Biofilm 8 (2024) 100231

Contents lists available at ScienceDirect

Biofilm

journal homepage: www.sciencedirect.com/journal/biofilm

Collateral susceptibility-guided alternation of ceftolozane/tazobactam with imipenem prevents resistance development in XDR *Pseudomonas aeruginosa* biofilms

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ARTICLE INFO

Keywords:
Pseudomonas aeruginosa
XDR
Biofilms
Antagonistic resistance mechanisms to antibiotics

ABSTRACT

Objectives: New combinations of β-lactams and β-lactamase inhibitors, such as ceftolozane/tazobactam could be useful to combat biofilm-driven chronic infections by extensively resistant (XDR) *Pseudomonas aeruginosa* but resistance development by mutations in the Ω-loop of AmpC has been described. However, these mutations confer collateral susceptibility to carbapenems. Thus we aimed to evaluate the therapeutic efficacy and the prevention of resistance development of regimen alternating ceftolozane/tazobactam and imipenem.

Methods: A carbapenem-resistant XDR *P. aeruginosa* clinical strain (ST175, 104-B7) and its isogenic imipenem-susceptible ceftolozane/tazobactam-resistant mutant derivative (AmpC T96I, 104-I9) were used. Experiments of single strains and mixed (104-B7 and 104-I9, 1:0.01 ratio) biofilms were performed. 48h biofilms (flow cell system) were treated for 6 days with either ceftolozane/tazobactam, 4/4 mg/L or the alternation of ceftolozane/tazobactam (2 days)-imipenem 4 mg/L (2 days) - ceftolozane/tazobactam (2 days). After treatment, biofilms were collected and plated on Mueller-Hinton agar± ceftolozane/tazobactam 4/4 mg/L. Structural dynamics were monitored using confocal laser scanning microscopy and images were processed with IMARIS software. At least, three independent triplicate experiments per condition were performed. Emerging resistant mutants were characterized through whole genome sequencing (Illumina).

Results: Ceftolozane/tazobactam monotherapy failed to reduce the biofilms of the 104-B7 XDR strain and led to the selection of resistant mutants that showed AmpC Ω-loop mutations (T96I, L244R or aa236Δ7). On the contrary, alternation with imipenem enhanced activity (3 Logs reduction at day 6) and prevented the emergence of ceftolozane/tazobactam-resistant mutants. Likewise, treatment with ceftolozane/tazobactam dramatically amplified the resistant strain 104-I9 in mixed biofilms (>90 % of the population), while the alternation regimen counterselected it.

Conclusions: Collateral susceptibility-guided alternation of ceftolozane/tazobactam with imipenem effectively prevented the selection of resistant mutants and thus could be a potential therapeutic strategy for the treatment of *P. aeruginosa* XDR chronic infections.

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https://doi.org/10.1016/j.biofilm.2024.100231
Received 6 June 2024; Received in revised form 19 September 2024; Accepted 21 October 2024
Available online 22 October 2024

1. Introduction

Chronic bacterial infections are complicated to overcome despite the action of immune system or adequate antibiotic treatments. Typically, persistent infections are related to the formation of bacterial biofilms. Updated definition of biofilm could be "group of bacteria clumped together in a dense colony embedded in a self-produced matrix composed by extracellular polymeric substances" [1]. Biofilm associated infections account for 65–80 % of all infections in humans and represent a major cause of concern for the healthcare systems [2]. Chronic biofilm infections include a wide range, from those related to indwelling medical devices (e.g., catheters, prosthetic joints, and surgical implants) to tissue infections, such as otitis media, osteomyelitis, rhinosinusitis, wound infections, or chronic respiratory infections in cystic fibrosis (CF) patients [3,4]. In general, these infections have increased their incidence over time, especially in the context of an aging population and the widespread use of new technologies and medical devices. Biofilms are up to 100–1000 times more resistant to antibiotics than planktonic cells [5]. The basis for resistance to antimicrobials in biofilms is complex and involves mechanisms of tolerance and conventional resistance. Physical, physiological, adaptive, and in vivo tolerance mechanisms together with the involvement of persister cells and the expression of specific genes are sources of the intrinsic recalcitrant nature of the biofilm to antibiotics [6]. On the other hand, mutational resistance and horizontal gene transfer determine the genetic changes leading to genuine resistance to antimicrobials [6,7]. Considering all this, strategies to treat biofilms should be directed to combat the different key points of their peculiarity as well as being individualized according to the nature of the infection [4]. For example, in the case of device-associated chronic infections, replacing the implant to remove biofilms is often, an effective interventionist strategy. However, for tissue or respiratory chronic biofilm infections, the only available therapy to date is antibiotic treatment. Long-term therapies with antibiotics, despite *in vitro* susceptibility, frequently lead to a longstanding inflammatory response, together with a high risk of antibiotic resistance development, thus triggering an unavoidable chronic infection extremely difficult to eradicate. Currently, the management of these infections represents a challenge for doctors, demanding the participation of multidisciplinary teams specialized in this type of pathologies. Some strategies to optimize anti-biofilm efficacy include administration in continuous or extended infusion, combination of antibiotics and sequential treatment [8,9].

To worsen the situation, the proportion of multidrug (MDR), extensively drug-resistant (XDR) and difficult to treat resistant (DTR) strains, as defined by the European Centre for Diseases Control (ECDC) or the Infectious Diseases Society of America (IDSA), are increasing their prevalence overtime [10,11]. In this case, biofilm-associated infections caused by MDR Gram-negative bacilli (GNB) represent even more complicated clinical scenarios, mostly nosocomial (post-surgical, etc.), that usually affect an aging and fragile population. For instance, in the case of prosthetic joint infections multidrug resistant GNB represent 21 % of GNB infections, with *Pseudomonas aeruginosa* being one of the most prevalent [12,13]. This is partly due to the special idiosyncrasy of the bacteria *P. aeruginosa* that involves an intrinsic antibiotic resistance and a notable capacity to acquire new resistance mechanisms by mutation, like those leading to overexpression of the chromosomal beta-lactamase AmpC, the inactivation of the carbapenem porin OprD, or the upregulation of efflux pumps [14,15]. *P. aeruginosa* clones associated with MDR/XDR/DTR phenotypes (high-risk clones) are a growing problem in hospitals around the world [16,17].

Currently, first-line treatment against XDR/DTR clones of *P. aeruginosa* must be based on new beta-lactams (with/without beta-lactamase inhibitors), such as ceftolozane-tazobactam or ceftazidime-avibactam. While these novel β-lactam–β-lactamase inhibitor combinations seemed a promising option they are not exempt from mutational resistance development [18,19]. Indeed, resistance development to ceftolozane/tazobactam is mostly driven by specific mutations in the Ω-loop of AmpC, which frequently confer cross-resistance to ceftazidime-avibactam. However, on the positive side, this resistance mechanism entails an increased (collateral) susceptibility to carbapenems, such as imipenem.

