) against a collection of both ceftazidime-avibactam (CZA) susceptible and resistant KPC-producing (KPC-EB) isolates. Secondly, to assess its synergistic activity in combination with different antibiotics.

Methods: One hundred KPC-EB isolates were tested: 60 CZA susceptible and 40 CZA resistant. Among them, 17 pairs of CZA susceptible and resistant KPC-producing *Klebsiella pneumoniae* (KPC-Kp) isolates were collected from 17 distinct patients before and after CZA treatment, respectively.

Results: CFDC susceptibility was evaluated by both broth microdilution (lyophilized panels; Sensititre; Thermo Fisher) and disk diffusion testing. Results were interpreted using EUCAST breakpoints. Synergistic activity of CFDC in combination with CZA, meropenem-vaborbactam, imipenem, and amikacin against six characterized KPC-Kp strains, before and after acquisition of CZA resistance, was evaluated using gradient diffusion strip crossing method.

CFDC resistance rate was significantly higher in CZA resistant EB subset than in the susceptible one ($p<0.001$): 82.5% *vs* 6.7%. MIC 50 and MIC90 values were 0.25 and 2 mg/L, 8 and 64 mg/L in CZA-susceptible and CZA-resistant subset, respectively. KPC-Kp isolates harboring KPC-D179Y or KPC-Δ242-GT-243 variants showed CFDC MICs ranging from 4 to 64 mg/L. *CFDC* showed *in vitro* synergistic effect mostly with CZA, against both CZA susceptible and resistant isolates, resulting in a synergy rate of 66.7%.

Conclusions: CZA resistance mechanisms in KPC-EB impair the *in vitro* activity of CFDC, often leading to co-resistance. CFDC in combination with the new β-lactamases inhibitors might represent a strategy to enhance its activity.

Keywords: cefiderocol; cross-resistance; KPC; *Klebsiella pneumoniae*; ceftazidime-avibactam; multidrug resistance; synergistic activity

1. Introduction

Carbapenemase-producing Enterobacterales (CPE) have emerged as a major public health threat worldwide due to both their difficult-to-treat resistance phenotypes and ability to rapidly disseminate in healthcare facilities (1). The most clinically relevant carbapenemases encountered in Enterobacterales (EB) belong to Ambler class A, mostly *Klebsiella pneumoniae* carbapenemase (KPC) type, Ambler class B (metallo-β-lactamases such as IMP, VIM and NDM types) and Ambler class D (OXA-48-like enzymes) (2). KPC-producing EB (KPC-EB) are endemic in several countries, such as the United States, Latin America, and Southern Europe (1), and are associated with high rates of morbidity and hospital mortality (3).

Ceftazidime-avibactam (CZA) showed to improve clinical outcomes in patients suffering from KPC-EB infections, especially if compared to older treatment options such as polymyxins (4, 5). Despite it was recently introduced several reports attested the *in vivo* selection of CZA resistant KPC-EB strains. Several mechanisms were described, including presence of β-lactamase enzymes and alterations of *ompK35/*36 porins (6, 7). Indeed, presence of KPC variants exhibiting single amino acid substitutions in the Ω-loop region (aminoacid positions 164-179), and particularly the Asp179Tyr substitution, was the most spread mechanism in CZA resistant strains, leading to enhanced affinity toward ceftazidime, together with a concomitant reduced binding to avibactam. Moreover, KPC variants encoding mutations outside the Ω-loop region and associated with CZA resistance (e.g. KPC-41, KPC-23, KPC-14, KPC-8, KPC-50) have been recently isolated from patients with or without history of CZA treatment (8-11).

Cefiderocol (CFDC) (formerly S-649266), a novel siderophore cephalosporin recently approved by the Food and Drug Administration and European Medicines Agency showed broad activity against multidrug-resistant Gram-negative organisms, including carbapenem-resistant EB, *Pseudomonas*

aeruginosa and *Acinetobacter baumannii*. Its broad activity is explained by its distinctive mechanism of penetrations via the iron transport system of Gram-negative bacteria overcoming resistance mechanisms including efflux pump up-regulation and porin channel loss. Moreover, the side-chain properties render high stability against hydrolysis by β-lactamases, including serine β-lactamases (KPC and OXA) and metallo-β-lactamases (NDM, VIM, IMP) (12).

Antimicrobial susceptibility testing of CFDC presents considerable challenge for microbiology laboratories since reference broth microdilution requires iron-depleted Mueller-Hinton, whose preparation is cumbersome. Disk diffusion and commercial broth microdilution panels has been proposed as alternative methods (13).

Activity of CFDC has been investigated in large international surveillance studies, revealing promising results on the *in vitro* activity against meropenem-non-susceptible Gram-negative isolates, including KPC-EB strains (14). Multiple studies covering approximately 1,000 KPC-EB isolates reported MIC50 and MIC90 ranging from 0.25 to 2 mg/L and from 2 to 8 mg/L, respectively (15-18). However, limited evidence on both the *in vitro* activity of CFDC against notable collections of CZA resistant KPC-EB strains and effects of CZA resistance mechanisms on CFDC efficacy are available so far. Additionally, the role of CFDC-based regimes has not been explored to evaluate the potential for *in vitro* synergy against CZA susceptible and resistant KPC-EB.

This study aimed at evaluating the *in vitro* activity of CFDC against a collection of KPC-EB, including both CZA susceptible and resistant clinical isolates. Secondly, synergistic activity of CFDC in combination with different antibiotics against KPC-producing *Klebsiella pneumoniae* (KPC-Kp) clinical strains, before and after acquisition of CZA resistance, was assessed.

2. Material and Methods

2.1 Clinical isolates and antimicrobial susceptibility testing

In vitro activity of CFDC against a collection of KPC-EB clinical isolates was evaluated. Then, *in vitro* susceptibility to CFDC of a collection of KPC-Kp clinical isolates, before and after acquisition of CZA resistance, was also tested. Review of medical records of patients who presented with CZA susceptible to resistant KPC-Kp isolates was carried out to investigate clinical context and CZA exposure.

