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Efficacy of ceftolozane/tazobactam, alone and in combination with colistin, against multidrug-resistant *Pseudomonas aeruginosa* in an *in vitro* biofilm pharmacodynamic model

Joan Gómez-Junyent[a], Eva Benavent[a], Yanik Sierra[b], Cristina El Haj[a], Laura Soldevila[a], Benjamín Torrejón[c], Raul Rigo-Bonnin[d], Fe Tubau[b], Javier Ariza[a,e], Oscar Murillo[a,e,*]

[a] *Infectious Diseases Service, Laboratory of Experimental Infection, Hospital Universitari de Bellvitge, IDIBELL, Universitat de Barcelona, Barcelona, Spain*
[b] *Department of Microbiology, Hospital Universitari de Bellvitge, IDIBELL, Universitat de Barcelona, Barcelona, Spain*
[c] *Centres Científics i Tecnològics, Universitat de Barcelona, Barcelona, Spain*
[d] *Department of Clinical Laboratory, Hospital Universitari de Bellvitge, IDIBELL, Universitat de Barcelona, Barcelona, Spain*
[e] *Spanish Network for the Research in Infectious Diseases (REIPIRD12/0015), Instituto de Salud Carlos III, Madrid, Spain*

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ABSTRACT

Objectives: Ceftolozane/tazobactam is a potential tool for infections caused by multidrug-resistant (MDR) *Pseudomonas aeruginosa* (*P. aeruginosa*), but its efficacy against some difficult-to-treat infections has not been well defined.

Methods: Using an *in vitro* pharmacodynamic biofilm model, this study evaluated the comparative efficacy of ceftolozane/tazobactam against MDR/extensively drug-resistant (XDR) *P. aeruginosa* strains, alone and in combination with colistin. Simulated regimens of ceftolozane/tazobactam (2 g/1 g every 8 h), meropenem (2 g every 8 h) and ceftazidime (2 g every 8 h), alone and in combination with colistin (continuous infusion) were evaluated against three colistin-susceptible and ceftazidime-resistant strains: MDR-HUB1, ceftolozane/tazobactam-susceptible and meropenem-susceptible; XDR-HUB2, ceftolozane/tazobactam-susceptible and meropenem-resistant; MDR-HUB3, ceftolozane/tazobactam-resistant and meropenem-susceptible. Antibiotic efficacy was evaluated by decreases in bacterial counts (Δlog CFU/mL) from biofilm-embedded bacteria over 54 h. Resistance emergence was screened.

Results: Among monotherapies, ceftolozane/tazobactam had low killing but no resistance appeared, ceftazidime was ineffective, colistin was initially effective but regrowth and resistance occurred, and meropenem was bactericidal against carbapenem-susceptible strains. Ceftolozane/tazobactam plus colistin was the most effective combination against the meropenem-resistant XDR-HUB2 strain (Δlog CFU/mL 54–0 h = −4.42 vs. −3.54 for meropenem-colistin; $P = 0.002$), whereas this combination against MDR-HUB1 (−4.36) was less effective than meropenem-colistin (−6.25; $P < 0.001$). Ceftolozane/tazobactam plus colistin was ineffective against the ceftolozane/tazobactam-resistant strain; meropenem plus colistin was the most bactericidal therapy (−6.37; $P < 0.001$ vs. others). Combinations of active beta-lactams plus colistin prevented the emergence of colistin-resistant strains.

Conclusions: Combinations of colistin plus ceftolozane/tazobactam and meropenem were the most appropriate treatments for biofilm-related infections caused by XDR and MDR *P. aeruginosa* strains, respectively. These combinations could be considered as potential treatment options for these difficult to treat infections.

1. Introduction

Osteoarticular and orthopaedic device-related infections are among the most frequent concerns and therapeutic challenges of infectious diseases. While staphylococci are the most common microorganisms responsible for these infections, a significant rise in the proportion of device-related infections caused by

* Corresponding author. Oscar Murillo, Infectious Diseases Service, Hospital Universitari de Bellvitge. IDIBELL, Universitat de Barcelona, FeixaLlarga s/n, 08907 L'Hospitalet de Llobregat, Barcelona, Spain. Tel.: 34 93 260 76 25; fax: 34 93 260 76 37.
E-mail address: omurillo@bellvitgehospital.cat (O. Murillo).

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Gram-negative bacilli (GNB) has been observed in recent years [1]. Moreover, the emergence of multidrug-resistant (MDR) GNB and, particularly, the global spread of MDR *Pseudomonas aeruginosa* (*P. aeruginosa*) is worrisome in the setting of device-related infections due to the presence of biofilms. Bacterial biofilms impair the activity of most antibiotics [2,3], and very limited options exist for the treatment of these MDR *P. aeruginosa* strains, which are commonly resistant to fluoroquinolones and have a decreased susceptibility to beta-lactams [4].

Colistin is often the only active drug that can be used against these MDR microorganisms [5,6]. Colistin may have notable activity against biofilm-embedded bacteria present in the inner layers of biofilms [7,8], but its clinical efficacy can be threatened by its toxicity and the ability to select for resistant subpopulations when given in monotherapy. Thus, its administration in combination with other antibiotics, such as beta-lactams, may provide a synergistic effect and protect against the emergence of resistant strains [9–11].