Thus, in this work we tested the hypothesis that treatment of carbapenem-resistant XDR/DTR *P. aeruginosa* biofilms with the alternation of ceftolozane/tazobactam, (that leads to ceftolozane/tazobactam resistance development but increases susceptibility to imipenem), followed by imipenem could avoid the development of ceftolozane/tazobactam resistant mutants maybe being a promising therapeutic strategy on *P. aeruginosa* chronic infections. From a more fundamental point of view, this work applies for the first time collateral susceptibility principles to design alternate antibiotic regimens to avoid resistance development in XDR *P. aeruginosa* biofilms.

2. Materials and methods

2.1. P. aeruginosa strains

The wild-type, reference strain PAO1 was obtained from the Danish collection (PAO1-DK, Systems Biology-DTU) [20]. Clinical strains were obtained from a previously characterized collection of 8 pairs (susceptible and resistant to ceftolozane/tazobactam) of MDR *P. aeruginosa* obtained from patients treated with ceftolozane/tazobactam at Son Espases Hospital (Palma de Mallorca, Spain) between July 2016 and April 2017 [19] were screened for biofilm formation on microtiter plates using crystal violet (CV) assay (data not shown) [21]. A pair of isogenic XDR clinical strains, belonging to ST175 clone, (104-B7 and 104-I9) showing biofilm formation, were selected for the study. 104-B7 strain exhibited an XDR and DTR phenotype according to ECDC and IDSA definitions. It was susceptible to ceftolozane/tazobactam but showed an OprD mutation (Q142X) responsible for carbapenem resistance, and an AmpR (G154R) mutation leading to AmpC overexpression resulting in β-lactam resistance. During the treatment of the patient with ceftolozane/tazobactam, the susceptible strain became resistant to ceftolozane/tazobactam by a mutation in AmpC (T96I) turning into 104-I9 [19]. The minimum inhibitory concentration (MIC) of β-lactam antibiotics determined for the strains of the study, as well as the β-lactam resistance genotype of the clinical strains, are summarized in Table 1.

The clinical isolate 104-B7 was tagged at the *att* intergenic neutral chromosomal locus with EYFP (enhanced yellow fluorescent protein) using a mini-Tn7 constructs containing streptomycin (200 mg/L), while the clinical isogenic resistant 104-I9 strain was tagged with ECFP (enhanced cyan fluorescent protein) containing streptomycin (200 mg/L) [22–24]. Plasmids containing mini-Tn7 constructs were electropored, as previously described [23,24].

2.2. The PK/PD model of biofilm treatment

Biofilms of XDR/DTR clinical strain 104-B7 were grown at 30 °C using the flow cell system supplied with modified FAB [20], which is a minimal medium composed by FB [Milli-Q H_2O, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 10.000x trace metals [$CaSO_4{\cdot}2H_2O$ (200 mg/L), $FeSO_4{\cdot}7H_2O$ (200 mg/L), $MnSO_4{\cdot}H_2O$ (20 mg/L), $CuSO_4{\cdot}5H_2O$ (20 mg/L), $ZnSO_4{\cdot}7H_2O$ (20 mg/L), $CoSO_4{\cdot}7H_2O$ (10 mg/L), $NaMoO_4{\cdot}H_2O$ (5 mg/L), H_3BO_3 (5 mg/L)] plus A-10 [$(NH_4)_2SO_4$ (20 g/L) Na_2HPO_4 (60 g/L) KH_2PO_4 (30 g/L) NaCl (30 g/L) pH 6.4±0.1] and a carbon source (20 % glucose 0.3 mM).

48 h-old biofilms, were challenged during 2 days with ceftolozane/tazobactam 4/4 mg/L in monotherapy, from there the treatments were divided in two branches: either with monotherapy (ceftolozane/tazobactam during 4 days), or with the alternation of imipenem 4 mg/L (during 2 days) and ceftolozane/tazobactam (during 2 days). Fig. 1 summarizes these treatments in a diagram. Antibiotic concentrations used were based on EUCAST 2024 clinical breakpoints (www.eucast.org).

Table 1
Antibiotic susceptibility profiles and resistome of strains used in this work.

Strain	MIC (μg/ml)											β-Lactam resistance genotype
	CAZ	FEP	TZP	CZA	TL/TZ	ATM	IPM	MEM	CIP	TOB	AMK	COL	
	S ≤ 0.001 R > 8	S ≤ 0.001 R > 8	S ≤ 0.001 R > 16	S ≤ 8	S ≤ 4	S ≤ 0.001 R > 16	S ≤ 0.001 R > 4	S ≤ 2 R > 8	S ≤ 0.001 R > 0.5	S ≤ 2	S ≤ 16	S ≤ 4	
PAO1-DK	2	2	<4/4	2/4	1/4	4	2	1	0.125	1	<2	2	
104-B7	16	16	64/4	4/4	4/4	16	16	16	>16	32	4	1	OprD Q142X, AmpR G154R
104-I9	32	16	8/4	32/4	>32/4	16	2	4	>16	32	4	1	OprD Q142X, AmpR G154R, **AmpC T96I**
104-B7-1	16	8	16/4	8/4	8/4	8	4	8	>16	32	2	1	OprD Q142X, AmpR G154R **AmpC L244R**
104-B7-2	16	8	8/4	16/4	16/4	8	4	4	>16	>32	8	1	OprD Q142X, AmpR G154R **AmpC T96I**
104-B7-3	64	16	16/4	32/4	32/4	16	2	8	>16	32	4	2	OprD Q142X, AmpR G154R **AmpC aa236Δ7**
104-B7-4	32	16	16/4	32/4	32/4	16	2	4	>16	32	4	1	OprD Q142X, AmpR G154R **AmpC T96I**
104-B7-5	64	16	16/4	32/4	32/4	32	2	8	>16	32	8	1	OprD Q142X, AmpR G154R **AmpC L244R**
104-B7-6	32	8	32/4	16/4	32/4	32	4	8	>16	32	4	2	OprD Q142X, AmpR G154R **AmpC L244R**
104-B7-7	64	16	32/4	16/4	32/4	32	4	8	>16	32	4	1	OprD Q142X, AmpR G154R **AmpC L244R**
104-B7-8	64	16	32/4	16/4	32/4	32	4	8	>16	>32	8	1	OprD Q142X, AmpR G154R **AmpC L244R**
104-B7-9	64	16	64/4	16/4	32/4	32	4	16	>16	>32	8	1	OprD Q142X, AmpR G154R **AmpC L244R**

CAZ, ceftazidime; FEP, cefepime; TZP, piperacillin/tazobactam; CZA, ceftazidime/avibactam; TL/TZ, ceftolozane/tazobactam; ATM, aztreonam; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; TOB, tobramycin; AMK, amikacin; COL, colistin. [a]EUCAST breakpoints indicated. Modified from Fraile-Ribot [18].