The isolates of KPC-EB tested in this study (n=100) were selected from a collection obtained from the program of surveillance and control of healthcare-associated MDR Gram-negative infections based at the Microbiology Laboratory of the "University Hospital Città della Salute e della Scienza di Torino", a 2300-bed tertiary care teaching hospital in Turin, North-western Italy. The isolates selection was performed evaluating antimicrobial pattern differences, hospital, department, and date of isolation to minimize the number of clonal isolates (see Supplementary material, Table S1). The isolates tested were mostly isolated from blood, rectal swabs, respiratory specimens, wound exudate and urines of patients admitted from 2017 to 2020. All isolates were stored at -80 °C in cation-adjusted Muller-Hinton broth (Teknova, Hollister, CA) with 20% glycerol and were subcultured twice on blood agar plates prior to use.

Species identification was carried out by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker DALTONIK GmbH, Bremen, Germany). Detection of carbapenemase genes was carried out using the Xpert Carba-R assay (Cepheid, Sunnyvale, CA, USA) and confirmed with an in-house real-time PCR. Carbapenemase activity was investigated by modified carbapenem inactivation method (mCIM), following CLSI guidelines (*http://www.iacld.ir/DL/public/96/CLSI-2017.pdf*).

Susceptibility to ceftazidime (CAZ), cefepime (FEP), meropenem (MEM), imipenem (IMP), ertapenem (ERT), were determined by a commercially available microdilution assay (Panel NMDR, Microscan WalkAway 96 Plus, Beckman Coulter, Switzerland) according to the manufacturer's instructions. CZA MICs were determined by gradient diffusion method (Liofilchem®, Roseto degli

Abruzzi, Italy). CFDC antimicrobial susceptibility was carried out using lyophilized panel (Sensititre™, ThermoFisher Scientific, Waltham, MA, USA) and disk diffusion testing (Liofilchem®, Roseto degli Abruzzi, Italy). The broth microdilution panel contained CFDC concentrations ranging from 0.03 to 64 mg/L and a proprietary chelator in the wells, bypassing the requirement for iron-depleted cation-adjusted Mueller-Hinton broth. The CFDC lyophilized panel has been shown to be substantially equivalent to reference broth microdilution and has received FDA clearance (15). The disk diffusion method was carried out on standard Mueller-Hinton agar incubated for 18 to 24 h at 35 ± 2 °C following EUCAST guidelines (19). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603 were used as quality control strains on each day of testing, checking that all quality control results were within the specified ranges. Susceptibility data were interpreted according to current EUCAST breakpoints (https://eucast.org). Resistance breakpoints were as follows: for CAZ, FEP and MEM: >8 mg/L; for ERT and IMP: >1 mg/L, and for CFDC: >2 mg/L and <22 mm.

2.2 Evaluation of synergistic activity

Synergistic activity of CFDC in combination with CZA, meropenem-vaborbactam (MEV), IMP, and amikacin (AK) against six KPC-Kp clinical strains, before and after acquisition of CZA resistance, was also evaluated. These three paired CZA susceptible and resistant KPC-EB isolates were previously characterized by whole genome sequencing (WGS) to identify potential resistance mechanisms and multilocus sequence typing (20). All isolates were tested for CFDC susceptibility by broth microdilution and disk diffusion testing as above described.

Synergy testing was carried out using gradient diffusion strip crossing, as previously described (21). Briefly, CFDC strips (BioMérieux, Marcy-l'Ètoile, France) were placed perpendicularly to CZA, MEV, IMP, and AK strips (Liofilchem®, Roseto degli Abruzzi, Italy) at their respective MICs onto Mueller-Hinton agar plates using a 0.5 MacFarland inoculum and incubated at 37 °C overnight. MICs were read at the point at which the elliptical inhibition area touched the strips. The mean fractional

inhibitory concentration index (FICI) was calculated by dividing the mean MIC of each drug in combination by the MIC of each drug alone and adding the results. FICI results were interpreted as follows: ≤0.5 as synergy; >0.5 to ≤4 as no interaction; and >4 as antagonism.

2.3 Statistical analysis

Comparison involving dichotomous variables were tested using X^2 test. Comparison involving MICs distributions were tested using the Mann-Whitney test (2-tailed). Summary statistics used for MIC values included the MIC range (MIC_{min} [lowest detected MIC] and MIC_{max} [highest detected MIC]), MIC_{50} and MIC_{90}. The CFDC disk diffusion results were analyzed by mean and range of inhibition zones. For all tests, a $p \leq 0.05$ was considered significant.

3. Results

3.1 *In vitro* activity

Sixty of the KPC-EB isolates included in the study were susceptible to CZA (*K. pneumoniae* n=57, *E. coli* n=1, *Klebsiella oxytoca* n=1, *Serratia marcescens* n=1) and the remaining 40 were CZA resistant *K. pneumoniae*. All the isolates tested positive to bla_{KPC} and negative to the other carbapenemase genes detectable by the molecular assay used (NDM, IMP, VIM and OXA-48 like). Moreover, 25 out of 40 (62.5%) CZA resistant isolates resulted negative to mCIM suggesting impaired carbapenemase activity.

MIC ranges, MIC50 and MIC90 values of CFDC and comparators, as well as resistance rates were summarized in Table 1. CFDC resistance rate was significantly higher in CZA resistant EB subset (CZA-R) than in the susceptible one (CZA-S) ($p<0.001$): 82.5% *vs* 6.7% and 82.5% *vs* 13.3% according to broth microdilution and disk diffusion results, respectively. Conversely, carbapenems resistance rates were significantly higher in CZA-S ($p<0.001$; 86.6-96.6% *vs* 32.5-72.5%) and a lower rate of carbapenemase activity was detected by mCIM in CZA-R (15/40, 37.5%) *vs* CZA-S (60/60, 100%) (data not shown). Significant discrepancy in the pattern of CFDC MICs and inhibition zones

distributions was observed in the two subsets ($p< 0.001$). MIC 50 and MIC90 values were 0.25 and 2 mg/L, 8 and 64 mg/L in CZA-S and CZA-R, respectively. Of note, full concordance in CFDC susceptibility results tested by broth microdilution and disk diffusion methods in CZA-R was observed, while five discordant results among CZA susceptible KPC-EB isolates were observed (MIC 2 mg/L n=5 *vs* inhibition zones 19 mm n=2 or 20 mm n=3).

