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Pharmacodynamic evaluation of suppression of in vitro resistance in *Acinetobacter baumannii* strains using polymyxin B-based combination therapy

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The emergence of polymyxin resistance in Gram-negative bacteria infections has motivated the use of combination therapy. This study determined the mutant selection window (MSW) of polymyxin B alone and in combination with meropenem and fosfomycin against *A. baumannii* strains belonging to clonal lineages I and III. To evaluate the inhibition of in vitro drug resistance, we investigate the MSW-derived pharmacodynamic indices associated with resistance to polymyxin B administrated regimens as monotherapy and combination therapy, such as the percentage of each dosage interval that free plasma concentration was within the MSW (%T_{MSW}) and the percentage of each dosage interval that free plasma concentration exceeded the mutant prevention concentration (%T_{MPC}). The MSW of polymyxin B varied between 1 and 16 µg/mL for polymyxin B-susceptible strains. The triple combination of polymyxin B with meropenem and fosfomycin inhibited the polymyxin B-resistant subpopulation in meropenem-resistant isolates and polymyxin B plus meropenem as a double combination sufficiently inhibited meropenem-intermediate, and susceptible strains. T_{>MPC} 90% was reached for polymyxin B in these combinations, while %T_{MSW} was 0 against all strains. T_{MSW} for meropenem and fosfomycin were also reduced. Effective antimicrobial combinations significantly reduced MSW. The MSW-derived pharmacodynamic indices can be used for the selection of effective combination regimen to combat the polymyxin B-resistant strain.

Polymyxins are an old class of antibiotics first discovered in the 1950s, but have regained interest over the past few years due to the emergence of multidrug-resistant Gram-negative bacteria (MDR-GNB) worldwide, particularly the carbapenem-resistant *Acinetobacter baumannii*, CR-Ab¹. Although in recent years, the pharmaceutical industry with government initiatives has launched several new drugs in the market with activity against MDR-GNB; none of them, except cefiderocol and eravacycline, have antimicrobial activity against CR-Ab².

The clinical use of polymyxin B is not thoroughly understood owing to the insufficient information on efficacy at the site of infection, pharmacokinetic limitation (potential nephrotoxicity) and the lack of clinically relevant susceptibility breakpoints^{3,4}. The Clinical and Laboratory Standards Institute (CLSI) recently updated polymyxin B breakpoints only for intermediate (≤ 2 µg/mL) and resistant (≥ 4 µg/mL) interpretative categories, whereas European Committee on Antimicrobial Susceptibility Testing (EUCAST) maintained this interpretative category (susceptible ≤ 2 and resistant > 4 µg/mL)^{5,6}. Polymyxin B demonstrates in vitro activity against CR-Ab^{7,8}, however its use as monotherapy often results in clinical failure and emergence of drug resistance in the microorganism

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ID code	Strain name	Year	MLST	Non-susceptible antimicrobial profile	MIC/MPC in µg/mL			Carbapenemase genes
					PMB	MEM	FOF	
ATCC 19606	–	1948	ST52	AMP, AMC, CFZ, FOX, NIT, STX	1/4	2/16	128/2048	–
Ac56-HUEL	Ac-PMBs/Mi	1996	ST374	TZP, CAZ, FEP, CIP, LEV, CN, AMK	2/8	4/32	256/2048	–
Ac56-HUM	Ac-PMBs/Mr	2012	ST983	TZP, SAM, CAZ, FEP, CIP, LEV, GEN, AMK, IPM, MEM, SXT, TGC	2/16	16/64	128/2048	<i>bla</i> _{VIM-like}
Ac576-HUEL	Ac-PMBr/Mr	2011	ST1	TZP, SAM, CAZ, FEP, CIP, LEV, GEN, AMK, IPM, MEM, SXT, TGC, PMB	32/64	64/ > 512	128/2048	<i>bla</i> _{OXA-23^a}