Fig. 1. Diagram of control and treatments. After the inoculation (t-2), 48 h-old biofilms (t0), were either followed as controls without treatment during 6 days (t2, t4 and t6) or challenged with ceftolozane/tazobactam 4/4 mg/L (t2), to be divided, afterwards, in two subbranches: with ceftolozane/tazobactam in monotherapy during 4 days (t4 and t6), or with the alternation of imipenem 4 mg/L (2 days, t4) and ceftolozane/tazobactam (2 days, t6). Cell counts and images were monitored at time points t0, t2, t4 and t6 with, at least, three independent experiments per condition. INOC stands for inoculation; CTRL stands for control biofilms (without treatment); TL/TZ stands for ceftolozane/tazobactam and IM stands for imipenem.

At time points t0 (2 days-old biofilm), t2 (2 days of treatment or 4-day-old biofilm), t4 (4 days of treatment or 6-day-old biofilm), and t6 (6 days of treatment or 8-day-old biofilm) of biofilm follow-up according previous works [8,25,26] biofilms were detached and collected by washing the flow cell channels with 1 ml of glass beads (Sigma) suspension in 0.9 % NaCl and direct and/or serial dilutions were plated in Mueller-Hinton agar (MHA) to determine the numbers of viable cells and in MHA with ceftolozane/tazobactam 4/4 mg/L to determine the ceftolozane/tazobactam resistant mutants. The detection limit was 2.5 total CFU (<1 CFU on agar plates after plating 100 μl of direct biofilm extracts).

In all cases, the results from at least three independent experiments per condition were considered.

2.3. Microscopic analysis

Biofilm structural dynamics were monitored by confocal laser scanning microscopy (CLSM) at time points 0, 2, 4, and 6. All microscopic observations were performed by using a Zeiss LSM710 CLSM (Carl Zeiss, Jena, Germany) equipped with a multiline argon laser, detector, and filter sets to monitoring EYFP expression (excitation 514 nm, emission 527 nm) as well as ECFP (excitation 434 nm, emission 474 nm) and NeHe laser for simultaneous monitoring of the red fluorescence emit from the PI (excitation, 543 nm; emission filter, 565–615 nm). Images were obtained by using 63/1.4 oil Plan Apo objective lenses.

Simulated three-dimensional (3D) images and sections were generated by using the IMARIS software package (Bitplane AG, Zurich, Switzerland). Biofilm biomass was extracted from the CLSM images by COMSTAT [19].

2.4. Competition experiments

Biofilm competition experiments were performed between 104-B7 (EYFP-tagged) and its isogenic resistant mutant 104-I9 (ECFP-tagged). Treatment was started at 1:0.01 proportions (104-B7: 104-I9, respectively) to effectively monitor the selection and amplification of resistant mutants according to previous works [25,26]. In the same way as with individual biofilms, after 48 h of incubation (t0), mixed biofilms were challenged during 2 days with ceftolozane/tazobactam, 4/4 mg/L in monotherapy (t2) to branch treatments, afterwards, with either

monotherapy (ceftolozane/tazobactam during 4 days, t4 and t6) or the alternation of imipenem 4 mg/L (during 2 days, t4) and ceftolozane/-tazobactam (during 2 days, t6). Non-treated mixed (1:0.01, 104-B7: 104-I9, respectively) biofilms controls were also studied. Biofilms were monitored by CLSM over time and pictures were taken at time-points t0, t2, t4 and t6. At the end of the experiments, (t0, t2, t4 and t6), biofilms were detached, collected and plated, as described above, to determine the CFU numbers of 104-B7, and its isogenic resistant mutant, 104-I9. Percentages of CFUs resistant to ceftolozane/tazobactam were evaluated by plating in MHA containing ceftolozane/tazobactam 4/4 μg/mL and on a MHA plates without antibiotic, in parallel. The results from at least three independent experiments were considered.

2.5. Characterization of ceftolozane/tazobactam resistant mutants

Nine representative ceftolozane/tazobactam resistant mutants from three different independent experiments were characterized through antimicrobial susceptibility profiling and whole genome sequencing.

2.6. Antibiotic susceptibility

The resistance profiles were evaluated by determining the MICs by broth microdilution of the following panel of antipseudomonal agents: ceftazidime, cefepime, piperacillin/tazobactam, ceftazidime/avibactam, ceftolozane/tazobactam, aztreonam, imipenem, meropenem, ciprofloxacin, tobramycin, amikacin, and colistin, following the EUCAST recommendations (www.Eucast.org). PAO1 DK was used as control.

2.7. Whole genome sequencing

For the library preparation and sequencing, protocols previously implemented by our group were used [27,28]. Genomic DNA was obtained by using a commercial extraction kit (High Pure PCR template preparation kit; Roche Diagnostics). Indexed paired-end libraries were prepared with the Illumina DNA Prep Kit (Illumina, Inc., USA) and then sequenced on an Illumina MiSeq benchtop sequencer with the MiSeq Reagent Kit v3 (Illumina, Inc.), resulting in 300-bp paired-end reads. For variant calling, paired-ended reads were aligned to the *P. aeruginosa* PAO1 reference genome (GenBank accession no. NC_002516.2) with Bowtie 2 v2.2.4 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). Pileup and raw files were obtained by using SAMtools v0.1.16 (https://sourceforge.net/projects/samtools/files/samtools/) and PicardTools v1.140 (https://github.com/broadinstitute/picard). The Genome Analysis Toolkit (GATK) v3.4-46 (https://www.broadinstitute.org/gatk/) was used for realignment around indels. SNPs and indels for each isolate were annotated by using SnpEff software v4.2 (http://snpeff.sourceforge.net/index.html), with default options. A set of 164 genes chromosomal genes related to antibiotic resistance (mutational resistome) previously defined by our Group [27–29] was analysed.

2.8. Statistical analysis

GraphPad Prism 7 was used for statistical analysis. Quantitative variables were analysed through the repeated measures ANOVA (with the post-hoc Tukey's multiple comparison test) pairing data obtained under different experimental conditions from the experimental replicates and/or the Student's t-test (two-tailed) or Mann Mann-Whitney *U* test (non-normal distribution) as appropriate. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Therapeutic efficacy based on the determination of viable cells and antibiotic-resistant mutants

Fig. 2 shows the dynamics over time of 104-B7 biofilms controls and treated with ceftolozane/tazobactam or ceftolozane/tazobactam and imipenem sequential treatment.

Ceftolozane/tazobactam in monotherapy was not able to reduce biofilm viable cells of 104-B7 after 6 days, compared to non-treated biofilm controls and, even worse, selection of ceftolozane/tazobactam resistant mutants was document at approximately 3 logs at day 6. In contrast with ceftolozane/tazobactam monotherapy, the sequential treatment of ceftolozane/tazobactam followed by imipenem and then again ceftolozane/tazobactam, during 6 days, was able to achieve a statistically significant ($p = 0.0079$) reduction of biofilm viable cells, that reached 3 log compared to monotherapy at t6. Furthermore, the alternation regime did not select for ceftolozane/tazobactam resistant mutants.