Among the KPC-EB isolates selected, 17 paired KPC-Kp isolates, susceptible and resistant to CZA, were collected from 17 distinct hospitalized patients before and after CZA treatment, respectively. Clinical characteristics of patients, characterization of the isolates and pairwise comparison of CZA and CFDC susceptibility results were shown in Table 2. Amongst the CZA resistant strains, 10 out of 17 were previously investigated for CZA resistance mechanisms (20, 22): expression of *bla*KPC harbouring D179Y amino acid mutation was the main mechanism identified (n= 5) followed by expression of KPC-2 variant carrying Δ242-GT-243 deletion (n=2). Moreover, the first 5 paired of CZA susceptible and resistant isolates were previously investigated by Whole Genome Sequencing and pairwise comparisons in the phylogenetic analysis showed tight clustering of isolates (0-1 single nucleotide polymorphism) in pairs 1-A/1-B, 2-A/2-B, 3-A/3-B and 5-A/5-B (20).

Paired isolates, susceptible and resistant to CZA, exhibited CZA MICs ranging from 0.064 to 8 and from 12 to >256 mg/L, respectively. CFDC MICs in CZA susceptible strains were significantly lower than those observed in the respective CZA resistant isolates ($p<0.001$; ranges 0.12-16 mg/L *vs* 4 to >64 mg/L, respectively), as also documented by the reduction of inhibition zones diameters for the isolates which developed CZA resistance.

Overall, 16 out of 17 CZA resistant isolates were also resistant to CFDC according to both broth microdilution and disk diffusion results, while 16 out of 17 CZA susceptible isolates were susceptible to CFDC. As such, isolate 3-A, collected by a transplant recipient with bloodstream infection, was CZA susceptible and CFDC resistant (MICs 4 mg/L and 16 mg/L, respectively), while isolate 12-B,

collected by a patient treated for pneumonia, remained susceptible to CFDC (MIC 0.5 mg/L) despite CZA resistance development (MIC 12 mg/L).

3.2 Evaluation of synergistic activity

Synergistic activity of CFDC in combination with CZA, MEV, IMP, and AK evaluated against paired ceftazidime-avibactam susceptible (1-A, 2-A, 5-A) and resistant (1-B, 2-B, 5-B) KPC-Kp isolates was shown in Table 3. Of note, CFDC MICs determined by gradient diffusion testing were lower than those obtained with reference broth microdilution method, leading to consider isolates 1-B, 2-B, and 5-B as false susceptible to CFDC in comparison to both broth microdilution and disk diffusion testing.

Acquisition of CZA resistance mechanisms conferred restoration of susceptibility to both MEV and IMP and did not affect MEV susceptibility. Paired isolates n°1 and n°2 were resistant to AK exhibiting high off-scale MIC values.

Overall, rate of synergistic activity of CFDC-based combinations was 29.2% (7/24). In detail, combinations with β-lactamases inhibitors (CZA and MEV) showed FICI ≤1 against all isolates tested. *In vitro* synergistic effect was mostly observed with CZA, against both CZA susceptible and resistant isolates (1-A/1-B and 5-A/5-B), resulting in a synergy rate of 66.7%. Synergistic activities with MEV against isolates 1-B and 5-B and IMP against isolate 1-B were also observed.

4. Discussion

Evidence on *in vitro* CFDC activity available so far included less than ten KPC-EB isolates resistant to CZA, of which three VIM-carbapenemase co-producers (15-18). To the best of our knowledge, this is the first study that evaluated the *in vitro* activity of CFDC against a KPC-EB collection that included a notable number of clinical isolates resistant to CZA. Our results showed potent antimicrobial activity of CFDC against CZA susceptible KPC-EB isolates and were consistent with literature (15-19). As such, the largest surveillance study involving KPC-producing EB strains, named

ARGONAUT, tested the *in vitro* activity of CDFC against 738 KPC-EB isolates and reported MIC50/MIC90 of 1/8 mg/L and 0.25/2 mg/L for KPC-2 and KPC-3 producers, respectively (16).

The main finding of this study was showing how CFDC resistance rate significantly increased from 6.7% in CZA-S to 82.5% in CZA-R subset. Indeed, a 32-fold increase in both CFDC MIC50 and MIC90 values (8 mg/L and 64 mg/L, respectively) and a reduction of 6.2 mm of the mean inhibition zone were also observed. Herein, we observed that CZA treatment may lead to select subpopulations resistant to this β-lactam/β-lactamase inhibitor combination but also co-resistant to the new cephalosporin CFDC. Data on the development of CFDC resistance in Gram-negative bacteria are lacking and a clear correlation between β-lactamase production and increased CFDC MICs has not yet been found (23). Tiseo *et al* recently reported the *in vivo* selection of CZA and CFDC co-resistant *K. pneumoniae* isolate harboring KPC-D179Y mutant following 14 days of empirical treatment with CZA (24). Likewise, Shields *et al* described a case of clinical evolution of AmpC-mediated CZA and CFDC co-resistance in *Enterobacter cloacae* following FEP exposure (25). The AmpC sequence contained a 2-amino acid deletion of alanine and leucine at positions 292 and 293, respectively (A292_L293DEL), not present in the AmpC carried by the CZA susceptible ancestor strain. Among characterized isolates included in this study, KPC-Kp isolates harboring KPC-D179Y and KPC-Δ242-GT-243 variants showed CFDC MICs ranging from 4 to 64 mg/L and from 16 to 64 mg/L, respectively. We may hypothesize that these mutations might be associated with an increased hydrolytic activity against CFDC. This hypothesis was recently supported by *in vitro* experiments; Hobson *et al* observed 4-32-fold increases of CFDC MICs on 37 *in vitro* mutants harboring various blaKPC variants with increased CZA MICs (26).