Recently, the appearance of ceftolozane/tazobactam, a novel cephalosporin in combination with a beta-lactamase inhibitor, has represented a promising opportunity for the treatment of serious infections by MDR *P. aeruginosa* [12]. It is approved for the treatment of intraabdominal and urinary tract infections, but in the current global era of multiresistance there is a need to improve the knowledge about its efficacy against other infections caused by MDR and XDR strains of *P. aeruginosa*, such as biofilm-related infections, in which scarce experience exists.

Thus, the objective of this study was to evaluate the activity of ceftolozane/tazobactam, in comparison with that of meropenem and ceftazidime, alone and in combination with colistin against MDR and XDR *P. aeruginosa* in an *in vitro* pharmacodynamic biofilm model. It also aimed to investigate the protection of resistance to colistin and ceftolozane/tazobactam after exposure to these antibiotics.

2. Materials and methods

2.1. Bacterial isolates

Three clinical isolates of *P. aeruginosa*, all colistin-susceptible but ceftazidime-resistant strains, were used: HUB1, a ceftolozane/tazobactam-susceptible and meropenem-susceptible MDR strain (ST308); HUB2, a ceftolozane/tazobactam-susceptible and meropenem-resistant XDR strain (ST175); and HUB3, a ceftolozane/tazobactam-resistant and meropenem-susceptible MDR strain (ST274). The three strains have spread worldwide and are considered to be high-risk clones [13]; mechanisms of resistance are AmpC hyperproduction for all strains (MDR-HUB3 having the AmpR mutation G154R), plus OprDporin deletion in XDR-HUB2. Multidrug resistant and XDR were defined in accordance with previous criteria [14]; antibiotic susceptibility was interpreted according to EUCAST criteria.

2.2. Antibiotics

Ceftolozane was provided by MSD (Merck Sharp & Dohme, Spain), whereas the remaining drugs were purchased from the manufacturers' laboratory (Sigma-Aldrich, Madrid, Spain). Stock solutions of antibiotics were re-suspended immediately prior to each experiment following the laboratories' recommendations.

2.3. Determination of MIC, MBC, and minimum biofilm inhibitory and eradication concentrations

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by broth microdilution method using standard recommendations [15]. The minimum biofilm inhibitory (MBIC) and eradication (MBEC) concentrations were determined based on previously described methodology [16], using an MBEC™ device (Innovotech Inc., Canada). All tests were performed at least in triplicate.

2.4. In vitro pharmacokinetics/pharmacodynamics (PK/PD) biofilm model

A CDC Biofilm Reactor (CBR) (BioSurface Technologies, USA) was used, which consists of a glass vessel with an effluent spout giving place to an operational volume of 350 mL in continuous mixing by a magnetic baffled stir bar. Antibiotics and media can be added through the ports in the top lid of the reactor, from where eight rods descend, each housing three removable Teflon coupons (biofilm growth surfaces), for a total of 24 sampling opportunities throughout the experiment.

The protocol followed previously reported methods [17–19], and consisted of a biofilm conditioning phase, in which the biofilm was formed for 48 h, followed by a therapeutic phase. Briefly, the biofilm conditioning phase started with the bacteria inoculation into the reactor (initial inoculum of 7 log CFU/mL), followed by a 24-h batch culture at 37 °C in drug-free 20% TSB. Then, fresh sterile 20% TSB was infused into the model for 24 h using a peristaltic pump (Masterflex, Cole-Parmer, USA), to achieve a bacterial residence time within the reactor shorter than the generation time for the suspended bacteria. The generation time (g) was calculated according to the following equation:

$$g = \mathrm{Ln}\,2/\mu;$$

where μ is the growth rate,

$$\mu = \mathrm{Ln}\,N - \mathrm{Ln}\,N_0/t - t_0;$$

where N is the number of bacteria at time t, and N_0 is the number of bacteria at time t_0.

Thus, the generation times, infusion rates and estimated bacterial residence times within the reactor were as follows: 45 min, 8 mL/min and 43.75 min, respectively, for MDR-HUB3, and 60 min, 6 mL/min and 58.33 min, respectively, for MDR-HUB1 and XDR-HUB2.

Once the biofilm was formed, the therapeutic phase started (time zero, 0 h). For the three beta-lactam regimens, a bolus dose was injected into the model every 8 h to achieve the desired free-drug C_{max} (fC_{max}; in accordance with the protein binding for each drug). Then, fresh media (20% TSB) was pumped at a flow rate reproducing the respective beta-lactam $t_{1/2}$.

Evaluated regimens were as follows: ceftazidime, 2 g every 8 h (fC_{max} 134 mg/L, $t_{1/2}$ 2 h, flow rate 2 mL/min, protein binding considered 16%); meropenem, 2 g every 8 h (fC_{max} 90 mg/L, $t_{1/2}$ 1 h, flow rate 4 mL/min, protein binding considered 10%); ceftolozane/tazobactam, 2 g/1 g every 8 h (fC_{max} 111 mg/L, $t_{1/2}$ 2.5 h, flow rate 1.61 mL/min, protein binding considered 21%/fC_{max} 25 mg/L, $t_{1/2}$ 2.5 h, flow rate 1.61 mL/min, protein binding considered 30%, respectively) [20–22].

