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Combination of polymyxin B and meropenem eradicates persister cells from *Acinetobacter baumannii* strains in exponential growth

Stephanie W. Gallo, Carlos Alexandre S. Ferreira and Sílvia D. de Oliveira*

Acinetobacter baumannii is an important opportunistic pathogen that causes several healthcare-associated infections that have high rates of morbidity and mortality in hospital settings, particularly in intensive care units [1]. Treatment of infections caused by *A. baumannii* has become increasingly limited due to the emergence of multidrug-resistant isolates, including resistance to carbapenems, leaving polymyxins as the last therapeutic resource. Although the standard antimicrobial scheme against *A. baumannii* infections has not been fully established, evidence supports the fact that combination therapies including polymyxin B are more effective than polymyxin B monotherapies. Moreover, polymyxin B combined with a second antimicrobial agent was able to reduce hospital mortality rates, and has been widely recommended due to resistance emerging in some *A. baumannii* strains during treatments [2–4]. Nevertheless, antimicrobial therapy failure has been reported, especially in recalcitrance of chronic infections, which may be due to the tolerance mediated by persister cells [5, 6] – a microbial subpopulation able to survive to normally lethal concentrations of bactericidal antimicrobials [7], as has been previously described in *Acinetobacter* spp. exposed to polymyxin B [8]. This tolerance appears to be a transient phenotype that may be understood as arising from a combination of epigenetic inheritance and cellular noise [9], whose regulation involves a multiplicity of pathways [10]. Thus, eradication of persister cells is unusually challenging, and, to the best of our knowledge, the influence of combined therapy has not been investigated in *A. baumannii* persister cells. In this context, we evaluated the effect of polymyxin B and meropenem in combination to eradicate the persister cells produced by clinical *A. baumannii* strains.

A. baumannii strains (Acb-1, Acb-8 and Acb-20) were isolated from tracheal aspirate and sputum by the Department of Microbiology of the Clinical Pathology Laboratory of São Lucas Hospital, Porto Alegre, Brazil. The strains were previously identified as *A. baumannii* by multiplex PCR according to Higgins *et al.* [11], as well as being characterized as susceptible to polymyxin B and meropenem by MIC assessment

according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [12]. Polymyxin B and meropenem MIC values were determined as 1 $\mu\text{g ml}^{-1}$ for both antimicrobials for Acb-1, 0.5 and 0.25 $\mu\text{g ml}^{-1}$ for Acb-8, and 1 and 0.5 $\mu\text{g ml}^{-1}$ for Acb-20, respectively. The strains were previously characterized as producing persisters at different levels after exposure to meropenem [13]. To evaluate the persister levels, strains were cultured at 37 °C for 18 h in Lysogeny broth (LB), diluted 1:30 with a fresh medium, and incubated until the late exponential phase. Afterwards, the initial cell concentration was determined by decimal serial dilutions and drop-plating onto nutrient agar. To determine the killing curves, the cultures were exposed for 48 h to 15, 10 and 5 $\mu\text{g ml}^{-1}$ of polymyxin B or meropenem, and were then treated with both antimicrobials at different concentrations, as follows: polymyxin B at 15 $\mu\text{g ml}^{-1}$ plus meropenem at 15 $\mu\text{g ml}^{-1}$; polymyxin B at 10 $\mu\text{g ml}^{-1}$ plus meropenem at 10 $\mu\text{g ml}^{-1}$; polymyxin B at 5 $\mu\text{g ml}^{-1}$ plus meropenem at 5 $\mu\text{g ml}^{-1}$; polymyxin B at 10 $\mu\text{g ml}^{-1}$ plus meropenem at 5 $\mu\text{g ml}^{-1}$; and polymyxin B at 5 $\mu\text{g ml}^{-1}$ plus meropenem at 10 $\mu\text{g ml}^{-1}$. The combination of polymyxin B at 5 $\mu\text{g ml}^{-1}$ plus meropenem at 5 $\mu\text{g ml}^{-1}$ was evaluated only for Acb-1. After 6, 24 and 48 h of antimicrobial exposure, aliquots were collected, washed with 0.85 % saline, serially diluted and spotted onto nutrient agar. The number of c.f.u. per millilitre (ml^{-1}) was measured in order to determine the persister cell fractions. The lower limit for the quantification of persister cells was 10^2 c.f.u. ml^{-1} . All experiments were performed in triplicate. To confirm the persistence phenotype and exclude the selection of resistant cells, persister fractions obtained after 48 h of antimicrobial exposure were evaluated for polymyxin B and meropenem susceptibility by MIC determination, and also re-cultivated into a sterile LB with the same antimicrobial concentrations. All experiments to quantify persister cells were performed using three biological replicates. Persister cell fractions obtained after exposure to the polymyxin B plus meropenem combinations were compared, as well as with each antimicrobial used alone, employing an unpaired *t*-test in GraphPad Prism software version 7.00

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Keywords: persistence; persisters eradication; chronic infection; *Acinetobacter baumannii*; polymyxin B; carbapenems.