However, KPC-Kp isolates both resistant to CZA and harboring no mutation in bla_{KPC} showed a range of CFDC MICs of 4-32 mg/L. Therefore, other resistance mechanisms such as co-production of other beta-lactamases, high number of bla_{KPC} copies, expression of efflux pumps and porins loss could also lead to CZA and CFDC co-resistance and variability in the MIC values.

The lack of a full molecular characterization of resistance genes content and cloning typing of all bacterial isolates represents the main limitation of our study. Studies in-depth of the entire resistance genes content and investigations of steady-state kinetic parameters of KPC mutants are warranted to understand the mechanisms of co-resistance in detail.

Knowledge of resistance mechanisms represents the starting point for setting the role of CFDC-based combination therapies, especially in cases of reduced susceptibility or resistance to this new antibiotic. Although clinical relevance of synergistic testing remains controversial at best, our data on synergistic activity of CFDC suggest that combination of CFDC with formulations that include avibactam or vaborbactam β-lactamases inhibitors might represent an appropriate choice to act synergistically against KPC-EB isolates.

In conclusion, CZA resistance mechanisms in KPC-EB may impair the *in vitro* activity of CFDC leading to co-resistance. CFDC in combination with the new β-lactamases inhibitors might represent a strategy to enhance its activity. Our data support the needing for further investigations on CFDC activity alone and in combination, against worldwide CZA resistant KPC-EB.

Declarations

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This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interests

No competing financial interests exist.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article.

Code availability

Not applicable

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki. Formal ethical approval was obtained by Center's institutional review board.

Consent to participate

Not applicable

Consent for publication

Not applicable

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In vitro activity of cefiderocol against ceftazidime-avibactam susceptible and resistant KPC-producing Enterobacterales: cross-resistance and synergistic effects

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Abstract

Purpose: To assess the *in vitro* activity of cefiderocol (CFDC) against a collection of both ceftazidime-avibactam (CZA) susceptible and resistant KPC-producing (KPC-EB) isolates. Secondly, to assess its synergistic activity in combination with different antibiotics.

Methods: One hundred KPC-EB isolates were tested: 60 CZA susceptible and 40 CZA resistant. Among them, 17 pairs of CZA susceptible and resistant KPC-producing *Klebsiella pneumoniae* (KPC-Kp) isolates were collected from 17 distinct patients before and after CZA treatment, respectively.

Results: CFDC susceptibility was evaluated by both broth microdilution (lyophilized panels; Sensititre; Thermo Fisher) and disk diffusion testing. Results were interpreted using EUCAST breakpoints. Synergistic activity of CFDC in combination with CZA, meropenem-vaborbactam, imipenem, and amikacin against six characterized KPC-Kp strains, before and after acquisition of CZA resistance, was evaluated using gradient diffusion strip crossing method.

CFDC resistance rate was significantly higher in CZA resistant EB subset than in the susceptible one ($p<0.001$): 82.5% *vs* 6.7%. MIC 50 and MIC90 values were 0.25 and 2 mg/L, 8 and 64 mg/L in CZA-susceptible and CZA-resistant subset, respectively. KPC-Kp isolates harboring KPC-D179Y or KPC-Δ242-GT-243 variants showed CFDC MICs ranging from 4 to 64 mg/L. *CFDC* showed *in vitro* synergistic effect mostly with CZA, against both CZA susceptible and resistant isolates, resulting in a synergy rate of 66.7%.

Conclusions: CZA resistance mechanisms in KPC-EB impair the *in vitro* activity of CFDC, often leading to co-resistance. CFDC in combination with the new β-lactamases inhibitors might represent a strategy to enhance its activity.

Keywords: cefiderocol; cross-resistance; KPC; *Klebsiella pneumoniae*; ceftazidime-avibactam; multidrug resistance; synergistic activity

1. Introduction

Carbapenemase-producing Enterobacterales (CPE) have emerged as a major public health threat worldwide due to both their difficult-to-treat resistance phenotypes and ability to rapidly disseminate in healthcare facilities (1). The most clinically relevant carbapenemases encountered in Enterobacterales (EB) belong to Ambler class A, mostly *Klebsiella pneumoniae* carbapenemase (KPC) type, Ambler class B (metallo-β-lactamases such as IMP, VIM and NDM types) and Ambler class D (OXA-48-like enzymes) (2). KPC-producing EB (KPC-EB) are endemic in several countries, such as the United States, Latin America, and Southern Europe (1), and are associated with high rates of morbidity and hospital mortality (3).

Ceftazidime-avibactam (CZA) showed to improve clinical outcomes in patients suffering from KPC-EB infections, especially if compared to older treatment options such as polymyxins (4, 5). Despite it was recently introduced several reports attested the *in vivo* selection of CZA resistant KPC-EB strains. Several mechanisms were described, including presence of β-lactamase enzymes and alterations of *ompK35/*36 porins (6, 7). Indeed, presence of KPC variants exhibiting single amino acid substitutions in the Ω-loop region (aminoacid positions 164-179), and particularly the Asp179Tyr substitution, was the most spread mechanism in CZA resistant strains, leading to enhanced affinity toward ceftazidime, together with a concomitant reduced binding to avibactam. Moreover, KPC variants encoding mutations outside the Ω-loop region and associated with CZA resistance (e.g. KPC-41, KPC-23, KPC-14, KPC-8, KPC-50) have been recently isolated from patients with or without history of CZA treatment (8-11).

Cefiderocol (CFDC) (formerly S-649266), a novel siderophore cephalosporin recently approved by the Food and Drug Administration and European Medicines Agency showed broad activity against multidrug-resistant Gram-negative organisms, including carbapenem-resistant EB, *Pseudomonas*

aeruginosa and *Acinetobacter baumannii*. Its broad activity is explained by its distinctive mechanism of penetrations via the iron transport system of Gram-negative bacteria overcoming resistance mechanisms including efflux pump up-regulation and porin channel loss. Moreover, the side-chain properties render high stability against hydrolysis by β-lactamases, including serine β-lactamases (KPC and OXA) and metallo-β-lactamases (NDM, VIM, IMP) (12).

Antimicrobial susceptibility testing of CFDC presents considerable challenge for microbiology laboratories since reference broth microdilution requires iron-depleted Mueller-Hinton, whose preparation is cumbersome. Disk diffusion and commercial broth microdilution panels has been proposed as alternative methods (13).

