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Short Communication

In vitro antibacterial activity of ceftazidime/avibactam in combination against planktonic and biofilm carbapenemase-producing *Klebsiella pneumoniae* isolated from blood

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ABSTRACT

Objectives: The aim of this study was to report on in vitro tests of antibacterial activity of ceftazidime/avibactam in combination against planktonic or biofilm KPC carbapenemase-producing *Klebsiella pneumoniae* (KPC-Kp), the rate of KPC-Kp blood isolates in University of Perugia Hospital over a 5-year period, and their antimicrobial susceptibility patterns.
Methods: The antibacterial activity of ceftazidime/avibactam in combination with other antimicrobials was assessed against planktonic and biofilm bacteria by Etest and checkerboard assay. A retrospective review of laboratory data was performed to evaluate the rate of KPC-Kp from blood samples and their antimicrobial susceptibility patterns.
Results: Between 2014 and 2019, 130/4241 (3.1%) KPC-Kp were identified from blood cultures. Their rate increased from 2.3% in 2014–2015 to 4.5% over the last 3 years. Overall, 4.6% (6/130) of KPC-Kp isolates were susceptible to meropenem, 65.4% (85/130) to colistin, 65.1% (84/129) to tigecycline, 34.6% (45/130) to amikacin, 36.2% (42/116) to gentamicin, 40.2% (39/97) to fosfomycin and 91.5% (65/71) to ceftazidime/avibactam. Five of six ceftazidime/avibactam-resistant KPC-Kp were isolated from patients not treated with ceftazidime/avibactam. Synergism was detected both by Etest and checkerboard assay for the combination of ceftazidime/avibactam plus meropenem against planktonic isolates, whilst lower bactericidal activity was observed in biofilm KPC-Kp isolates.
Conclusions: Our in vitro data suggest that the combination of ceftazidime/avibactam plus meropenem has a synergistic antibacterial activity against planktonic bacteria, whilst a lower activity was detected against biofilm, suggesting worse clinical outcomes whenever biofilm infections are present. Further analyses are required to confirm these results before extending them to clinical practice.

1. Introduction

Over the last decade, the prevalence of KPC carbapenemase-producing *Klebsiella pneumoniae* (KPC-Kp) infections has increased worldwide [1]. KPC-Kp isolates are characterised by several antimicrobial resistances and these bacteria are often multi-drug-resistant (MDR) [2]. The multiple broad-spectrum resistance pattern along with the burden of co-morbidities in patients diagnosed with KPC-Kp infections poses therapeutic challenges and contributes to elevated mortality rates. Mortality rates have been reported to be highest for those patients diagnosed with bloodstream infection (BSI) [3]. Several studies [4,5] have suggested treating patients with KPC-Kp infections using combination regimens that improve bactericidal activity and overcome the emergence of new resistance. Among these combinations, those associated with the most favourable clinical courses and the lowest mortality rates included a carbapenem [6]. However, some authors have suggested not to prescribe a regimen including a carbapenem in the presence of high KPC-Kp endemicity [7].

The aim of this study was to report on in vitro tests evaluating the antibacterial activity of ceftazidime/avibactam (CAZ/AVI) in

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combination against selected KPC-Kp blood isolates both in planktonic and biofilm phases of growth. Moreover, we defined the rate of KPC-Kp blood culture positivity among patients admitted to Perugia Hospital over 5 years as well as the antimicrobial susceptibility patterns of these bacteria.

2. Materials and methods

2.1. Bacterial isolates

Between June 2014 and June 2019, data from our microbiology laboratory were analysed retrospectively to determine the rate of blood culture positivity. For each pathogen/patient combination, only the first blood isolate was included in the analysis.

Blood cultures were collected using BD BACTEC™ Plus Aerobic/F and BD BACTEC™ Lytic/10 Anaerobic/F bottles and were incubated within 1 h from collection using a BD BACTEC™ FX instrument (Becton Dickinson, Sparks, MD, USA). Positive cultures were processed for Gram staining and subculture on solid media manually or, as for the last 2 years of the study, automatically with a BD Work Cell Automation (WCA) System (Becton Dickinson). Colonies were identified using a MALDI Biotyper instrument (Bruker Daltonik GmbH, Bremen, Germany). Antimicrobial susceptibility testing was performed using a BD Phoenix™ Automated Microbiology System (Becton Dickinson) and the results were interpreted according to current European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints [8]. *Klebsiella pneumoniae* organisms suspected of being MDR were tested by the Sensititre™ microdilution method (Thermo Fisher Scientific, Cleveland, OH, USA) [9,10]. *Klebsiella* isolates were tested for carbapenemases using an Xpert® Carba-R assay (Cepheid, Sunnyvale, CA, USA) [11].