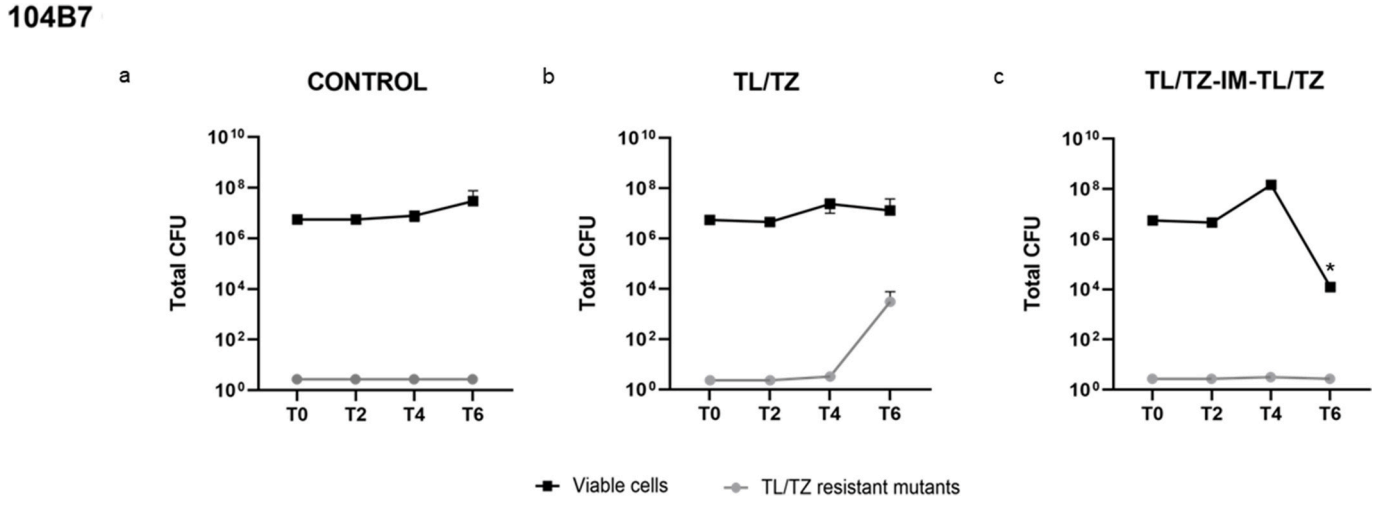

Fig. 2. Dynamics over time of the bacterial populations of 104-B7 biofilms: (a) control; (b) treated with 4/4 μg/ml ceftolozane/tazobactam (TL/TZ), 6 days; and (c) treated with 4/4 μg/ml TL/TZ (2 days), 2 μg/ml imipenem (IM) (2 days) and TL/TZ (2 days). The results represent the averages (symbols) and standard deviations (error bars) from at least three independent experiments. *Stands for statistically significant differences ($p < 0.05$) in total CFU reduction between treatments (Mann Whithney *U* test).

3.2. Characterization of ceftolozane/tazobactam resistant mutants

Table 1 summarizes the antibiotic susceptibility profile as well as β-lactam resistance genotype of the resistant mutants studied.

Nine ceftolozane/tazobactam resistant mutants, from three different experiments, selected during monotherapy were studied (104-B7-1-9). The characterized mutants shared the same susceptibility pattern of the parental strain 104-B7 but became resistant to ceftazidime/avibactam and ceftolozane/tazobactam and recovered susceptibility to carbapenems imipenem and meropenem. The resistant isolates showed changes in the Ω-loop of AmpC associated with ceftolozane/tazobactam resistance and imipenem collateral susceptibility, including the mutations in T96I (n = 2), in L244R (n = 6), and the deletion aa236Δ7 (n = 1). The T96I mutation, developed in two of the mutants, was the same that led to ceftolozane/tazobactam resistance during therapy of the strain 104-I9 isolated from a patient. These results therefore confirm that mechanisms of ceftolozane/tazobactam resistance development previously described *in vitro* and in vivo [18,19] also play a major role in biofilms.

3.3. Therapeutic efficacy based on biofilm structural dynamics

Fig. 3 shows biomass analyses for control, ceftolozane/tazobactam monotherapy and alternation regime, for biofilms of 104-B7 strain. Despite the intrinsic irregularities in biomass measurement, sequential treatment statistically reduced the biomass at all time points compared to controls ($p < 0.05$), so did ceftolozane/tazobactam monotherapy. However, comparing both treatments, alternation regime achieved the greatest biomass reduction especially at t6, being statistically significant ($p < 0.05$) at t4 and t6. Thus, in terms of biomass decline, sequential treatment with ceftolozane/tazobactam and imipenem was the best therapeutic strategy for the biofilms of the XDR/DTR strain 104-B7.

Three-dimensional images obtained with IMARIS software exhibited in Fig. 4 show a fairly close correlation with biomass analyses. Thus, in the XDR/DTR strain 104-B7; the most intense filamentation effect was achieved with the alternation regime where, practically, all the biofilm is observed in red, therefore dead. Ceftolozane/tazobactam treatment also led to the filamentation of the biofilm cells but the proportion of living (yellow) areas seems importantly higher.

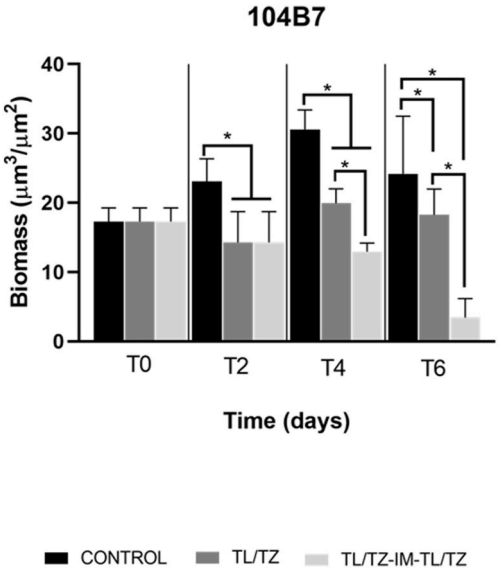

Fig. 3. Biomass ($\mu m^3/\mu m^2$) analysis for control (black bars) and treated with ceftolozane/tazobactam (TL/TZ) (dark grey bars) and alternation regime TL/TZ-imipenem (IM)-TL/TZ (light grey bars) biofilms formed by 104-B7 obtained with the COMSTAT program for the quantification of three-dimensional biofilm structures. The results represent the averages (bars) and standard deviations (error bars) from at least three independent experiments. *Stands for statistically significant differences ($p < 0.05$) in biomass (ANOVA).