Activity of CFDC has been investigated in large international surveillance studies, revealing promising results on the *in vitro* activity against meropenem-non-susceptible Gram-negative isolates, including KPC-EB strains (14). Multiple studies covering approximately 1,000 KPC-EB isolates reported MIC50 and MIC90 ranging from 0.25 to 2 mg/L and from 2 to 8 mg/L, respectively (15-18). However, limited evidence on both the *in vitro* activity of CFDC against notable collections of CZA resistant KPC-EB strains and effects of CZA resistance mechanisms on CFDC efficacy are available so far. Additionally, the role of CFDC-based regimes has not been explored to evaluate the potential for *in vitro* synergy against CZA susceptible and resistant KPC-EB.

This study aimed at evaluating the *in vitro* activity of CFDC against a collection of KPC-EB, including both CZA susceptible and resistant clinical isolates. Secondly, synergistic activity of CFDC in combination with different antibiotics against KPC-producing *Klebsiella pneumoniae* (KPC-Kp) clinical strains, before and after acquisition of CZA resistance, was assessed.

2. Material and Methods

2.1 Clinical isolates and antimicrobial susceptibility testing

In vitro activity of CFDC against a collection of KPC-EB clinical isolates was evaluated. Then, *in vitro* susceptibility to CFDC of a collection of KPC-Kp clinical isolates, before and after acquisition of CZA resistance, was also tested. Review of medical records of patients who presented with CZA susceptible to resistant KPC-Kp isolates was carried out to investigate clinical context and CZA exposure.

The isolates of KPC-EB tested in this study (n=100) were selected from a collection obtained from the program of surveillance and control of healthcare-associated MDR Gram-negative infections based at the Microbiology Laboratory of the "University Hospital Città della Salute e della Scienza di Torino", a 2300-bed tertiary care teaching hospital in Turin, North-western Italy. The isolates selection was performed evaluating antimicrobial pattern differences, hospital, department, and date of isolation to minimize the number of clonal isolates (see Supplementary material, Table S1). The isolates tested were mostly isolated from blood, rectal swabs, respiratory specimens, wound exudate and urines of patients admitted from 2017 to 2020. All isolates were stored at -80 °C in cation-adjusted Muller-Hinton broth (Teknova, Hollister, CA) with 20% glycerol and were subcultured twice on blood agar plates prior to use.

Species identification was carried out by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker DALTONIK GmbH, Bremen, Germany). Detection of carbapenemase genes was carried out using the Xpert Carba-R assay (Cepheid, Sunnyvale, CA, USA) and confirmed with an in-house real-time PCR. Carbapenemase activity was investigated by modified carbapenem inactivation method (mCIM), following CLSI guidelines (*http://www.iacld.ir/DL/public/96/CLSI-2017.pdf*).

Susceptibility to ceftazidime (CAZ), cefepime (FEP), meropenem (MEM), imipenem (IMP), ertapenem (ERT), were determined by a commercially available microdilution assay (Panel NMDR, Microscan WalkAway 96 Plus, Beckman Coulter, Switzerland) according to the manufacturer's instructions. CZA MICs were determined by gradient diffusion method (Liofilchem®, Roseto degli

Abruzzi, Italy). CFDC antimicrobial susceptibility was carried out using lyophilized panel (Sensititre™, ThermoFisher Scientific, Waltham, MA, USA) and disk diffusion testing (Liofilchem®, Roseto degli Abruzzi, Italy). The broth microdilution panel contained CFDC concentrations ranging from 0.03 to 64 mg/L and a proprietary chelator in the wells, bypassing the requirement for iron-depleted cation-adjusted Mueller-Hinton broth. The CFDC lyophilized panel has been shown to be substantially equivalent to reference broth microdilution and has received FDA clearance (15). The disk diffusion method was carried out on standard Mueller-Hinton agar incubated for 18 to 24 h at 35 ± 2 °C following EUCAST guidelines (19). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603 were used as quality control strains on each day of testing, checking that all quality control results were within the specified ranges. Susceptibility data were interpreted according to current EUCAST breakpoints (https://eucast.org). Resistance breakpoints were as follows: for CAZ, FEP and MEM: >8 mg/L; for ERT and IMP: >1 mg/L, and for CFDC: >2 mg/L and <22 mm.

2.2 Evaluation of synergistic activity

Synergistic activity of CFDC in combination with CZA, meropenem-vaborbactam (MEV), IMP, and amikacin (AK) against six KPC-Kp clinical strains, before and after acquisition of CZA resistance, was also evaluated. These three paired CZA susceptible and resistant KPC-EB isolates were previously characterized by whole genome sequencing (WGS) to identify potential resistance mechanisms and multilocus sequence typing (20). All isolates were tested for CFDC susceptibility by broth microdilution and disk diffusion testing as above described.

Synergy testing was carried out using gradient diffusion strip crossing, as previously described (21). Briefly, CFDC strips (BioMérieux, Marcy-l'Ètoile, France) were placed perpendicularly to CZA, MEV, IMP, and AK strips (Liofilchem®, Roseto degli Abruzzi, Italy) at their respective MICs onto Mueller-Hinton agar plates using a 0.5 MacFarland inoculum and incubated at 37 °C overnight. MICs were read at the point at which the elliptical inhibition area touched the strips. The mean fractional

inhibitory concentration index (FICI) was calculated by dividing the mean MIC of each drug in combination by the MIC of each drug alone and adding the results. FICI results were interpreted as follows: ≤0.5 as synergy; >0.5 to ≤4 as no interaction; and >4 as antagonism.

2.3 Statistical analysis

Comparison involving dichotomous variables were tested using X^2 test. Comparison involving MICs distributions were tested using the Mann-Whitney test (2-tailed). Summary statistics used for MIC values included the MIC range (MIC_{min} [lowest detected MIC] and MIC_{max} [highest detected MIC]), MIC_{50} and MIC_{90}. The CFDC disk diffusion results were analyzed by mean and range of inhibition zones. For all tests, a $p \leq 0.05$ was considered significant.