For the particular case of ceftolozane/tazobactam combination, with different $t_{1/2}$ (2.5 h and 1 h, respectively), the $t_{1/2}$ of ceftolozane was reproduced and it was assumed that tazobactam would be eliminated at the same $t_{1/2}$, thus providing tazobactam concentrations during the whole 8-h period always in adequate proportion with ceftolozane (at least 2:1). In all cases, flow rates were calibrated prior to each experiment and monitored throughout to ensure that the system was performing optimally.

Colistin was pumped into the CBR as a continuous infusion at 3.50 mg/L, which mimicked the plasma steady-state concentration observed in humans by 6–9 MU colistin every 24 h [23–24]. This

Table 1
Minimum inhibitory concentrations, minimum bactericidal concentrations, minimum biofilm inhibitory concentrations, and minimum eradication concentrations for the different antibiotics among all *Pseudomonas aeruginosa* strains.

Antibiotics	MDR-HUB1				XDR-HUB2				MDR-HUB3			
	MIC	MBC	MBIC	MBEC	MIC	MBC	MBIC	MBEC	MIC	MBC	MBIC	MBEC
CST	1	4	8	> 64	2	2	8	64	2	2	8	> 64
CAZ	64	128	> 256	> 256	32	32	> 256	> 256	64	> 256	> 256	> 256
MEM	2	4	2	> 256	16	16	16	> 256	2	4	2	> 256
*TOL/TZB	2	4	8	> 256	4	4	16	> 256	8	8	16	> 256

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MBIC = minimum biofilm inhibitory concentration; MBEC = minimum biofilm eradication concentration; CST = colistin; CAZ = ceftazidime; MEM = meropenem; TOL/TZB = ceftolozane/tazobactam.

* The MIC, MBC, MBIC and MBEC values refer to the concentration of ceftolozane in the presence of a fixed concentration of tazobactam at 4 mg/L.

was achieved by bolus administration at 0 h followed by infused medium with colistin at the appropriate concentration.

For all strains, the therapeutic regimens evaluated were ceftazidime, meropenem, ceftolozane/tazobactam and colistin, as monotherapies, the respective beta-lactams in combination with colistin, and controls (no antibiotic). All the experiments were performed at least in duplicate.

2.5. Pharmacodynamic analysis

One sample from medium (free-floating bacteria) and three coupons from a rod (biofilm-embedded bacteria) were collected at 0, 6, 24, 30, 48, and 54 h (two extra coupons were collected at the last time point). The removed coupons were processed following previously described methodology [17,19]; medium and coupon samples were serially diluted (10-fold), plated on agar plates (Beckton Dickinson, Spain), and incubated at 37 °C for 24–48 h.

Bacterial counts were expressed as log CFU/mL (means and standard deviations [SD]). Efficacy was evaluated against biofilm-embedded and free-floating bacteria using the log change method from 0 h to each *t* timepoint (Δlog CFU/mL *t*h-0h). Treatments were considered to be bactericidal (or to have bactericidal effect) when they led to a ≥ 3 log CFU/mL reduction, compared with the corresponding counts at zero time. Monotherapy or combination regimens causing a reduction of ≥ 1 log CFU/mL at a specified time were considered active. Synergy (or synergistic effect) was defined as ≥ 2 log CFU/mL killing for the combination relative to the most active monotherapy at a specified time; additivity was defined as 1–2 log CFU/mL greater killing for the combination.

2.6. Pharmacokinetic studies

For these studies, the CBR was filled with saline serum, antibiotic boluses were injected into the CBR, and peristaltic pumps were set up in accordance with simulated $t_{1/2}$ (described above). Samples were then collected into 1 mL polypropylene test tubes at different time points and stored at −20 °C until analysis. All antibiotics and tazobactam concentrations were analysed by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC/MS-MS), following a methodology previously standardised by the current group [25]; a prior standardisation of this method was developed for ceftolozane and tazobactam.

2.7. Colistin population analysis profiles, and resistance studies

Baseline heteroresistance to colistin was studied by the screening of subpopulations (population analysis profiles, PAPs) able to grow in the presence of ≥ 2, 4, 8, and 16 mg/L of colistin, applying previously reported methods [9,17]. To evaluate the emergence of resistance to ceftolozane/tazobactam and colistin during the therapeutic experiments, samples from coupons at all time points were plated onto nutrient agar plates containing 4–4 mg/L of ceftolozane/tazobactam and 2 mg/L of colistin. Results were interpreted as positive if any macroscopic growth was observed. For the particular case of XDR-HUB2 strain, the PAPs of colistin from isolates recovered at the end of experiments were also analysed.

2.8. Confocal laser scanning microscopy

Coupons were evaluated by confocal laser scanning microscopy (CLSM) to confirm biofilm infection (0 h) and treatment activity (54 h). Images of the biofilms stained with LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher Scientific, USA) were acquired using a Leica TCS-SL filter-free spectral confocal laser scanning microscope (Leica Microsystems, Germany) equipped with a 488 nm argon laser and 543 nm He/Ne laser (Centres CientíficsiTecnològics, Universitat Barcelona, Spain) using a 63x oil immersion objective (1.4 numerical aperture). Different image stacks were acquired with a 0.5 microns' distance between planes and the pinhole size was kept at 1 AU. The number of total planes was calculated according with the thickness of each biofilm. Three different stacks were obtained randomly of each coupon. Selected fields were acquired with zoom 4 and an image resolution of 1024 × 1024 pixels. The images obtained were processed with IMARIS software (Bitplane AG, Switzerland).