Table 1. Antimicrobial susceptibility profile and molecular characteristics of selected *A. baumannii* strains. Susceptibility antimicrobial profile results were performed by automated systems. CLSI 2019 *A. baumannii* breakpoints (µg/mL) for PMB: susceptible (S) ≤ 2 and resistant (R) ≥ 4; and MEM: S ≤ 2, intermediate 4, R ≥ 8. EUCAST 2020 *A. baumannii* breakpoints (µg/mL) for colistin: S ≤ 2 and R > 2; and MEM: S ≤ 2 and R ≥ 8. FOF breakpoints (µg/mL) for Enterobacteriales according to the CLSI 2019 guidelines: S ≤ 64, intermediate 128, R ≥ 256. MLST multilocus sequence typing, ST sequence type, AMP ampicillin, AMC amoxicillin-clavulanic acid, CFZ cefazolin, FOX cefoxitin, NIT nitrofurantoin, SXT trimetoprim-sulfamethoxazole, TZP piperacillin-tazobactam, SAM ampicillin-sulbactam, CAZ ceftazidime, FEP cefepime, IPM imipenem, MEM meropenem, CIP ciprofloxacin, LEV levofloxacin, AMK amikacin, GEN gentamicin, PMB polymyxin B, TGC tigecycline, FOF fosfomicin. ^a*bla*_{OXA-23-like} carbapenemase gene is associated with the strong promoter *ISAbal* located upstream of the gene.

during therapy^{9,10}, in part because therapeutic concentrations that kill the majority of susceptible cells may be insufficient to restrict the growth of resistant mutant subpopulation¹¹.

The mutant selection window (MSW) hypothesis postulates this critical concentration range where resistant mutant subpopulation is selectively amplified. The upper limit of the window is the mutant prevention concentration (MPC) and the lower boundary is the minimum inhibitory concentration (MIC)¹². According to this hypothesis, keeping drug concentrations above MPC throughout therapy will restrict the emergence of resistance and achieve its therapeutic effect. The high bacterial inocula used in the MPC measurements are also more representative of the bacterial burden that is present at the infection sites, such as pneumonia¹³. Given that polymyxin B has a very narrow therapeutic window, administration of this drug as monotherapy failed to attain MPC safely, especially in patients with renal impairment¹⁴.

Even though clinical data that explored the advantages of polymyxins combination therapy versus monotherapy presented conflicting results¹⁵, combination therapy is still relevant because the use of less-toxic and broad-spectrum antibiotics can restore polymyxin B activities^{10,16–18}. Several in vitro studies demonstrated the synergistic effect of combinations with meropenem and fosfomicin^{19–21}. Fosfomicin acts by blocking the MurA enzyme that is involved in the first steps of the peptidoglycan biosynthetic pathway²². Meropenem binds to penicillin-binding proteins in the periplasmic space, preventing peptidoglycan biosynthesis and viable cell wall production²³. Polymyxins are cationic agents that bind to the anionic lipopolysaccharide and phospholipids molecules in the outer membrane, leading to a detergent-type effect that disrupts membrane integrity²⁴. Concomitant use of antimicrobials acting on different cellular targets can theoretically result in the synergism between them and possibly restrict the selection of polymyxin B-resistant mutant bacteria.

There is an urgent need to investigate the effects of drug combination with the usual polymyxin B regimens on the resistant mutant subpopulation of both susceptible and non-susceptible strains. The current study determines the MPC of polymyxin B against *A. baumannii* and investigates the effect of combination therapy on pharmacodynamic indices associated with resistance suppression.

Results

Sequence type distribution of isolates and in vitro antimicrobial susceptibility. Antimicrobial susceptibility profiles and molecular characteristics of all strains are described in Table 1. The population structure of the 1,557 sequence types available in the PubMLST *A. baumannii* database is shown in Fig. 1 (data accessed on December 8, 2020), based on the Pasteur scheme using the goeBURST algorithm implemented in PhyloViz Online²⁵.

The MIC, MPC, and MPC:MIC ratio of polymyxin B alone and in combination with meropenem and fosfomicin for each strain are shown in Fig. 2. For Ac-PMBs/Mi isolate, the MPC value was four times higher than MIC (MSW from 2 to 8 µg/mL). The ATCC 19606 strain had similar MSW compared with this clinical isolate. The Ac-PMBs/Mr showed a wide MSW covering concentrations between 2 and 16 µg/mL. On the other hand, a narrow MSW was observed for Ac-PMBr/Mr isolate, whose concentration ranged between 32 and 64 µg/mL.