3. Results

3.1 *In vitro* activity

Sixty of the KPC-EB isolates included in the study were susceptible to CZA (*K. pneumoniae* n=57, *E. coli* n=1, *Klebsiella oxytoca* n=1, *Serratia marcescens* n=1) and the remaining 40 were CZA resistant *K. pneumoniae*. All the isolates tested positive to bla_{KPC} and negative to the other carbapenemase genes detectable by the molecular assay used (NDM, IMP, VIM and OXA-48 like). Moreover, 25 out of 40 (62.5%) CZA resistant isolates resulted negative to mCIM suggesting impaired carbapenemase activity.

MIC ranges, MIC50 and MIC90 values of CFDC and comparators, as well as resistance rates were summarized in Table 1. CFDC resistance rate was significantly higher in CZA resistant EB subset (CZA-R) than in the susceptible one (CZA-S) ($p<0.001$): 82.5% *vs* 6.7% and 82.5% *vs* 13.3% according to broth microdilution and disk diffusion results, respectively. Conversely, carbapenems resistance rates were significantly higher in CZA-S ($p<0.001$; 86.6-96.6% *vs* 32.5-72.5%) and a lower rate of carbapenemase activity was detected by mCIM in CZA-R (15/40, 37.5%) *vs* CZA-S (60/60, 100%) (data not shown). Significant discrepancy in the pattern of CFDC MICs and inhibition zones

distributions was observed in the two subsets ($p< 0.001$). MIC 50 and MIC90 values were 0.25 and 2 mg/L, 8 and 64 mg/L in CZA-S and CZA-R, respectively. Of note, full concordance in CFDC susceptibility results tested by broth microdilution and disk diffusion methods in CZA-R was observed, while five discordant results among CZA susceptible KPC-EB isolates were observed (MIC 2 mg/L n=5 *vs* inhibition zones 19 mm n=2 or 20 mm n=3).

Among the KPC-EB isolates selected, 17 paired KPC-Kp isolates, susceptible and resistant to CZA, were collected from 17 distinct hospitalized patients before and after CZA treatment, respectively. Clinical characteristics of patients, characterization of the isolates and pairwise comparison of CZA and CFDC susceptibility results were shown in Table 2. Amongst the CZA resistant strains, 10 out of 17 were previously investigated for CZA resistance mechanisms (20, 22): expression of *bla*KPC harbouring D179Y amino acid mutation was the main mechanism identified (n= 5) followed by expression of KPC-2 variant carrying Δ242-GT-243 deletion (n=2). Moreover, the first 5 paired of CZA susceptible and resistant isolates were previously investigated by Whole Genome Sequencing and pairwise comparisons in the phylogenetic analysis showed tight clustering of isolates (0-1 single nucleotide polymorphism) in pairs 1-A/1-B, 2-A/2-B, 3-A/3-B and 5-A/5-B (20).

Paired isolates, susceptible and resistant to CZA, exhibited CZA MICs ranging from 0.064 to 8 and from 12 to >256 mg/L, respectively. CFDC MICs in CZA susceptible strains were significantly lower than those observed in the respective CZA resistant isolates ($p<0.001$; ranges 0.12-16 mg/L *vs* 4 to >64 mg/L, respectively), as also documented by the reduction of inhibition zones diameters for the isolates which developed CZA resistance.

Overall, 16 out of 17 CZA resistant isolates were also resistant to CFDC according to both broth microdilution and disk diffusion results, while 16 out of 17 CZA susceptible isolates were susceptible to CFDC. As such, isolate 3-A, collected by a transplant recipient with bloodstream infection, was CZA susceptible and CFDC resistant (MICs 4 mg/L and 16 mg/L, respectively), while isolate 12-B,

collected by a patient treated for pneumonia, remained susceptible to CFDC (MIC 0.5 mg/L) despite CZA resistance development (MIC 12 mg/L).

3.2 Evaluation of synergistic activity

Synergistic activity of CFDC in combination with CZA, MEV, IMP, and AK evaluated against paired ceftazidime-avibactam susceptible (1-A, 2-A, 5-A) and resistant (1-B, 2-B, 5-B) KPC-Kp isolates was shown in Table 3. Of note, CFDC MICs determined by gradient diffusion testing were lower than those obtained with reference broth microdilution method, leading to consider isolates 1-B, 2-B, and 5-B as false susceptible to CFDC in comparison to both broth microdilution and disk diffusion testing.

Acquisition of CZA resistance mechanisms conferred restoration of susceptibility to both MEV and IMP and did not affect MEV susceptibility. Paired isolates n°1 and n°2 were resistant to AK exhibiting high off-scale MIC values.

Overall, rate of synergistic activity of CFDC-based combinations was 29.2% (7/24). In detail, combinations with β-lactamases inhibitors (CZA and MEV) showed FICI ≤1 against all isolates tested. *In vitro* synergistic effect was mostly observed with CZA, against both CZA susceptible and resistant isolates (1-A/1-B and 5-A/5-B), resulting in a synergy rate of 66.7%. Synergistic activities with MEV against isolates 1-B and 5-B and IMP against isolate 1-B were also observed.

4. Discussion

Evidence on *in vitro* CFDC activity available so far included less than ten KPC-EB isolates resistant to CZA, of which three VIM-carbapenemase co-producers (15-18). To the best of our knowledge, this is the first study that evaluated the *in vitro* activity of CFDC against a KPC-EB collection that included a notable number of clinical isolates resistant to CZA. Our results showed potent antimicrobial activity of CFDC against CZA susceptible KPC-EB isolates and were consistent with literature (15-19). As such, the largest surveillance study involving KPC-producing EB strains, named

ARGONAUT, tested the *in vitro* activity of CDFC against 738 KPC-EB isolates and reported MIC50/MIC90 of 1/8 mg/L and 0.25/2 mg/L for KPC-2 and KPC-3 producers, respectively (16).