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; LB, Lysogeny broth; Mer, meropenem; MIC, Minimum Inhibitory Concentration; ND, not detected; PolB, polymyxin B.

Table 1. Persister cell fractions from *A. baumannii* strains exposed to polymyxin B (PolB) and meropenem (Mer) in combination or as monotherapy for 48 h

Antimicrobial concentrations ($\mu\text{g ml}^{-1}$)	<i>A. baumannii</i> strains - c.f.u. ml ⁻¹ (%)		
	Acb-1	Acb-8	Acb-20
PolB (5)	1.51×10^4 (0.0064)	8.97×10^3 (0.0053)	2.90×10^5 (0.1556)
PolB (10)	9.40×10^3 (0.0043)	7.17×10^3 (0.0040)	2.47×10^5 (0.1413)
PolB (15)	7.15×10^3 (0.0038)	5.10×10^3 (0.0031)	2.36×10^5 (0.1285)
Mer (5)	4.60×10^5 (0.2849)	2.23×10^4 (0.0124)	1.70×10^6 (0.9743)
Mer (10)	4.20×10^5 (0.2321)	1.67×10^4 (0.0094)	1.37×10^6 (0.8547)
Mer (15)	3.07×10^5 (0.2087)	6.67×10^3 (0.0042)	1.02×10^6 (0.7310)
PolB (5) plus Mer (5)	1.40×10^4 (0.0056)	-	-
PolB (5) plus Mer (10)	1.63×10^3 (0.0008)	0 (0)	4.73×10^3 (0.0023)
PolB (10) plus Mer (5)	0 (0)	0 (0)	0 (0)
PolB (10) plus Mer (10)	0 (0)	0 (0)	0 (0)
PolB (15) plus Mer (15)	0 (0)	0 (0)	0 (0)

-, Not evaluated.

(GraphPad, La Jolla, CA, USA). *P*-values <0.05 were considered significant.

All *A. baumannii* strains formed persister cells after 48 h of exposure to all concentrations of polymyxin B or meropenem as monotherapies (Table 1). As previously described [13], these strains produced different persister fractions after exposure to meropenem. Here, we could detect that Acb-20 exposed to polymyxin B formed the highest fractions, similarly when it was exposed to meropenem. Polymyxin B at $10 \mu\text{g ml}^{-1}$ plus meropenem at $5 \mu\text{g ml}^{-1}$ and a combination of

both antimicrobials at 10 or $15 \mu\text{g ml}^{-1}$ were able to eradicate persister cells after exposure for 48 h from all isolates. However, exposure to polymyxin B at $5 \mu\text{g ml}^{-1}$ plus meropenem at $10 \mu\text{g ml}^{-1}$ was not able to eliminate persister cells from Acb-1 and Acb-20, with fractions of 0.0008 and 0.0023 % remaining, respectively (Table 1 and Fig. 1a, b). In addition, the combination of $5 \mu\text{g ml}^{-1}$ of each antimicrobial also did not completely eradicate persister cells from Acb-1, with 0.0056 % of the initial population remaining (Fig. 1a). On the other hand, all combinations of the antimicrobial concentrations tested were able to eradicate persisters from Acb-8,