We evaluated the hypothesis that effective antimicrobial combinations will reduce or even close the MSW of polymyxin B, meropenem and fosfomicin. The MICs and MPCs were lower than those obtained from polymyxin B alone for all strains (Fig. 2). After the addition of meropenem, both MIC and MPC values decreased up to thirty-two times for polymyxin B (0.25 µg/mL) against ATCC 19606 and Ac-PMBs/Mi strains, closing the MSW and conferring susceptibility to polymyxin B based on the breakpoint value of ≤ 2 µg/mL^{6,26}. The MPC:MIC ratio indicated that polymyxin B in combination with meropenem restricted the emergence of polymyxin B resistance against these strains. For Ac-PMBs/Mr isolate, a MSW varying between 0.25 and 4 µg/mL was maintained even when polymyxin B was combined with meropenem. The Ac-PMBr/Mr showed a greater reduction of MIC and MPC values (sixty-four times) with the two-agent combination than the polymyxin B-susceptible strains,

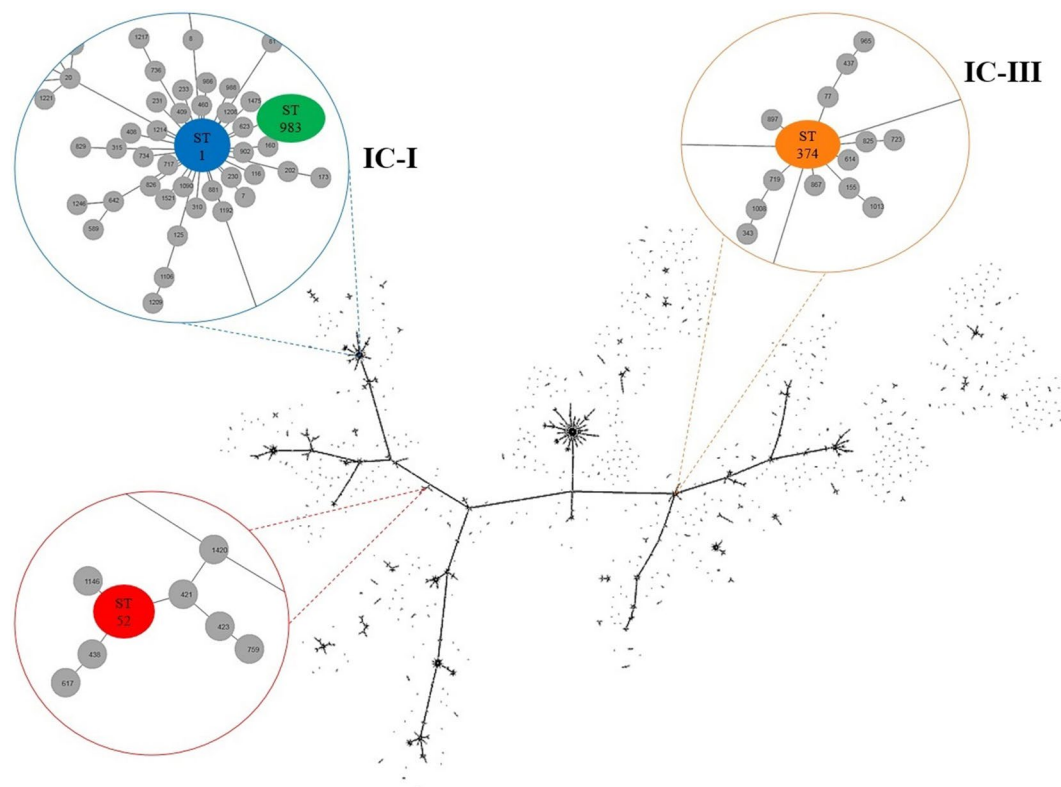


Figure 1. Diagram constructed by the goeBURST algorithm and using the Phyloviz software version 2.0 (<http://www.phyloviz.net/>) indicating the similarity among sequence type (ST) and international clone (IC) corresponding to *A. baumannii* clinical isolates studied. Each line indicates that the connected circles correspond to STs sharing 6/7 of the alleles. The STs observed in the present study are enlarged and highlighted in color.