Identification of KPC-Kp in blood cultures in patients with symptoms/signs of systemic infection was regarded as true BSI, given the high positive predictive value of having KPC-Kp isolated from blood.

KPC-Kp were stored in our microbiology laboratory using glycerol broth at −80 °C. Isolate numbers 147/17, 85/18 and 38/18 were selected for further study. Of the three isolates, 3/3 were resistant to meropenem and tigecycline, 1/3 to CAZ/AVI and 1/3 to fosfomycin [8]. Given this pattern of susceptibility, they were representative of difficult-to-treat bacteria.

2.2. Biofilm formation and quantification

The ability of the selected isolates to develop mature biofilm after 48 h of incubation was evaluated before bactericidal studies were performed.

Biofilm formation was performed as previously described by Naparstek et al. with some modifications [12]. Loops of overnight cultures were suspended in Mueller–Hinton broth, were adjusted to a 0.5 McFarland standard and, after a 1:10 dilution, 200 μL was inoculated in each well of a 96-well plate. The number of bacteria inoculated was 1–5 × 10^7 CFU/mL. Plates were incubated for 48 h at 35 °C, with medium renewal after the first 24 h. After the medium was discarded, plates were washed twice with saline solution, fixed with 99% methanol and stained for 15 min with 0.1% crystal violet (CV). Excess CV was rinsed twice with saline and then 200 μL of 33% acetic acid was added and the biofilm was quantified using a microplate reader at 570 nm (OD_{570}) (Tecan Infinite M200; Tecan Trading AG, Männedorf, Switzerland). The experiments were carried out in triplicate, repeated on two different days and averaged. Results are expressed as mean ± standard deviation (S.D.). Interpretation of the level of biofilm formation was done accordingly to Stepanović et al. [13].

2.3. Antimicrobial susceptibility testing

Ceftazidime/avibactam (Zavicefta®; Pfizer, Ringaskiddy, County Cork, Ireland), meropenem (Hikma Farmaceutica, Terrugem, Portugal), tigecycline (Tygacil®; Pfizer, Sandwich, UK) and fosfomycin (InfectoFos®; InfectoPharm, Heppenheim, Germany) powders from commercial preparations were used for the experiments. Each antibiotic was diluted and stock solutions were prepared and maintained at −80 °C until used. Minimum inhibitory concentrations (MICs) were determined both by Etest and the microdilution method and the results were interpreted according to EUCAST guidelines [8]. Minimum bactericidal concentrations (MBCs) were determined by subculturing on antibiotic-free plates 10 μL from each well without visible growth. The MBC was defined by the concentration without colony growth. The experiments were repeated on two different days and the results were concordant.

2.4. Antimicrobial synergy evaluation

Synergism between antibiotic combinations was evaluated by Etest and the checkerboard assay for planktonic bacteria, and the latter method was used when biofilm bacteria were investigated. The antibiotics tested were CAZ/AVI in combination with meropenem, tigecycline or fosfomycin.

2.4.1. Etest synergism

Etest strips were placed perpendicularly crossing them at the respective MIC of antibiotics and the results were defined as previously described and expressed as the fractional inhibitory concentration index (FICI) [14]. A FICI of ≤0.5 was considered synergistic, 0.5 < FICI ≤ 1 as additive, 1 < FICI ≤ 4 as indifferent and FICI > 4 as antagonistic.

2.4.2. Checkerboard assay

The checkerboard assay against planktonic bacteria was used to evaluate CAZ/AVI in combination with meropenem, tigecycline or fosfomycin. CAZ/AVI concentrations ranged from 0.063–2× the MIC of each isolate, whilst those of meropenem, tigecycline and fosfomycin ranged from 0.031–2× MIC. After 24 h of incubation, the FICI was evaluated in wells sited at the turbidity/non-turbidity interface of bacterial growth [10]. Isobologram curves were plotted through the extrapolation of MICs of antibiotic alone and in combination.