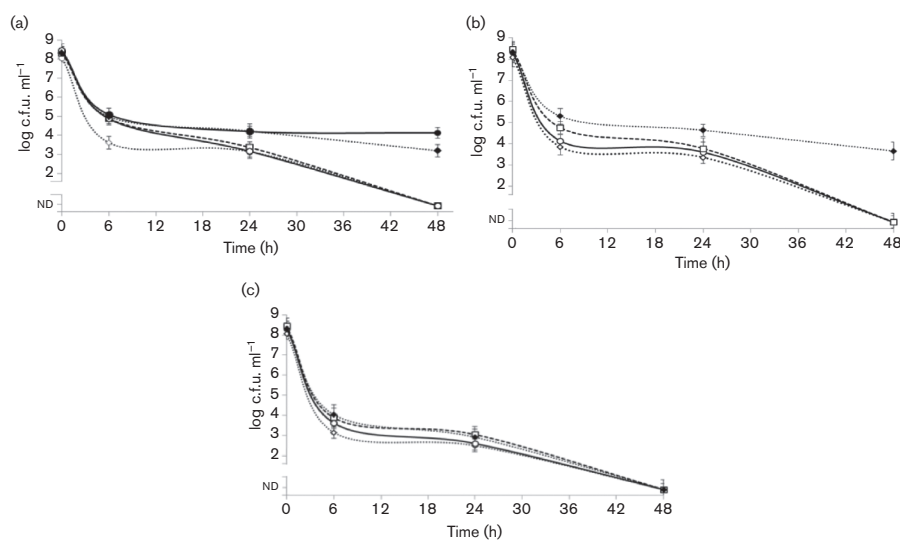


Fig. 1. Killing curves of *A. baumannii* strains after 48 h exposure to meropenem and polymyxin B in combination. Acb-1 (a), Acb-20 (b) and Acb-8 (c) strains were cultured until the late exponential phase and exposed to polymyxin B at $15 \mu\text{g ml}^{-1}$ plus meropenem at $15 \mu\text{g ml}^{-1}$ (open diamonds); polymyxin B at $10 \mu\text{g ml}^{-1}$ plus meropenem at $10 \mu\text{g ml}^{-1}$ (open circles); polymyxin B at $10 \mu\text{g ml}^{-1}$ plus meropenem at $5 \mu\text{g ml}^{-1}$ (open squares); and polymyxin B at $5 \mu\text{g ml}^{-1}$ plus meropenem at $10 \mu\text{g ml}^{-1}$ (filled diamonds); polymyxin B at $5 \mu\text{g ml}^{-1}$ plus meropenem at $5 \mu\text{g ml}^{-1}$ (filled circles). ND (not detected).

raising the possibility that lower-producing persisters can be eliminated more easily (Fig. 1c). Significant differences were found between the persister cell fractions remaining after treatment with antimicrobial combinations when compared to monotherapies ($P < 0.05$), with the exception of the $5 \mu\text{g ml}^{-1}$ combinations of each antimicrobial in comparison with polymyxin B at $5 \mu\text{g ml}^{-1}$ ($P = 0.7847$), polymyxin B at $10 \mu\text{g ml}^{-1}$ ($P = 0.5083$) and polymyxin B at $15 \mu\text{g ml}^{-1}$ ($P = 0.2712$) in the Acb-1 strain. Likewise, persister cell fractions remaining after exposure to combinations that did not eradicate Acb-1 were not statistically different ($P = 0.0073$). Persister cells obtained after 48 h of polymyxin B and meropenem exposure remained susceptible when regrown in LB supplemented with the antimicrobials, and the MIC evaluated after the treatment remained unchanged for both antimicrobials, confirming the drug-tolerance phenotype. Furthermore, we observed that combining the highest doses tested of both antimicrobials was more efficient even after 6 h exposure, and rapidly reduced the bulk of cells in comparison with the other combinations. Thus, taking into account the present data and our previous findings that polymyxin B ($15 \mu\text{g ml}^{-1}$) [8] or meropenem alone ($15, 30, 100$ and $200 \mu\text{g ml}^{-1}$) were not able to eradicate *A. baumannii* persisters [13], we may highlight that there is a synergistic bactericidal effect of this drug combination. Simultaneous treatment employing high doses of conventional antimicrobials has also been reported as being able to completely eradicate *Staphylococcus aureus* persister cells within a few hours of antibiotic combination exposure [14]. Furthermore, the treatment grounded in antimicrobial combination could be an effective alternative to eradicating these cells, especially taking into account the heterogeneous pattern of persister cell levels obtained after exposure to distinct antibiotics, even those belonging to the same class [8, 15–17]. So, a multidrug persistence phenotype is suggested, as well as the existence of different types of persisters in the bacterial population [16, 18]. Another strategy implemented to improve killing persisters is to use conventional antibiotics in a pulse-dosing regimen, as verified for *Borrelia burgdorferi* treated with ceftriaxone [17], but a similar regimen failed in the eradication of *Acinetobacter calcoaceticus-baumannii* persisters when exposed to high doses of meropenem [13]. Additionally, eradication of persister cells formed by other bacteria has been achieved using experimental antimicrobial compounds as monotherapy or combined with drugs commonly employed to treat infections caused by them [15, 17, 19]. In conclusion, our results provide additional knowledge for the design of persister eradication strategies using antimicrobials commonly employed to treat chronic and recurrent infections caused by *A. baumannii*.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This study was approved by the Research Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) under protocol number 483469.

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