Strain	Antimicrobial	Checkerboard test (μg/mL)		Polymyxin B concentrations (μg/mL)												MPC:MIC ratio [†]
		MIC	MPC	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64		
ATCC 19606	PMB	1	4	[Dark Blue]												4
	PMB/MEM	0.25/1	0.25/4	[Light Blue]												0.25
	PMB/MEM/FOF	0.25/0.25/8	0.25/0.25/128	[Orange]												0.25
Ac-PMBs/Mi	PMB	2	8	[Dark Blue]												4
	PMB/MEM	0.25/2	0.25/16	[Light Blue]												0.125
	PMB/MEM/FOF	0.25/1/16	0.25/4/64	[Orange]												0.125
Ac-PMBs/Mr	PMB	2	16	[Dark Blue]												8
	PMB/MEM	0.25/2	4/16	[Light Blue]												2
	PMB/MEM/FOF	0.25/0.25/128	0.25/16/512	[Orange]												0.125
Ac-PMBr/Mr	PMB	32	64	[Dark Blue]												2
	PMB/MEM	0.5/16	1/32	[Light Blue]												0.031
	PMB/MEM/FOF	0.25/0.25/64	0.25/4/128	[Orange]												0.007

- Sub-MIC: no selective enrichment of resistant mutant subpopulation is expected
- MSW: the resistant mutant subpopulation is likely to be enriched
- Above MPC: the resistant mutant subpopulation is unlikely to be enriched
- MPC and MIC values of PMB in combination were equal

Figure 2. MIC, MPC, and MIC/MPC ratio of polymyxin B alone and in combination with meropenem and fosfomycin against *A. baumannii* strains. MIC minimum inhibitory concentration, MPC mutant prevention concentration, MSW mutant selection window, PMB polymyxin B, MEM meropenem, FOF fosfomycin, PMB/MEM PMB combined with MEM, PMB/MEM/FOF the combination of all three antimicrobials. [†]MPC:MIC ratio is defined as the ratio of the MPC obtained to the original MIC and smaller values indicate a better ability to prevent the emergence of mutants.

Strain	Polymyxin B dosage regimen ^a	Monotherapy		Double-combination of polymyxin B regimen/2 g q8h meropenem ^b		Triple-combination of polymyxin B regimen/2 g q8h meropenem/8 g q8h fosfomicin ^b	
		%T _{MSW} ^c	%T _{>MPC} ^c	%T _{MSW}	%T _{>MPC}	%T _{MSW}	%T _{>MPC}
ATCC 19606	1 mg/kg q12h	12.5 ± 12.7	0.23 ± 1.26	0	48.7 ± 19.3	0	48.7 ± 19.3
	1.5 mg/kg q12h	68.1 ± 31.1	14.5 ± 24.8	0	96.0 ± 11.5	0	96.0 ± 11.5
	LD plus 0.5 mg/kg q12h	22.0 ± 12.7	2.94 ± 4.82	0	56.9 ± 19.7	0	56.9 ± 19.7
	LD plus 1 mg/kg q12h	57.5 ± 29.5	6.75 ± 14.5	0	97.6 ± 6.7	0	97.6 ± 6.7
	LD plus 100 mg q12h	27.6 ± 13.6	4.25 ± 6.6	0	64.4 ± 19.4	0	64.4 ± 19.4
Ac-PMBs/Mi	1 mg/kg q12h	10.8 ± 16.2	NC	0	94.1 ± 15.7	0	94.1 ± 15.7
	1.5 mg/kg q12h	24.4 ± 25.8	0.1 ± 1.1	0	98.2 ± 8.08	0	98.2 ± 8.08
	LD plus 0.5 mg/kg q12h	34.1 ± 31.2	1.2 ± 4.8	0	99.3 ± 4.8	0	99.3 ± 4.8
	LD plus 1 mg/kg q12h	40.3 ± 33.9	1.3 ± 4.2	0	99.6 ± 3.5	0	99.6 ± 3.5
	LD plus 100 mg q12h	44.5 ± 34.3	1.5 ± 4.6	0	99.8 ± 2.6	0	99.8 ± 2.6
Ac-PMBs/Mr	1 mg/kg q12h	10.8 ± 16.3	NC	93.3 ± 15.8	0.8 ± 3.3	0	94.1 ± 15.7
	1.5 mg/kg q12h	24.5 ± 26.1	NC	93.6 ± 12.1	4.7 ± 9.8	0	98.2 ± 8.1
	LD plus 0.5 mg/kg q12h	35.4 ± 32.9	NC	89.2 ± 16.3	10.1 ± 16.0	0	99.3 ± 4.8
	LD plus 1 mg/kg q12h	41.7 ± 35.5	NC	87.2 ± 19.6	12.4 ± 19.4	0	99.6 ± 3.5
	LD plus 100 mg q12h	46.0 ± 35.8	NC	85.4 ± 20.8	14.3 ± 20.7	0	99.8 ± 2.6
Ac-PMBr/Mr	1 mg/kg q12h	NC	NC	35.2 ± 21.4	37.5 ± 31.6	0	94.1 ± 15.7
	1.5 mg/kg q12h	NC	NC	29.2 ± 23.3	58.5 ± 33.8	0	98.2 ± 8.1
	LD plus 0.5 mg/kg q12h	NC	NC	22.1 ± 25.0	71.6 ± 32.3	0	99.3 ± 4.8
	LD plus 1 mg/kg q12h	NC	NC	18.2 ± 26.0	77.9 ± 31.1	0	99.6 ± 3.5
	LD plus 100 mg q12h	NC	NC	15.5 ± 24.8	81.7 ± 29.0	0	99.8 ± 2.9