3.4. Competition experiments

In order to gain insights on the structural dynamics of ceftolozane/tazobactam resistance development under monotherapy and sequential treatments, competition experiments between both isogenic strains, at initial ratio of 1:0.01, 104-B7:104-I9, respectively, were carried out. Fig. 5 shows the dynamic of mixed biofilms viable cells on controls without treatment and under ceftolozane/tazobactam monotherapy and sequential treatment (Fig. 5a). At time-points 2 and 4, both treatments significantly reduced the total amount of mixed biofilms whereas at t6, the reduction achieved with the alternation regimen was statistically higher than that with ceftolozane/tazobactam alone ($p < 0.05$). Percentages of the resistant strain were not significantly modified on control mixed biofilms, remaining in the range of 0.5–2 % across the 8 days experiments. However, treatment with ceftolozane/tazobactam strongly amplified the resistant strain 104-I9 in mixed biofilms reaching >90 % of the final population. On the other hand, the alternation regimen maintained the resistant strain at levels similar to the control biofilms at the end of the treatment (2.33 %) (Fig. 5b). Moreover the purging effect of ceftolozane/tazobactam resistant mutant with imipenem treatment was clearly patent, counter selecting the resistant strain to levels well below the control biofilms (0.11 %) ($p = 0.0043$).

These results were also in agreement with the re-constructed three dimensional images obtained during competition experiments challenged with the different treatments as shown in Fig. 6. In the mixed biofilm, consistently with Fig. 5 cell counts, treatment with ceftolozane/tazobactam monotherapy drove the visible increase in the proportion of the isogenic resistant strain, 104-I9 (blue) with respect to 104-B7 (yellow), already from t2, until almost covering the entire biofilm area. On the contrary, with sequential treatment, the colour was predominantly yellow according with the proportion of the strains (Fig. 6). The biomass lessening as well as filamentation effect of the alternation regime is also well-observed at t6 (Fig. 6).

Taken together, the results clearly indicated that the alternation strategy efficiently avoids selection and amplification of ceftolozane/tazobactam resistant mutants originated during ceftolozane/tazobactam monotherapy.

4. Discussion

Treatment of chronic biofilm-mediated infections by XDR/DTR *P. aeruginosa* poses an important challenge for different health professionals, including clinicians and microbiologists. While eradication of biofilms is extremely difficult once chronic infection has established, reduction of bacterial load and control of infection represent, in some circumstances, a more realistic goal. In the present study, monotherapy with ceftolozane/tazobactam was not able to reduce the biofilm bacterial loads in the XDR/DTR strain and, on the contrary, induced the selection and amplification of resistant mutants harbouring the same mutations that those naturally selected in vivo in the patients [19]. Although, initially, ceftolozane/tazobactam was more active and stable than other beta-lactam antibiotics, mutations in the catalytic centres of intrinsic β-lactamases that can importantly reduce its activity were documented [18]. The responsible AmpC mutations comprise substitutions or deletions in Ω-loop residues, or residues in the Ω-loop surroundings interacting with it [30,31]. Accordingly, in this work, the mutation in the Ω-loop of AmpC, T96I, the one carried by the isogenic clinical resistant strain, 104-I9, was also presented in two of the mutants obtained from biofilms treated with ceftolozane/tazobactam monotherapy. Actually, in competition experiments the resistant isogenic strain was amplified under monotherapy, doubly evidencing the generation of resistance in biofilm growth as well. Correspondingly, the rest of characterized mutants showed previously identified AmpC Ω-loop

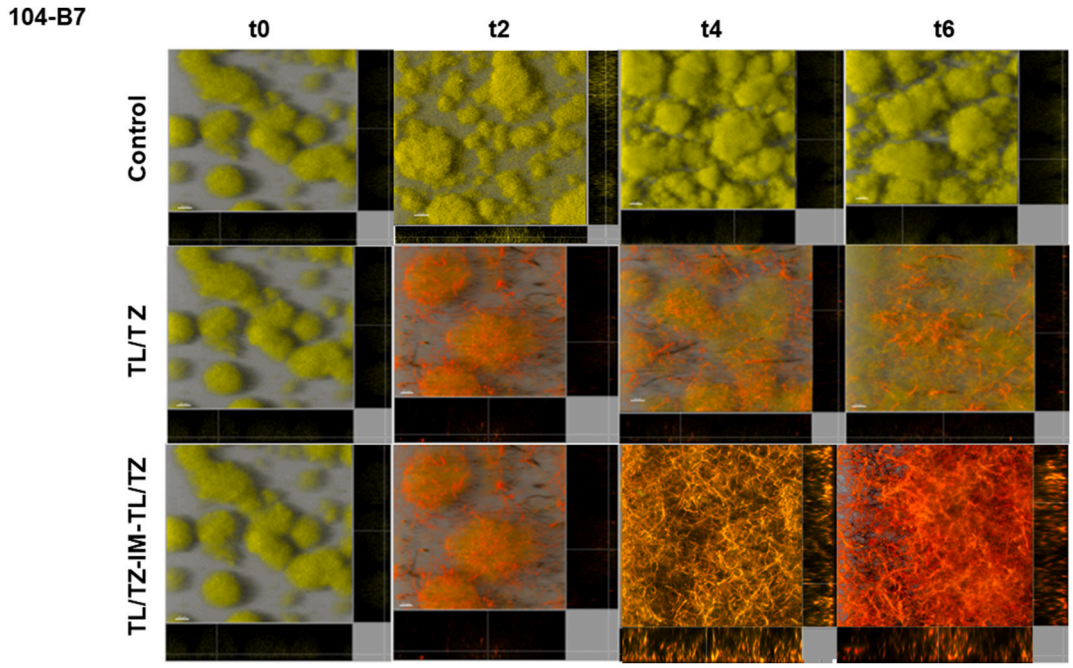

Fig. 4. Three-dimensional images and transversal sections of 104-B7 control biofilms, treated with ceftolozane/tazobactam (TL/TZ) and with alternation regime (TL/TZ-imipenem (IM)-TL/TZ), stained with propidium iodide (red). The images obtained at four time points (t0, t2, t4, and t6) are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