The main finding of this study was showing how CFDC resistance rate significantly increased from 6.7% in CZA-S to 82.5% in CZA-R subset. Indeed, a 32-fold increase in both CFDC MIC50 and MIC90 values (8 mg/L and 64 mg/L, respectively) and a reduction of 6.2 mm of the mean inhibition zone were also observed. Herein, we observed that CZA treatment may lead to select subpopulations resistant to this β-lactam/β-lactamase inhibitor combination but also co-resistant to the new cephalosporin CFDC. Data on the development of CFDC resistance in Gram-negative bacteria are lacking and a clear correlation between β-lactamase production and increased CFDC MICs has not yet been found (23). Tiseo *et al* recently reported the *in vivo* selection of CZA and CFDC co-resistant *K. pneumoniae* isolate harboring KPC-D179Y mutant following 14 days of empirical treatment with CZA (24). Likewise, Shields *et al* described a case of clinical evolution of AmpC-mediated CZA and CFDC co-resistance in *Enterobacter cloacae* following FEP exposure (25). The AmpC sequence contained a 2-amino acid deletion of alanine and leucine at positions 292 and 293, respectively (A292_L293DEL), not present in the AmpC carried by the CZA susceptible ancestor strain. Among characterized isolates included in this study, KPC-Kp isolates harboring KPC-D179Y and KPC-Δ242-GT-243 variants showed CFDC MICs ranging from 4 to 64 mg/L and from 16 to 64 mg/L, respectively. We may hypothesize that these mutations might be associated with an increased hydrolytic activity against CFDC. This hypothesis was recently supported by *in vitro* experiments; Hobson *et al* observed 4-32-fold increases of CFDC MICs on 37 *in vitro* mutants harboring various blaKPC variants with increased CZA MICs (26).

However, KPC-Kp isolates both resistant to CZA and harboring no mutation in bla_{KPC} showed a range of CFDC MICs of 4-32 mg/L. Therefore, other resistance mechanisms such as co-production of other beta-lactamases, high number of bla_{KPC} copies, expression of efflux pumps and porins loss could also lead to CZA and CFDC co-resistance and variability in the MIC values.

The lack of a full molecular characterization of resistance genes content and cloning typing of all bacterial isolates represents the main limitation of our study. Studies in-depth of the entire resistance genes content and investigations of steady-state kinetic parameters of KPC mutants are warranted to understand the mechanisms of co-resistance in detail.

Knowledge of resistance mechanisms represents the starting point for setting the role of CFDC-based combination therapies, especially in cases of reduced susceptibility or resistance to this new antibiotic. Although clinical relevance of synergistic testing remains controversial at best, our data on synergistic activity of CFDC suggest that combination of CFDC with formulations that include avibactam or vaborbactam β-lactamases inhibitors might represent an appropriate choice to act synergistically against KPC-EB isolates.

In conclusion, CZA resistance mechanisms in KPC-EB may impair the *in vitro* activity of CFDC leading to co-resistance. CFDC in combination with the new β-lactamases inhibitors might represent a strategy to enhance its activity. Our data support the needing for further investigations on CFDC activity alone and in combination, against worldwide CZA resistant KPC-EB.

Declarations

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Competing interests

No competing financial interests exist.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article.

Code availability

Not applicable

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki. Formal ethical approval was obtained by Center's institutional review board.

Consent to participate

Not applicable

Consent for publication

Not applicable

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Turin, 23th August 2021

Editor-in-Chief

European Journal of Clinical Microbiology and Infectious Disease

We would like to thank the Editorial Team for his helpful suggestions, which in our view have greatly enhanced the quality and strength of our study. We hope that in this revised version the manuscript is now suitable for publication in European Journal of Clinical Microbiology and Infectious Disease.

Please, note that the changes to the original manuscript have been highlighted in the text. The response to the Editor's comments and ensuing modifications in the manuscript are also clearly indicated in the rebuttal.

Comments from Reviewers and point-by-point answers

Reviewer #3: I'm afraid that the authors did not address my most important comment, which would not be difficult, at least for the 17 isolates' pairs that were submitted to WGS. These isolates could be analyzed by core-genome MLST or SNP analysis and phylogenetic trees could be constructed. I also suggest that the authors provide some data on the geographical and temporal distribution of the isolates and the varying or not resistotypes (although these are not very meaningful).

If, as I feel, the isolates were clonal, this has to be mentioned as a limitation, which hampers the generalizability but does not eliminate the validity of the results.

We thank the Reviewer for this comment.

Five pairs of the susceptible and resistant KPC-producing K. pneumoniae isolates were previously characterized by WGS. Pairwise comparisons in the phylogenetic analysis showed tight clustering of isolates (0-1 single nucleotide polymorphism, SNP) in 4 of the

5 patients. We added these data in the revised manuscript R1 (see lines 178-181). Unfortunately, we did not analyze by WGS all the other pairs of isolates.

In the revised version of the manuscript, we added TABLE S1 as Supplementary material (see line 103) to provide some data on the geographical, temporal distribution and antimicrobial susceptibility patterns of the isolates selected in our study.

The lack of a full molecular typing of all isolates represents the main limitation of our study and we added it in the text (see lines 240-241).

Editor-in-Chief: As for the comments of the other referees, I see that the Introduction and Discussion sections were not shortened at all (Referee 2, comment 1), a comment should be added in the text that "The clinical relevance of synergistic testing remains controversial at best" (Referee 2, comment 3).

We thank you for this appraisal. Accordingly, we shortened the Introduction and Discussion sections. We added in the revised manuscript a comment regarding the controversial clinical relevance of synergistic testing (see lines 246-247).

Table

Table 1. MIC ranges, MIC50, MIC90, inhibition zones means and ranges, resistance percentages of cefiderocol and comparators against ceftazidime/avibactam susceptible and resistant KPC-producing Enterobacterales tested.