Activity against biofilm bacteria was evaluated as follows: 200 μL of the antibiotic dilution alone or in combination was added to each well having a mature biofilm formed and plates were then incubated at 35 °C for 24 h. Metabolic activity was evaluated through the XTT reduction assay and the concentration producing a ≥50% reduction in metabolic activity with respect to the control was defined as the minimal biofilm eradication concentration ($MBEC_{50}$) [15]. Whenever the highest antibiotic concentration tested was unable to reach a 50% reduction in the metabolic activity, the $MBEC_{50}$ was defined by the concentration above the highest concentration tested [16]. Isobologram curves were plotted through the extrapolation of $MBEC_{50}$ values of antibiotics alone and in combination. Experiments were repeated on two different days and the results were concordant.

3. Results

Over the study period, a total of 4241 blood cultures resulted positive. Among these positive cultures, 130 (3.1%) grew KPC-producing *K. pneumoniae* from 113 patients. Ten patients presented 1 or more relapses, for a total of 17 relapses. The isolation rate of

KPC-Kp isolates increased from 2.3% in the years 2014–2015 to 4.5% over the remaining 3 years.

Of the 130 BSI episodes, 26.9% (35/130) were recorded in the intensive care unit (ICU), 35.4% (46/130) in the internal medicine department, 26.2% (34/130) in the onco-haematology unit and 11.5% in the surgical unit (15/130). Moreover, 37 (28.5%) were related to a central venous catheter. The first time a KPC-Kp blood culture positivity was detected occurred at a mean of 20.5 days after admission, whilst relapses occurred on average 88.5 days after the first episode. A total of 68/113 patients (60.2%) with a positive KPC-Kp blood culture were male and the mean age was 65 years.

Regarding antimicrobial susceptibility, overall 4.6% (6/130) of the isolates were susceptible to meropenem, 65.4% (85/130) to colistin, 65.1% (84/129) to tigecycline, 34.6% (45/130) to amikacin, 36.2% (42/116) to gentamicin, 40.2% (39/97) to fosfomycin and 91.5% (65/71) to CAZ/AVI. Five of six CAZ/AVI-resistant KPC-Kp were detected in patients who had never been treated with CAZ/AVI.

Regarding the antimicrobial resistance patterns of relapsing BSI isolates, we evidenced higher rates of resistance to colistin (+7.1%), gentamicin (+17.8%) and fosfomycin (+7.8%). These relapsing patients had been treated with different antimicrobial combinations that included the abovementioned.

MICs and MBCs for the antibiotics studied are listed in Table 1. Isolate 38/18 was resistant to CAZ/AVI, meropenem, tigecycline and fosfomycin, whereas isolates 147/17 and 85/18 were resistant to meropenem and tigecycline [8].

Regarding the synergism studies on planktonic bacteria, CAZ/AVI combined with meropenem produced a synergistic activity against isolates 147/17 and 85/18 by Etest. Etest synergy assay was not performed for *K. pneumoniae* 38/18 as the meropenem concentrations in the strip were lower than the MIC of this isolate. CAZ/AVI and meropenem were synergistic by the microdilution checkerboard assay against the three isolates in planktonic growth (Fig. 1 a, d, f). The combination CAZ/AVI plus tigecycline resulted in indifference by Etest against all three isolates, whilst by the checkerboard assay an additive interaction was detected for isolates 147/17 and 85/18 and indifference for isolate 38/18. The combination CAZ/AVI and fosfomycin was synergistic by Etest against the three isolates and was additive by the checkerboard assay against isolates 147/17 and 38/18 and indifferent against isolate 85/18.

The CV staining method was used to evaluate biofilm formation. In our experimental conditions, after 48 h of incubation with medium renewal, strains 147/17, 38/18 and 85/18 reached mean ± S.D. values of OD_{570} of 1.78 ± 0.21, 0.54 ± 0.05 and 0.49 ± 0.11, respectively. Following the classification of Stepanović et al. [13], the strains were considered strong biofilm producers.

When biofilm bacteria were evaluated, the regimen of CAZ/AVI combined with meropenem evidenced synergistic activity against isolates 147/17 and 38/18 (Fig. 1b and e) and an additive effect for isolate 85/18. The combination CAZ/AVI with tigecycline had a synergistic effect only for isolate 147/17 (Fig. 1c). For all the other antibiotic combinations tested, indifference was observed.