2.9. Statistical analysis

Data were analysed using Stata 13.1 (Stata Corporation, USA). An analysis of variance with Tukeyś post hoc test was performed for each treatment regimen to evaluate changes in the log CFU/mL for free-floating and biofilm-embedded bacteria. A *P*-value of ≤ 0.05 was considered statistically significant.

3. Results

Table 1 summarises the MIC, MBC, MBIC and MBEC for all strains. Targeted values of PK parameters for intermittent administration of beta-lactams were well reproduced; observed fC_{max} concentrations (mean ± SD) were within 15% of the targeted values: 115 mg/L ± 2.1 for ceftazidime ($t_{1/2}$ 2 h), 94 mg/L ± 1 for meropenem ($t_{1/2}$ 1 h), 100 mg/L ± 1.9 and 24 mg/L ± 0.4 for ceftolozane and tazobactam, respectively ($t_{1/2}$ 2.5 h).

3.1. Microbiological response

The bacterial growth of biofilm-embedded and free-floating cells in the absence of antibiotics for all strains is illustrated in Fig. 1. Mean inoculums for biofilm-embedded cells at 0 h were higher for MDR strains (HUB1 and HUB3) than for XDR-HUB2.

Bacterial counts (log changes) of biofilm-embedded in the presence of antibiotics throughout the experiments are shown in

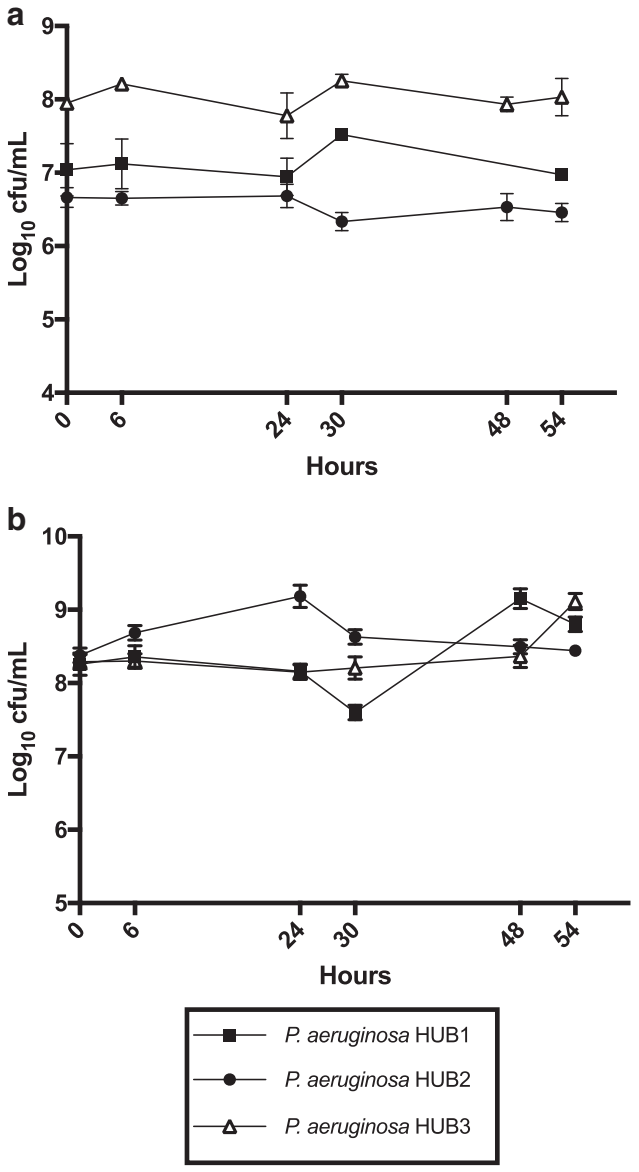

Fig. 1. Bacterial growth in the absence of antibiotics for biofilm-embedded (a) and free floating (b) cells for the three strains of *Pseudomonas aeruginosa*.
Time on the *x*-axis begins immediately after the 48-hour conditioning phase
Data presented as means ± SD
P. aeruginosa = *Pseudomonas aeruginosa*.

Fig. 2. Among monotherapies, at 54 h, ceftolozane/tazobactam achieved a low killing only against susceptible strains (MDR-HUB1 and XDR-HUB2), which was only greater than controls for MDR-HUB1 (Δlog CFU/mL = –0.91; $P = 0.002$), whereas ceftazidime was ineffective in all strains. Colistin therapy, overall, resulted in an initial killing against all strains, but regrowth appeared later in a different manner (Δlog CFU/mL at 54 h = –1.33 in MDR-HUB1, –1.85 in XDR-HUB2, and –2.07 in MDR-HUB3), this leading colistin to be the only effective monotherapy at 54 h against XDR-HUB2 ($P < 0.001$ vs. controls and other monotherapies). Of interest, meropenem alone was the most effective monotherapy and the only bactericidal regimen at 54 h against both carbapenem-susceptible strains (Δlog CFU/mL = –4.55 in MDR-HUB1 and –3.96 in MDR-HUB3; $P < 0.001$ vs. controls and other monotherapies).