Table 2. MSW-derived PD indices of polymyxin B alone and in combination against *A. baumannii* strains. MSW mutant selection window, MPC mutant prevention concentration, LD loading dose of 3.0 mg/kg, NC not computable because polymyxin B MIC is higher than maximum. Values reported as mean ± SD. ^aAll regimens were 1-h infusions. ^bMeropenem and fosfomicin regimens were administered as 3-h prolonged infusions. ^cT_{MSW} or T_{>MPC} was calculated as the percentage of the first 24 h.

resulting in attainable plasma levels for polymyxin B (MIC ≤ 2 µg/mL), despite a narrow MSW ranging between 0.5 and 1 µg/mL was present.

The addition of fosfomicin in combination with polymyxin B plus meropenem further decreased the MIC and MPC values of polymyxin B to 0.25 µg/mL against Ac-PMBs/Mr and Ac-PMBr/Mr isolates, closing the MSW and significantly restricting the selective amplification of polymyxin B-resistant subpopulation (Fig. 2). There was a greater effect of the two drugs on MPC:MIC ratio of polymyxin B than that tested individually. Furthermore, the three-drug combination recovered the carbapenem susceptibility with MICs ≤ 2 µg/mL for these strains^{5,26}. Only one out of four strains did not show a reduction in MPC of meropenem in the triple combination (Ac-PMBs/Mr).

Pharmacokinetic/pharmacodynamic (PK/PD) analysis and suppression of resistance. Table 2 shows the PK/PD analysis for five dosing regimens of polymyxin B in monotherapy and combined with meropenem and fosfomicin against four strains. None of the polymyxin B monotherapy regimens achieved plasma concentrations sufficient to attain at least a MIC of 32 µg/mL for Ac-PMBr/Mr, whereas, for the polymyxin B-susceptible strains, a T_{MSW} up to 68% of the dosing interval was observed.

All polymyxin B dose regimens as double combination reduced the T_{MSW} to 0% against ATCC 19606 and Ac-PMBs/Mi strains, while plasma concentrations were above the MPC of 0.25 µg/mL for a duration of at least 96% of the dosing interval at 1.5 mg/kg q12h (Table 2 and Fig. 3). Greater than 87% T_{MSW} was maintained for Ac-PMBs/Mr isolate even with the higher dose of 3 mg/kg followed by 1 mg/kg q12h. Concentrations of polymyxin B fell within the MSW in all dose regimens against Ac-PMBr/Mr isolate, although a reasonable T_{>MPC} up to 78% of the dosing interval was achieved at 3 mg/kg followed by 1 mg/kg q12h.

The triple combination provided a T_{MSW} of 0% for all strains, including those resistant to carbapenem and polymyxin B (Ac-PMBs/Mr and Ac-PMBr/Mr), in the five polymyxin B dosage regimens tested. Similarly, polymyxin B concentrations remained above 98% T_{>MPC} against both Ac-PMBs/Mr and PMBr/Mr isolates at 1.5 mg/kg q12h, which covered 99.13% of the virtual population free concentrations above 0.25 µg/mL (Fig. 3). There was no difference in T_{>MPC} between using 1.5 mg/kg q12h and the high dose of 3.0 mg/kg followed by 1 mg/kg at q12h (Table 2).

The polymyxin B regimen consisting of a loading dose (3 mg/kg) followed by a fixed dose of 100 mg q12h produced T_{MSW} and T_{>MPC} similar to those achieved with the regimen consisting of a loading dose plus a weight-based dose of 1 mg/kg at q12h for the four isolates tested (Table 2).