4. Discussion

The aim of this study was to report on in vitro assays evaluating the antibacterial activity of CAZ/AVI in combination with other antimicrobials against selected KPC-Kp blood isolates in planktonic and biofilm phases of growth. Moreover, we defined the rate of KPC-Kp blood culture positivity among patients admitted to Perugia Hospital over 5 years as well as the antimicrobial susceptibility patterns of these bacteria.

Our epidemiology, in accordance with national and international data [17], revealed an increase in the rate of KPC-Kp-positive blood cultures. Most of these occurred in males aged >65 years admitted to internal medicine department or ICU. A high rate (30%) was also recorded for onco-haematological patients. Being older, having several co-morbidities, the presence of devices, admission to the ICU or being diagnosed with an onco-haematological condition are recognised factors favouring colonisation and infection with KPC-Kp. Moreover, onco-haematological patients receiving fluoroquinolone prophylaxis have a greater risk for acquiring KPC-Kp infections. All of these risk factors are also negatively associated with clinical outcome in KPC-Kp infections, including BSI [18].

Antimicrobial susceptibility results for BSI KPC-Kp isolates evidenced a high rate of meropenem resistance (95.4%); all of the isolates had a meropenem MIC > 8 mg/L, suggesting a lower clinical efficacy when meropenem is used in combination with other antimicrobials [4,15]. CAZ/AVI resistance was observed in 6/71 (8.5%) isolates tested. However, five of six isolates had primary CAZ/AVI resistance. With regard to colistin and tigecycline susceptibility, overall these antibiotics had activity against ca. 65% of the strains (65.4% and 65.1%, respectively), yet over the last 2 years, the colistin resistance rate decreased from 45% to 23%, most likely reflecting a greater use of CAZ/AVI. In this setting, we highly recommend assessing for in vitro activity in all KPC-Kp isolates.

KPC-Kp isolates from patients with relapsing BSIs evidenced growing rates of resistance to antimicrobials that had been administered in the previous septic episodes.

An optimal therapeutic regimen for KPC-Kp infections has not yet been defined. Several studies have reported reduced mortality in combination therapy compared with monotherapy. Currently, the most recommended strategy is a combination including a carbapenem. In fact, Tumbarello et al. have reported an improved outcome with a combination including a carbapenem whenever isolates had a meropenem MIC ≤ 8 mg/L [6]. On the other hand, some authors have also obtained good clinical outcomes with isolates having meropenem MICs up to 64 mg/L [19]. Clinical data remain limited with regard to combination regimens including CAZ/AVI, however its use in combined regimens has been supported [20].

Table 1
Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of ceftazidime/avibactam, meropenem, tigecycline and fosfomycin against three selected KPC carbapenemase-producing *Klebsiella pneumoniae* blood isolates.

Antibiotic	147/17			38/18			85/18		
	MIC	I[a]	MBC	MIC	I[a]	MBC	MIC	I[a]	MBC
Ceftazidime/avibactam	4	S	4	32	R	64	4	S	6
Meropenem	16	R	16	512	R	1024	16	R	16
Tigecycline	2	R	128	4	R	32	2	R	64
Fosfomycin	8	S	8	256	R	1024	32	S	512

S, susceptible; R, resistant.
[a] Interpretation (I) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) [8].