Regarding drug combinations, the addition of colistin to ceftolozane/tazobactam significantly increased the activity of monotherapies against both ceftolozane/tazobactam-susceptible strains (MDR-HUB1 and XDR-HUB2) at 54 h ($P < 0.001$), this leading to a bactericidal and synergistic effect in both cases. Ceftolozane/tazobactam plus colistin was the most effective combination against the meropenem-resistant XDR-HUB2 strain (Δlog CFU/mL = –4.42 vs. –3.54 for meropenem-colistin; $P = 0.002$); whereas this combination against MDR-HUB1 (Δlog CFU/mL = –4.36) was less effective than meropenem-colistin (–6.25; $P < 0.001$) and showed similar efficacy as meropenem monotherapy ($P = 0.964$). In contrast, the combination ceftolozane/tazobactam-colistin was ineffective against the ceftolozane/tazobactam-resistant strain (MDR-HUB3), being meropenem plus colistin the most bactericidal therapy (Δlog CFU/mL = –6.37; $P < 0.001$ vs. other regimens). The combination ceftazidime-colistin was slightly effective against MDR-HUB1 and MDR-HUB3 (no synergism nor bactericidal effect), but it achieved a bactericidal effect against XDR-HUB2 (Δlog CFU/mL = –3.10).

Overall, low non-bactericidal activity was observed among free-floating cells of the three strains of *P. aeruginosa* (mean inoculums at 0 h around 8 log CFU/mL). Only meropenem and its combination with colistin showed activity at 54 h against MDR-HUB1 strain (Δlog CFU/mL = –2.67 and –2.23, respectively).

3.2. Resistance studies and colistin PAPs

Resistant strains to ceftolozane/tazobactam among biofilm-embedded cells were not detected with any treatment (monotherapy or combination) in ceftolozane/tazobactam-susceptible strains.

Colistin-heteroresistant subpopulations were detected at baseline in all strains (Fig. 3). The proportion of colonies able to grow at concentrations of colistin 2 mg/L, 4 mg/L and 8 mg/L was slightly higher for the XDR-HUB2 (from 1×10^{-4} to 1×10^{-6} CFU/mL) than for the MDR strains (from 1×10^{-5} to 1×10^{-8} CFU/mL). At the end of treatment (Fig. 4), colistin monotherapy led to the emergence of resistant subpopulations at 54 h among all strains. The combination of an active beta-lactam and colistin prevented the emergence of resistant subpopulations, in contrast with what occurred when the beta-lactam was non-active *in vitro*. For the XDR-HUB2 strain, the PAPs of cells recovered at the end of treatments with meropenem-colistin and ceftazidime-colistin showed the same proportion of colistin-resistant subpopulations than that obtained at baseline; in contrast, this proportion increased with colistin monotherapy (until 10^{-2} CFU/mL).

3.3. Confocal laser scanning microscopy images

Well-formed biofilms prior to the start of therapeutic experiments were observed in all strains. Treatment with beta-lactams altered the shape of isolates in both live and dead cells. Colistin in monotherapy mainly had activity within deeper layers of the biofilm structure, whereas beta-lactams plus colistin mainly resulted in activity against all the biofilm structure. Fig. 5 shows some CLSM images of the biofilm-embedded cells of *P. aeruginosa* HUB2, according to treatment regimens.

4. Discussion

The best treatment for osteoarticular and orthopaedic device-related infections caused by MDR *P. aeruginosa* is currently unknown. The presence of bacterial biofilms, where nutrient and oxygen penetration are limited, results in tolerance to antibiotics by expression of phenotypic changes and this impairs the activity of antibiotics, such as beta-lactams, which act against processes occurring in growing bacteria [2,3]. In this setting, the occurrence of MDR/XDR *P. aeruginosa* isolates dramatically limits the therapeutic