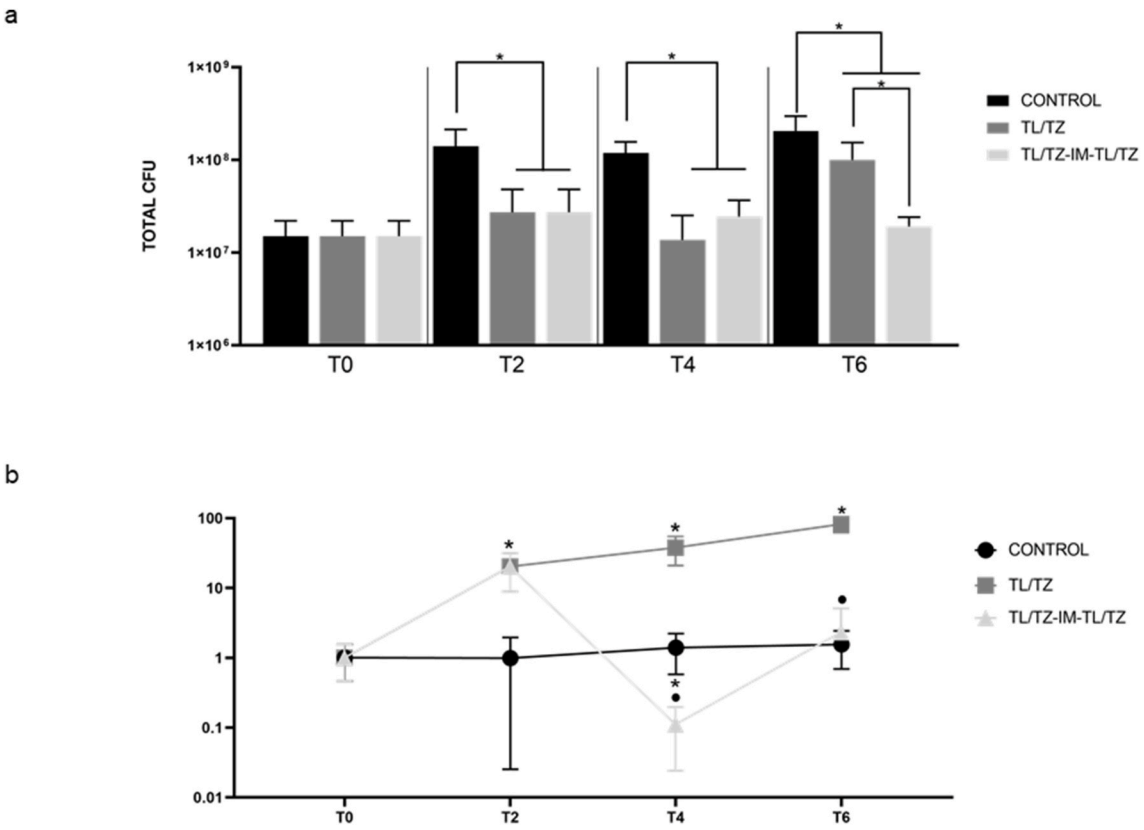

Fig. 5. a) Dynamics over time of mixed biofilms viable cells (initial 1:0.01 ratio; 104-B7:104-I9, respectively); control (black bars) and treated with ceftolozane/tazobactam (TL/TZ) (dark grey bars) and alternation regime TL/TZ-imipenem (IM)-TL/TZ (light grey bars). The results represent the averages (bars) and standard deviations (error bars) from at least three independent experiments. *Stands for statistically significant differences ($p < 0.05$, ANOVA). b) Proportion (%) of TL/TZ resistant mutants in control, treated with TL/TZ and with the alternation regime TL/TZ-IM-TL/TZ from mixed biofilms. The results represent the averages (symbols) and standard deviations (error bars) from at least three independent experiments. *Stands for statistically significant differences ($p < 0.05$) between treatments vs controls and •stands for statistically significant differences ($p < 0.05$) between treatments (Student' t-test).

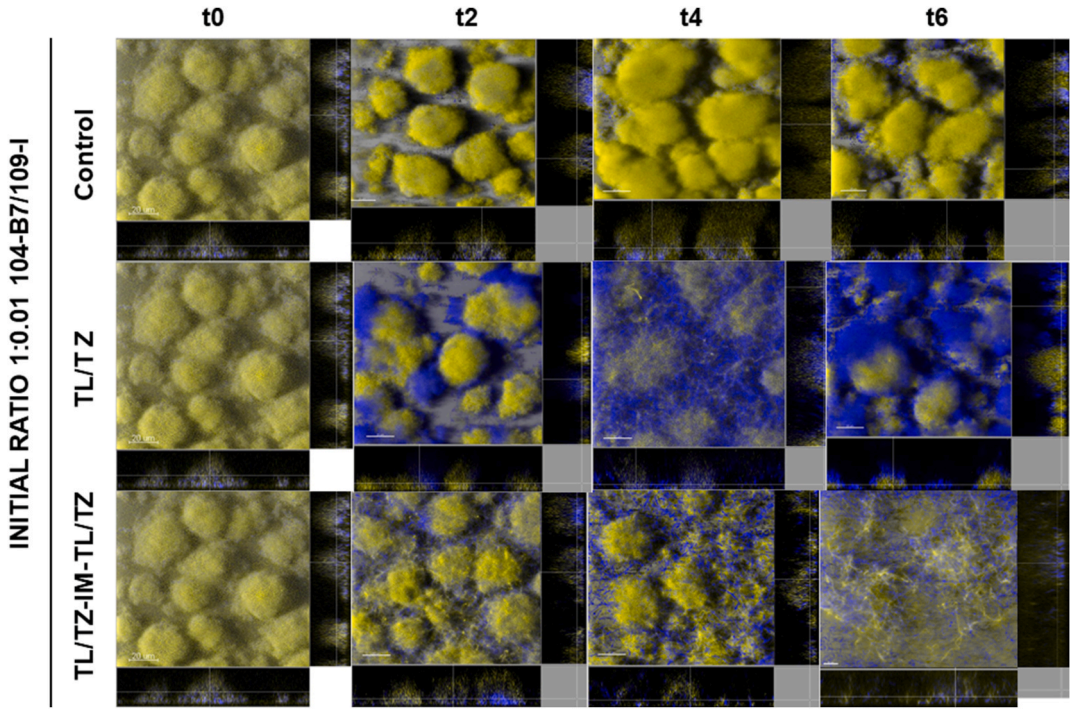

Fig. 6. Three-dimensional images and transversal sections of mixed control biofilms [initial 1:0.01 ratio; 104-B7 (EYFP, yellow):104-I9 (ECFP, blue), respectively] and along treatment with ceftolozane/tazobactam (TL/TZ) and with alternation regime (TL/TZ-imipenem (IM)-TL/TZ). The images obtained at four time points (t0, t2, t4, and t6) are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mutations, L244R (n = 6), and the deletion aa236Δ7 (n = 1) (https://arpbigidisba.com/pseudomonas-aeruginosa-derived-cephalosporinase-pdc-database/).

As reported before, cephalosporin-resistant *P. aeruginosa* strains harbouring those mutations in AmpC Ω-loop exhibited increased susceptibility to imipenem [18,19] which established the rational for the designing of the sequential treatment (ceftolozane/tazobactam - imipenem - ceftolozane/tazobactam) in this work. It has recently shown that the Ω-loop mutants demonstrate significantly reduced imipenem substrate specificity than the wild type leading to loss of efficiency for hydrolysing imipenem, thereby explaining the basis of imipenem collateral susceptibility [32]. Encouragingly, the alternation regimen was able to effectively reduce biofilm viable cells compared to monotherapy and successfully avoid selection and amplification of ceftolozane/tazobactam resistant mutants. Furthermore, in competition experiments, sequential treatment was able to counter-select the resistant isogenic mutant strain 104-I9. These therapeutic efficacy results based on viable cells and antibiotic-resistant mutants were mostly in accordance with the biofilm evolution through images monitored by CLSM.