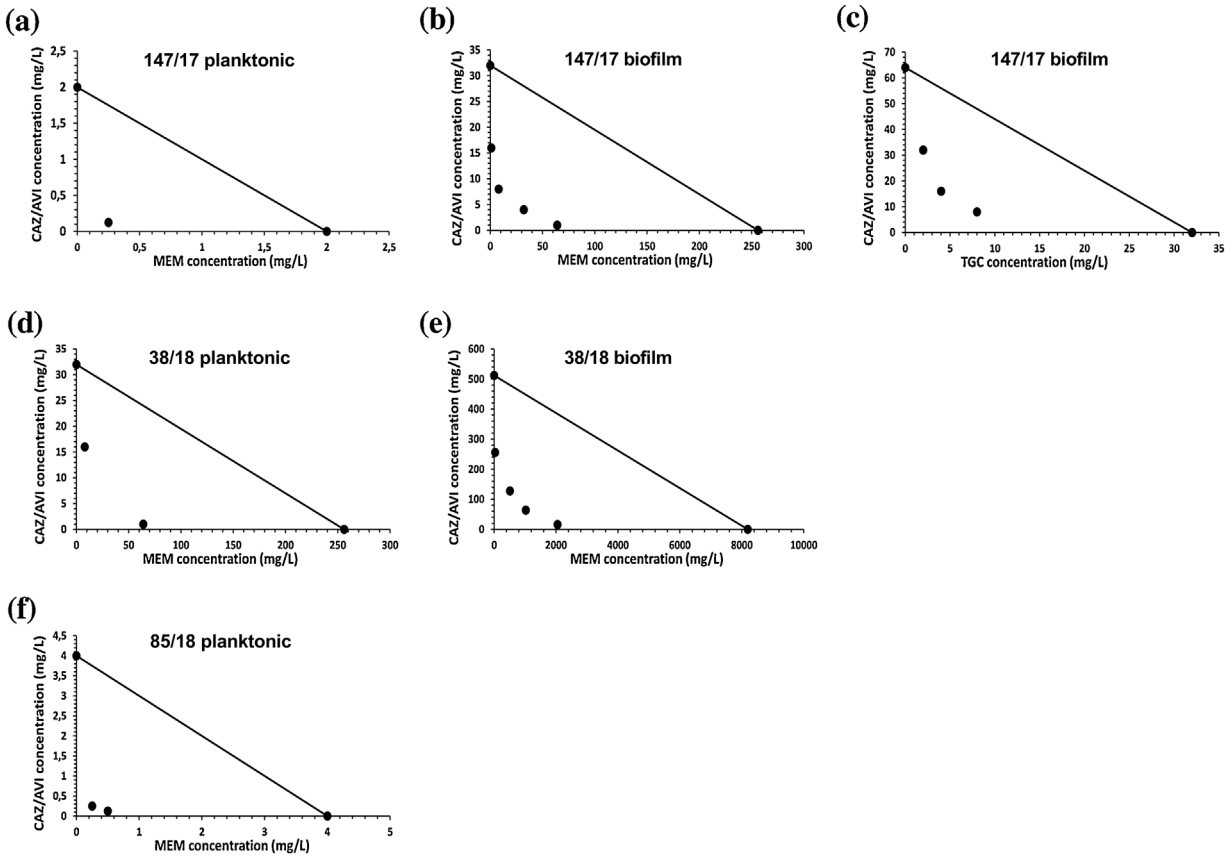

Fig. 1. Synergism of ceftazidime/avibactam (CAZ/AVI) in combination with meropenem (MEM) against planktonic bacteria isolates of strains 147/17, 38/18 and 85/18 (isobolograms a, d and f, respectively); CAZ/AVI in combination with MEM against biofilm isolates of strains 147/17 and 38/18 (isobolograms b and e, respectively); and CAZ/AVI in combination with tigecycline (TGC) against biofilm isolate 147/17 (isobologram c). All the experiments were repeated on two different days. The results were concordant and representative isobolograms are shown.

Nowadays, there are no data regarding the use of checkerboard assay with *K. pneumoniae* biofilm and the evaluation of CAZ/AVI combination therapies. Our in vitro results on CAZ/AVI activity, when combined with other antimicrobials, suggested different behaviours for bacteria in a planktonic or sessile state. Indeed, the combination of CAZ/AVI with meropenem was synergistic against all planktonic bacteria, even for the strain with high CAZ/AVI and meropenem MICs. On the other hand, synergism of CAZ/AVI in combination with meropenem was observed against 2/3 biofilm strains, but only very high antibiotic concentrations were able to affect the biofilm viability of strain 38/18. Furthermore, regarding the other combinations tested, we observed a positive effect both in planktonic and biofilm form only in the presence of tigecycline and against a single strain.

A limitation of this study includes the limited number of KPC-Kp assessed. Moreover, in vitro results might not be fully transferable to clinical practice given that is not possible to generalise the clinical usefulness of the combination of CAZ/AVI plus meropenem against MDR strains.

5. Conclusions

Our in vitro data suggest that the combination of CAZ/AVI plus meropenem enhances antibacterial activity against planktonic bacteria, but a lower activity was detected for biofilm bacteria since high antimicrobial concentrations were required to affect MDR strains. Similarly, CAZ/AVI antibiotic combinations with fosfomycin and tigecycline were not efficient in biofilm disruption. This preliminary study suggests a reduced clinical outcome whenever biofilm-associated infections are present. Further analyses are needed to confirm these results before extending them to clinical practice.

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None.

Competing interests

None declared.

Ethical approval

Not required; no patient information was stored in the study database.

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