Species (no. of isolates)	Antimicrobial agent	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Resistance (%)	Inhibition zone mean, range (mm)	Resistance (%)
CZA susceptible EB (n= 60)	Cefiderocol	0.12-16	0.25	2	6.7	24.9, 15-29	13.3
	Ceftazidime-avibactam	0.064-8	1.5	4	0	NA	NA
	Ceftazidime	>32	>32	>32	100	NA	NA
	Cefepime	>8	>32	>32	100	NA	NA
	Meropenem	1 to >32	32	>32	86.6	NA	NA
	Imipenem	2 to >8	>8	>8	93.3	NA	NA
	Ertapenem	0.5 to >1	>1	>1	96.6	NA	NA
CZA resistant EB (n= 40)							
	Cefiderocol	0.5 to >64	8	64	82.5	16.8, 13-25	82.5
	Ceftazidime-avibactam	12 to >256	>256	>256	100	NA	NA
	Ceftazidime	>32	>32	>32	100	NA	NA
	Cefepime	>8	>8	>8	100	NA	NA
	Meropenem	≤0.12 to >32	1	32	32.5	NA	NA
	Imipenem	≤1 to >8	≤1	>8	35	NA	NA
	Ertapenem	0.5 to >1	>1	>1	72.5	NA	NA

Ceftazidime-avibactam MICs were determinated by Etest method. Cefiderocol, ceftazidime, cefepime, meropenem, imipenem and ertapenem MICs were determined by broth microdilution panels. MICs values were interpreted following current EUCAST breakpoints.

Ranges of antimicrobial agents (mg/L) tested: cefiderocol (0.03-64), ceftazidime avibactam (0.06-256), ceftazidime (1-32), cefepime (0.5-8), meropenem (0.12-32), imipenem (1-8), ertapenem (0.5-1). MICs were interpreted according to current EUCAST breakpoints.

Abbreviations: MIC, minimum inhibitory concentration; CZA, ceftazidime-avibactam; EB, Enterobacterales; NA, non applicable.

Table 2. Cefiderocol susceptibility in ceftazidime-avibactam susceptible to resistant KPC-producing *Klebsiella pneumoniae* isolates in patients treated with ceftazidime-avibactam.

			CZA susceptible KPC-Kp isolate before CZA treatment					CZA resistant KPC-Kp isolate following CZA treatment					
Patient	Age (year), sex	Patient type	Isolate	Material	CZA MIC (mg/L)	CFDC MIC (mg/L)	CFDC inhibition zone (mm)	Isolate	Material	bla_{KPC} variant (amino acid mutation)	CZA MIC (mg/L)	CFDC MIC (mg/L)	CFDC inhibition zone (mm)
1	42, M	Surgical	1-A	sputum	1	0.25	27	1-B	blood	KPC-31 (D179Y)	96	4	20
2	54, F	Surgical	2-A	blood	8	0.25	28	2-B	blood	KPC-33 (D179Y)	>256	8	19
3	67, M	Transplant recipient	3-A	blood	4	16	19	3-B	blood	KPC-3	32	32	18
4	63, M	Medical	4-A	blood	4	1	22	4-B	blood	KPC-3	64	4	18
5	69, F	Medical	5-A	bronchoaspirate	3	1	27	5-B	blood	KPC-14 (Δ242-GT-243)	>256	16	19
6	22, M	Transplant recipient	6-A	rectal swab	2	0.12	27	KpBC01	blood	KPC-14 (Δ242-GT-243)	64	64	12
7	44, M	Surgical	7-A	rectal swab	1.5	0.25	26	KpBC04	blood	KPC-33 (D179Y)	>256	64	15
8	79, M	Surgical	8-A	rectal swab	1	0.25	26	KpBC07	blood	KPC-31 (D179Y)	>256	32	15
9	40, M	Surgical	9-A	bronchoaspirate	0.75	0.12	29	KpBC05	blood	KPC-31 (D179Y)	>256	4	18
10	55, M	Medical	10-A	sputum	1.5	1	28	KpBC09	blood	KPC-2	>256	32	14
11	45, M	Medical	11-A	rectal swab	1	1	23	11-B	bronchoaspirate	untyped	>256	16	14
12	25, F	Surgical	12-A	rectal swab	1.5	0.25	25	12-B	bronchoalveolar lavage	untyped	12	0.5	24
13	63, F	Medical	13-A	blood	4	2	21	13-B	urine	untyped	>256	32	15
14	57, F	Medical	14-A	rectal swab	1.5	1	25	14-B	rectal swab	untyped	>256	16	17
15	69, F	Medical	15-A	rectal swab	0.75	0.5	27	15-B	rectal swab	untyped	>256	>64	13
16	62, M	Medical	16-A	rectal swab	0.064	0.25	26	16-B	rectal swab	untyped	>256	4	19
17	72, M	Transplant recipient	17-A	urine	1	0.12	27	17-B	pus wound	untyped	16	4	20

Abbreviations: CZA, ceftazidime-avibactam; KPC-Kp, KPC-producing *K. pneumoniae*; CFDC, cefiderocol; M, male; F, female.

Table 3. Genotypic characterization, antimicrobial susceptibility and synergy testing results of KPC-producing *Klebsiella pneumoniae*.

Isolate	Sequence type	KPC variant	CFDC susceptibility by broth microdilution (mg/L)	Minimum inhibitory concentrations (mg/L) by gradient diffusion testing					Fractional inhibitory concentration index			
				CFDC	CZA	MEV	IMP	AK	CZA	MEV	IMP	AK
1-A	307	*bla*$_{KPC-3}$	0.25	0.004	1	0.064	>32	>256	**0.25**	0.75	1.25	1.75
1-B	307	*bla*$_{KPC-31}$	4	0.25	96	0.094	0.25	>256	**0.43**	**0.3**	**0.38**	1.51
2-A	101	*bla*$_{KPC-2}$	0.25	0.004	8	6	>32	>256	1	0.83	2	2
2-B	101	*bla*$_{KPC-33}$	8	0.25	>256	1.5	0.75	>256	0.62	0.71	0.71	2
5- A	1685	*bla*$_{KPC-2}$	1	0.008	3	1.5	>32	4	**0.5**	0.71	1.5	2
5-B	1685	*bla*$_{KPC-14}$	16	0.38	>256	1	0.25	4	**0.5**	**0.45**	1.26	1.41

Bold character indicates synergistic effect.

Abbreviations: CFDC, cefiderocol; CZA, ceftazidime-avibactam; MEV, meropenem-vaborbactam; IMP, imipenem; AK, amikacin.