A potential alternative to sequential treatments with cephalosporins and carbapenems, that has been previously explored, is combined therapy with both agents. Indeed, a previous work has shown that ceftolozane/tazobactam plus meropenem reduced bacterial density and suppressed resistance development in a Hollow-fiber model, being a useful combination for treating XDR *P. aeruginosa* [33]. The basis for this synergy could be related to an effect on several essential penicillin-binding proteins (PBP2 and PBP3 in *P. aeruginosa*), which can increase the bactericidal properties of the drugs and induce morphological changes in the bacteria [33]. However, while imipenem provides a positive advantage for sequential treatments compared to meropenem due to a much more pronounced collateral susceptibility of AmpC Ω-loop ceftolozane/tazobactam resistant mutants, its usefulness for combined treatments would be hampered by its strong induction of AmpC expression [18,19,32]. Furthermore, in general, the combination of two antibiotics has some other important disadvantages as compared to sequential treatment with each of them, such the economic and ecological impact of overall doubling the total amount of antibiotics administered as well as the possibility of providing a selective pressure for the emergence of resistance mechanisms that may simultaneously affect both antibiotics.

Thus, to the best of our knowledge this work is the first to apply the principles of collateral susceptibility-guided alternation of antibiotics to the successful treatment and prevention of resistance development in XDR *P. aeruginosa* biofilms [34–37].

The next step would necessarily require the evaluation of these regimens in vivo and the humanisation of the model to optimize PK/PD parameters to adapt the sequential strategy to the patients and to the specific site and nature of chronic infection.

From a critical point of view, the proposed sequential treatment, although achieved a great reduction of the population was not able to fully eradicate biofilms in any of the strains. This fact highlights the complex management of these infections and the need for the simultaneous use of further therapeutic and co-adjuvant strategies.

Also, as a limitation, although the study uses clinical strains obtained from a patient, it would be desirable to verify the general applicability of the results using a larger panel of strains.

In summary, the present work demonstrates for the first time that the alternation ceftolozane/tazobactam and imipenem, based on antagonistic resistance mechanism, effectively avoids the selection and amplification of ceftolozane/tazobactam resistant mutants in biofilms during treatment, thus representing a potential therapeutic alternative against XDR/DTR *P. aeruginosa* chronic infections that needs to be explored in clinical practice.

CRediT authorship contribution statement

María Fernández-Billón: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Elena Jordana-Lluch:** Supervision, Methodology, Investigation, Formal analysis, Data curation. **Aina E. Llambías-Cabot:** Methodology, Investigation, Formal analysis, Data curation. **María A. Gomis-Font:**

Methodology, Investigation, Formal analysis, Data curation. **Pablo Fraile-Ribot:** Methodology, Formal analysis. **Rosa I. Torrandell:** Methodology, Investigation. **Pamela J. Colman-Vega:** Methodology, Investigation. **Óscar Murillo:** Writing – review & editing, Conceptualization. **María D. Macià:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis, Conceptualization. **Antonio Oliver:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

EJL holds a "Juan de la Cierva-Incorporación" Fellowship (IJC2019-038836-I) granted by The Ministry of Science and Innovation (Spain). MFB holds a "Río Hortega" (CM23/00127) granted by The Ministry of Science and Innovation (Spain). MAGF holds a "PFIS" (FI22/00039) granted by the Ministry of Science and Innovation (Spain). MDM is the educational officer of the European Society for Clinical Microbiology and Infectious Disease Study Group for Biofilms (ESGB). Research of all authors is supported by The Ministry of Science and Innovation (PI23/01086 and PI21/00017), the Spanish Network for Research in Infectious Diseases (REIPI RD16/0016) and Centro de Investigación Biomédica en Red en Enfermedades Infecciosas, Instituto de Salud Carlos III (CIBERINFEC).

Data availability

No data was used for the research described in the article.