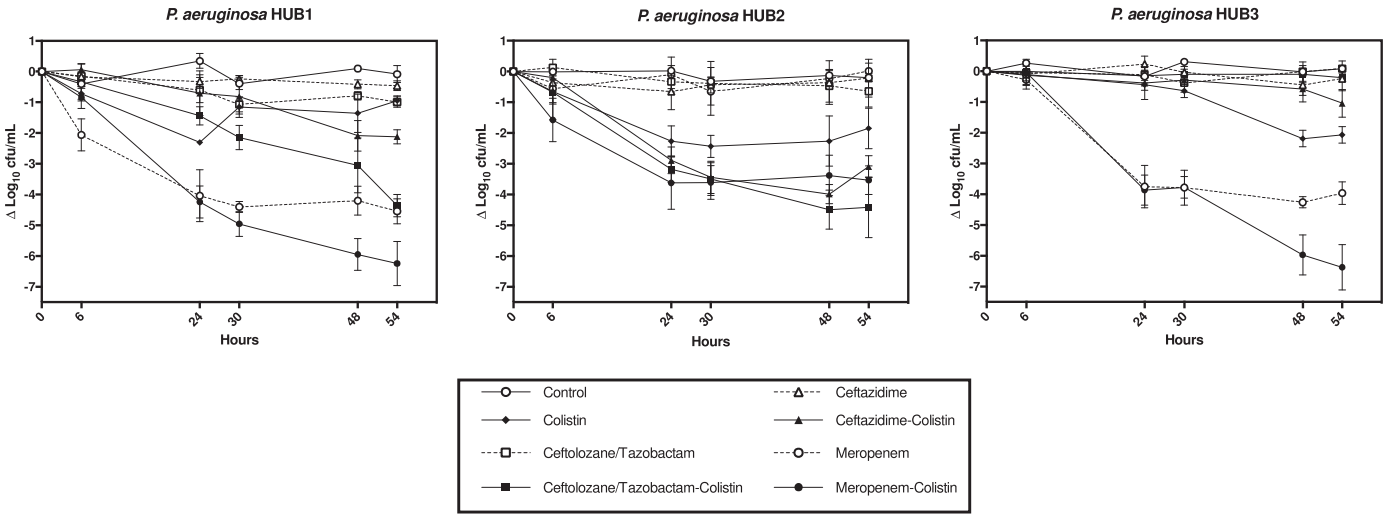

Fig. 2. Bacterial killing by monotherapies with colistin, ceftazidime, meropenem and ceftolozane-tazobactam, and the combination of colistin with beta-lactams against biofilm-embedded cells of three different *Pseudomonas aeruginosa* strains.
Results are expressed using the log change method
Data presented as means ± SD
P. aeruginosa = *Pseudomonas aeruginosa*.

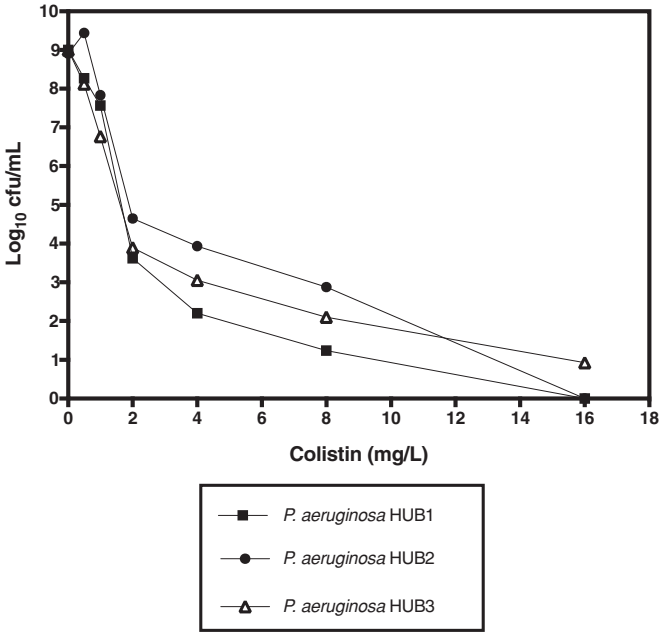

Fig. 3. Baseline Population Analysis Profiles of the three strains of *Pseudomonas aeruginosa* at an initial inoculum of 10^9 cfu/mL.
P. aeruginosa = *Pseudomonas aeruginosa*.

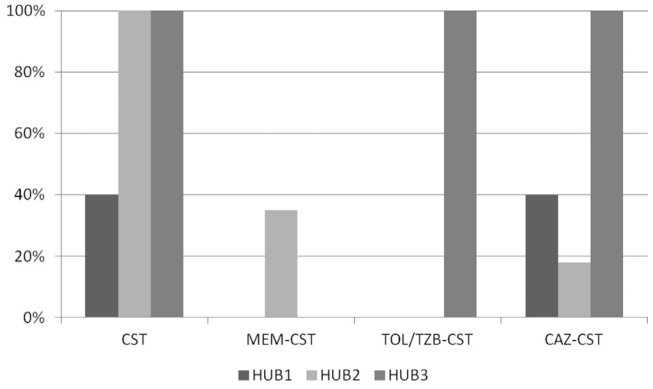

Fig. 4. Emergence of resistant colistin subpopulations among biofilm-embedded cells of the three *Pseudomonas aeruginosa* strains according to the treatment regimen at 54 hours.
Data expressed as proportion of samples with colonies growing at colistin concentration of 2 mg/L among all tested
CST = colistin; MEM = meropenem; TOL/TZB = ceftolozane-tazobactam; CAZ = ceftazidime.

alternatives, since these usually have a decreased susceptibility to beta-lactams and are often only susceptible to colistin.

The current study evaluated the comparative efficacy of ceftolozane/tazobactam, in monotherapy and in combination with colistin, using an *in vitro* pharmacodynamic biofilm model. This has previously been used to model *P. aeruginosa*, has been validated for evaluating the pharmacodynamic efficacy of antibiotics, and reasonably mimics foreign-body infections. Based on previous knowledge, antibiotic efficacy is evaluated by using free drug concentrations in order to reproduce the main PK/PD parameters achieved in human serum (i.e. AUC/MIC or $T_{>MIC}$), which are equivalent to those achieved in interstitial fluids [26]. However, it should be considered that antibiotic peak concentrations close to the biofilm infection within the reactor may be greater than those achieved locally in a human extravascular biofilm-related infection (i.e. prosthetic joint infection).