References

[1] Kragh KN, Richter K. Introduction: biofilms 101. In: Antibiofilm strategies: current and future applications to prevent, control and eradicate biofilms. Cham: Springer International Publishing; 2022. p. 3–15.
[2] Del Pozo JL. Biofilm-related disease. Expert Rev Anti Infect Ther 2018 Jan;16(1):51–65. https://doi.org/10.1080/14787210.2018.1417036. Epub 2017 Dec 19. PMID: 29235402.
[3] Costerton JW, et al. Bacterial biofilms: a common cause of persistent infections. Science 1999;284:1318–22.
[4] Høiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, Hall-Stoodley L, Holá V, Imbert C, Kirketerp-Møller K, Lebeaux D, Oliver A, Ullmann AJ, Williams C, ESCMID Study Group for Biofilms and Consulting External Expert Werner Zimmerli. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2015;21(Suppl 1):S1–25. https://doi.org/10.1016/j.cmi.2014.10.024.
[5] Mah TF. Biofilm-specific antibiotic resistance. Future Microbiol 2012;7(9):1061–72. https://doi.org/10.2217/fmb.12.76.
[6] Macià MD, Oliver A. In: Richter K, Kragh KN, editors. Antibiotic resistance development in bacterial biofilms BT - antibiofilm strategies: current and future applications to prevent, control and eradicate biofilms. Cham: Springer International Publishing; 2022. p. 37–58.
[7] Fernández-Billón M, Llambías-Cabot AE, Jordana-Lluch E, Oliver A, Macià MD. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Biofilm 2023;5:100129. https://doi.org/10.1016/j.bioflm.2023.100129.
[8] Rojo-Molinero E, Macià MD, Rubio R, Moyà B, Cabot G, López-Causapé C, Pérez JL, Cantón R, Oliver A. Sequential treatment of biofilms with aztreonam and tobramycin is a novel strategy for combating Pseudomonas aeruginosa chronic respiratory infections. Antimicrob Agents Chemother 2016;60(5):2912–22. https://doi.org/10.1128/AAC.00196-16.
[9] Ciofu O, Rojo-Molinero E, Macià MD, Oliver A. Antibiotic treatment of biofilm infections. APMIS 2017 Apr;125(4):304–19. https://doi.org/10.1111/apm.12673. PMID: 28407419.
[10] Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2012;18(3):268–81. https://doi.org/10.1111/j.1469-0691.2011.03570.x.
[11] Kadri SS, Adjemian J, Lai YL, Spaulding AB, Ricotta E, Prevots DR, Palmore TN, Rhee C, Klompas M, Dekker JP, Powers JH, Suffredini AF, Hooper DC, Fridkin S, Danner RL, National Institutes of Health Antimicrobial Resistance Outcomes Research Initiative (NIH–ARORI). Difficult-to-Treat resistance in gram-negative bacteremia at 173 US hospitals: retrospective cohort analysis of prevalence, predictors, and outcome of resistance to all first-line agents. Clin Infect Dis : an official publication of the Infectious Diseases Society of America 2018;67(12):1803–14. https://doi.org/10.1093/cid/ciy378.
[12] Benito N, et al. GEIO group. The different microbial etiology of prosthetic joint infections according to route of acquisition and time after prosthesis implantation, including the role of multidrug-resistant organisms. J Clin Med 2019;13:673.
[13] Benito N, et al. GEIO Group. Time trends in the aetiology of prosthetic joint infections: a multicentre cohort study. Clin Microbiol Infect 2016;22:732.e1–8.
[14] Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev 2009;22:582–610.
[15] Poole K. Pseudomonas aeruginosa: resistance to the max. Front Microbiol 2011;2:65.
[16] Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 2011;35:736–55.
[17] Oliver A, Mulet X, López-Causapé C, et al. The increasing threat of Pseudomonas aeruginosa high-risk clones. Drug Resist Updates 2015;21(22):41–59.
[18] Cabot G, Bruchmann S, Mulet X, Zamorano L, Moyà B, Juan C, Haussler S, Oliver A. Pseudomonas aeruginosa ceftolozane-tazobactam resistance development requires multiple mutations leading to overexpression and structural modification of AmpC. Antimicrob Agents Chemother 2014;58:3091–9. https://doi.org/10.1128/AAC.02462-13.
[19] Fraile-Ribot PA, Cabot G, Mulet X, Periañez L, Martín-Pena ML, Juan C, Pérez JL, Oliver A. Mechanisms leading to in vivo ceftolozane/tazobactam resistance development during the treatment of infections caused by MDR Pseudomonas aeruginosa. J Antimicrob Chemother 2018;73(3):658–63. https://doi.org/10.1093/jac/dkx424.
[20] Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersbøll BK, Molin S. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 2000;146:2395–407. https://doi.org/10.1099/00221287-146-10-2395.
[21] Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985;22:996–1006.
[22] Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S, Tolker-Nielsen T. Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol Microbiol 2003;48:1511–24. https://doi.org/10.1046/j.1365-2958.2003.03525.x.
[23] Lambertsen L, Sternberg C, Molin S. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. Environ Microbiol 2004;6(7):726–32. https://doi.org/10.1111/j.1462-2920.2004.00605.x.
[24] Christensen BB, Sternberg C, Andersen JB, Palmer Jr RJ, Nielsen AT, Givskov M, Molin S. Molecular tools for study of biofilm physiology. Methods Enzymol 1999;310:20–42. https://doi.org/10.1016/S0076-6879(99)10004-1.
[25] Macià MD, Pérez JL, Molin S, Oliver A. Dynamics of mutator and antibiotic-resistant populations in a pharmacokinetic/pharmacodynamic model of Pseudomonas aeruginosa biofilm treatment. Antimicrob Agents Chemother 2011 Nov;55(11):5230–7. https://doi.org/10.1128/AAC.00617-11. Epub 2011 Aug 22. PMID: 21859941; PMCID: PMC3195006.
[26] Rojo-Molinero E, Macià MD, Oliver A. Social behavior of antibiotic resistant mutants within Pseudomonas aeruginosa biofilm communities. Front Microbiol 2019 Mar 22;10:570. https://doi.org/10.3389/fmicb.2019.00570. PMID: 30967851; PMCID: PMC6438888.
[27] Cabot G, López-Causapé C, Ocampo-Sosa AA, Sommer LM, Domínguez MÁ, Zamorano L, Juan C, Tubau F, Rodríguez C, Moyà B, Peña C, Martínez-Martínez L, Plesiat P, Oliver A. Deciphering the resistome of the widespread Pseudomonas aeruginosa sequence type 175 international high-risk clone through whole-genome sequencing. Antimicrob Agents Chemother 2016;60(12):7415–23. https://doi.org/10.1128/AAC.01720-16.
[28] López-Causapé C, Sommer LM, Cabot G, Rubio R, Ocampo-Sosa AA, Johansen HK, Figuerola J, Cantón R, Kidd TJ, Molin S, Oliver A. Evolution of the Pseudomonas aeruginosa mutational resistome in an international Cystic Fibrosis clone. Sci Rep 2017;7(1):5555. https://doi.org/10.1038/s41598-017-05621-5.
[29] Del Barrio-Tofiño E, Zamorano L, Cortes-Lara S, López-Causapé C, Sánchez-Diener I, Cabot G, Bou G, Martínez-Martínez L, Oliver A, GEMARA-SEIMC/REIPI Pseudomonas study Group. Spanish nationwide survey on Pseudomonas aeruginosa antimicrobial resistance mechanisms and epidemiology. J Antimicrob Chemother 2019;74(7):1825–35. https://doi.org/10.1093/jac/dkz147.
[30] MacVane SH, Pandey R, Steed LL, Kreiswirth BN, Chen L. Emergence of ceftolozane-tazobactam-resistant Pseudomonas aeruginosa during treatment is mediated by a single AmpC structural mutation. Antimicrob Agents Chemother 2017;61. https://doi.org/10.1128/AAC.01183-17.
[31] Slater CL, Winogrodzki J, Fraile-Ribot PA, Oliver A, Khajehpour M, Mark BL. Adding insult to injury: mechanistic basis for how AmpC mutations allow Pseudomonas aeruginosa to accelerate cephalosporin hydrolysis and evade avibactam. Antimicrob Agents Chemother 2020;64. https://doi.org/10.1128/AAC.00894-20.

[32] Cabot G, Kim K, Mark BL, Oliver A, Khajehpour M. Biochemical insights into imipenem collateral susceptibility driven by ampC mutations conferring ceftolozane/tazobactam resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother 2023;67(2):e0140922. https://doi.org/10.1128/aac.01409-22.

[33] Montero M, VanScoy BD, López-Causapé C, Conde H, Adams J, Segura C, Zamorano L, Oliver A, Horcajada JP, Ambrose PG. Evaluation of ceftolozane-tazobactam in combination with meropenem against Pseudomonas aeruginosa sequence type 175 in a hollow-fiber infection model. Antimicrob Agents Chemother 2018 Apr 26;62(5). https://doi.org/10.1128/aac.00026-18.

[34] Hernando-Amado S, López-Causapé C, Laborda P, Sanz-García F, Oliver A, Martínez JL. Rapid phenotypic convergence towards collateral sensitivity in clinical isolates of Pseudomonas aeruginosa presenting different genomic backgrounds. Microbiol Spectr 2023;11(1):e0227622. https://doi.org/10.1128/spectrum.02276-22.

[35] Batra A, Roemhild R, Rousseau E, Franzenburg S, Niemann S, Schulenburg H. High potency of sequential therapy with only β-lactam antibiotics. Elife 2021 Jul 28;10:e68876. https://doi.org/10.7554/eLife.68876. PMID: 34318749; PMCID: PMC8456660.

[36] Hernando-Amado S, Sanz-García F, Martínez JL. Rapid and robust evolution of collateral sensitivity in Pseudomonas aeruginosa antibiotic-resistant mutants. Sci Adv 2020;6(32):eaba5493. https://doi.org/10.1126/sciadv.aba5493.

[37] Roemhild R, Gokhale CS, Dirksen P, Blake C, Rosenstiel P, Traulsen A, Andersson DI, Schulenburg H. Cellular hysteresis as a principle to maximize the efficacy of antibiotic therapy. Proc Natl Acad Sci U S A 2018 Sep 25;115(39):9767–72. https://doi.org/10.1073/pnas.1810004115. Epub 2018 Sep 12. PMID: 30209218; PMCID: PMC6166819.