Ceftolozane/tazobactam is a novel cephalosporin in combination with a beta-lactamase inhibitor that is active among most resistant GNB, including MDR/XDR *P. aeruginosa*. It is approved for clinical use, but its efficacy against osteoarticular and biofilm-related infections is not well known. In the pre-clinical setting, previous experiences have suggested poor activity and the clinical efficacy is limited to few cases with contradictory success [27–29]. In the current model, ceftolozane/tazobactam in monotherapy showed low anti-biofilm efficacy against susceptible strains and it was ineffective against a ceftolozane/tazobactam-resistant strain (MDR-HUB3). Of note, the latter strain was considered resistant (MIC = 8 mg/L) in accordance with the current susceptibility breakpoints (EUCAST criteria) for the 1 g/0.5 g every 8 h regimen, but it could likely be considered susceptible according to the simulated PK/PD parameters for the purpose of 2 g/1 g dosage. Regarding the

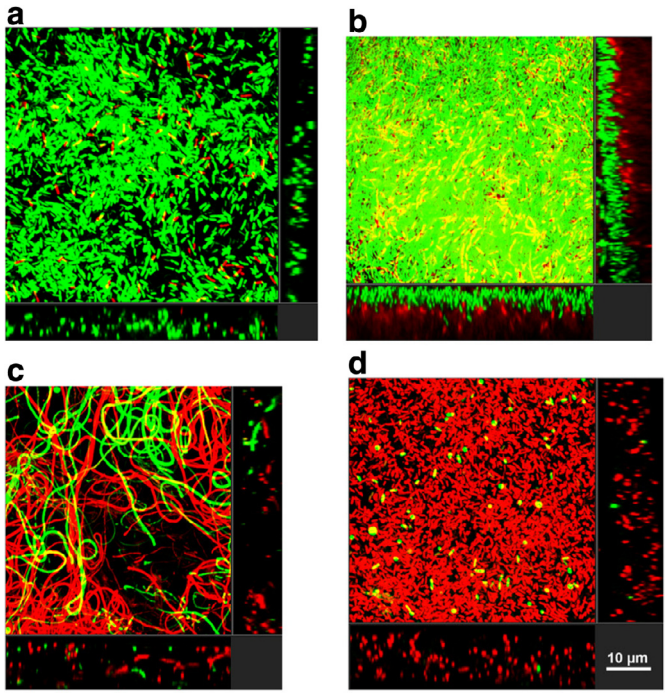

Fig. 5. Confocal laser scanning microscopy images of biofilm-embedded cells of *Pseudomonas aeruginosa* HUB2 at 0 hours (a), at 54 hours for the treatment of colistin in monotherapy (b), ceftolozane/tazobactam in monotherapy (c), and the combination of ceftolozane/tazobactam and colistin (d).
Live cells are green due to staining with Syto 9, whereas dead cells appear red due to staining with propidium iodide. Maximum intensity projection of confocal images of total biofilm thickness is represented as central image. Rectangle images below and to the right of the projection correspond to XZ and YZ planes, respectively.

low frequency of spontaneous resistant mutants previously reported [30], no ceftolozane/tazobactam-resistant strains among the biofilm-embedded population emerged. Overall, given conflicting data between this and other published *in vitro* studies and case reports, clinicians should be cautious when considering the use of ceftolozane/tazobactam in monotherapy against osteoarticular infections by MDR and XDR *P. aeruginosa*.

The current study also evaluated the comparative efficacy of other beta-lactams in monotherapy. All strains were resistant to ceftazidime and no significant activity was observed with this therapy. Interestingly, meropenem alone achieved bactericidal activity against the two meropenem-susceptible strains, suggesting a differential anti-biofilm activity by an unknown mechanism in comparison with other beta-lactams such as cephalosporins. Haagensen et al. used a dynamic biofilm model with flow cell technology and CLSM, and showed that meropenem initially targeted *P. aeruginosa* subpopulations present at the periphery of the biofilm structure but repeated doses resulted in progressive killing of cells in deeper layers [31,32].

Currently, colistin is often the only active drug for treating MDR-GNB and recent research suggests that it has a remarkable anti-biofilm effect mainly based on greater activity in anaerobic conditions and as a biofilm 'destabiliser' [7,8,33,34]. The current results with colistin in monotherapy showed initial killing against biofilm-embedded bacteria followed by regrowth and the progressive appearance of resistant strains; the final efficacy was variable but notable (almost 2 log CFU/mL killing). Interestingly, CLSM pictures from the current experiments showed how colistin has higher affinity for killing bacteria within inner layers of the biofilm population.

In agreement with previous reports [9,35,36], the current results have shown that combining beta-lactams with colistin substantially increases the activity of monotherapies against biofilm-related infections caused by MDR/XDR *P. aeruginosa* and also reinforce the opinion that combination therapy may prevent the emergence of colistin-resistant subpopulations. Moreover, the results also suggest that the anti-biofilm benefits of this combination extend to other subfamilies of beta-lactams, apart from carbapenems, but the efficacy of each beta-lactam plus colistin combination may significantly differ according to its prior activity and the strains' variability.

The combination of ceftolozane/tazobactam plus colistin achieved a bactericidal effect against susceptible strains, but it was ineffective against the ceftolozane/tazobactam-resistant strain. Overall, this combination was the most active treatment for the meropenem-resistant strain. It is believed that the combination ceftolozane/tazobactam-colistin has not been previously evaluated against biofilm-related infections by MDR *P. aeruginosa*, and few studies exist with time-kill analyses [37,38], which mainly reported a synergistic or additive effect even in the case of ceftolozane/tazobactam-resistant *P. aeruginosa* strains. This contrasts with the current results, which limited these beneficial effects against biofilm-embedded bacteria to the treatment of ceftolozane/tazobactam-susceptible strains. The combination of ceftazidime plus colistin was the least active in all strains, although bactericidal in the XDR strain. Finally, the combination of meropenem plus colistin was the most effective regimen for meropenem-susceptible MDR *P. aeruginosa* strains and, interestingly, this combination also achieved a synergistic and bactericidal effect against the meropenem-resistant XDR-HUB2 strain. It has previously been shown that doripenem plus colistin enhance the *in vitro* anti-biofilm killing of monotherapies against carbapenem-resistant MDR/XDR *P. aeruginosa* strains, which contained different mechanisms of resistance (VIM-2 metallo-beta-lactamase or PSE-1 beta-lactamase plus efflux pump) than the XDR-HUB2 strain used [17].

The synergy observed with the beta-lactam and colistin combination has been previously associated with mechanistic and subpopulation synergy effects [35], which may also be applied to biofilm-related infections by targeting different subpopulations. Whereas colistin may target subpopulations with low metabolic activity within inner layers of the biofilm [7,33,34], beta-lactams may act upon more metabolically active subpopulations present at the periphery of the biofilm structure [31,32]. In the particular setting of biofilm-related osteoarticular and orthopaedic device-related infections, clinical data have also emphasised the benefits of using colistin in combination, especially against *P. aeruginosa* isolates [39]. However, this combination has not been found to be superior to colistin alone among critically ill patients with other types of infection caused by MDR-GNB (not limited to *P. aeruginosa*) [40]. The different characteristics of biofilm-related infections and the particular activity of colistin in this field may explain these apparent contradictory results. The current study observed poor efficacy against free-floating bacteria for all monotherapies or combinations. Although it did not specifically study this bacteria population, it probably reflected a mix of microorganisms at high inoculums, forming small clusters or biofilm-like aggregates, either in a planktonic state or detached from biofilms. All these characteristics may impair the efficacy of treatments, which were clearly different from that obtained against biofilm-embedded cells, as also observed in a previous study [17].

Additionally, it was found that the combination of beta-lactams and colistin prevented the amplification of colistin-resistant subpopulations among heteroresistant strains in biofilm-embedded populations, depending on the strain's susceptibility to beta-lactams: protection was more likely if the strain was susceptible.

However, current analysis of colistin PAPs with XDR-HUB2 strain at the end of treatment showed a similar proportion of heteroresistant populations with the combined treatments compared to PAPs at baseline, thus suggesting a stochastic expression of resistance rather than the emergence of real mutants. In contrast, this proportion of heteroresistant strains did change at the end of treatment with colistin alone. Overall, this protective effect of combined therapies (independent of susceptibility or resistance to beta-lactam) should be evaluated for longer periods.

Although CBR can simulate the PK/PD profile of antibiotics similar to human dosage exposure, there is a clear limitation in mimicking the complex structures that biofilms constitute *in vivo*. Similarly, host-pathogen interactions were not considered and these may also affect the efficacy of treatments. Moreover, antibiotic concentrations near the biofilm infection site may differ depending on the biofilm location *in vivo*, which represents a limitation of the CBR. Even when ceftolozane/tazobactam was used in a 2:1 solution, the activity and dynamics of both compounds may have been different, as they were purchased from different companies. This study was also limited by the use of a small number of *P. aeruginosa* strains and, certainly, the use of more strains may provide a deeper understanding of the anti-biofilm activity of the treatments. However, these three strains are disseminated worldwide and very representative high-risk clones; specifically, ST274 clone is linked to chronic biofilm-related infections and ST175 is highly disseminated in Spanish hospitals. Finally, this model evaluated a 48-h-old biofilm, so different results may have been obtained with a more mature biofilm. For all these reasons, it is reasonable to be cautious when translating the results into clinical practice.

5. Conclusions

Monotherapies with beta-lactams mainly had little efficacy against biofilm-embedded MDR/XDR *P. aeruginosa*, with the exception of meropenem against susceptible strains. Colistin alone had notable efficacy, but it was influenced by the emergence of resistance. Based on the efficacy and protection against resistance, the results support the use of a beta-lactam plus colistin combination to treat foreign-body infections caused by MDR/XDR *P. aeruginosa*. Ceftolozane/tazobactam plus colistin was the most appropriate combination for meropenem-resistant (non-carbapenemase producer) *P. aeruginosa* strains, whereas the combination of meropenem-colistin was for the carbapenem-susceptible strains. More studies are needed to further evaluate the particular anti-biofilm activity of meropenem and its combination with colistin against carbapenem-resistant GNB and to provide more evidence for the use of these combinations in clinical practice.

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Competing Interests

All authors declare that they have no conflicts of interest to disclose. The funders of the study did not play any role in the design, analysis or reporting of the results.

Ethical Approval

Not required.

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