Table 3 shows the T_{MSW} and T_{>MPC} achieved by the monotherapy and combination therapy for meropenem and fosfomicin in various dosing regimens as 3-h infusions. None of the fosfomicin monotherapy regimens

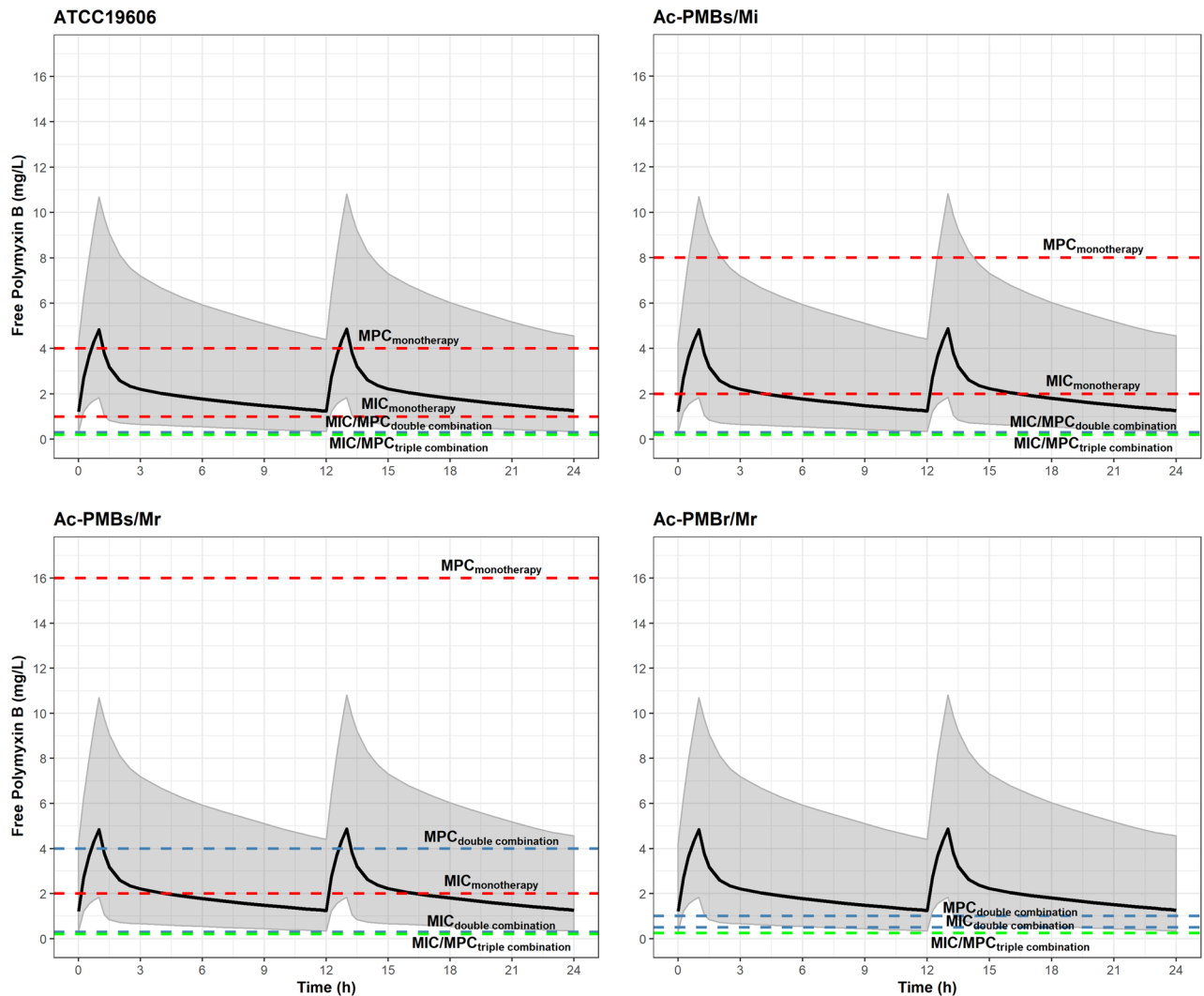


Figure 3. Simulated median and 95% prediction interval of free drug concentrations, as represented by solid black lines and grey shaded areas, respectively, in virtual patients against *A. baumannii* strains. The dosing regimen of polymyxin B at 1.5 mg/kg q12h with 1-h infusion was simulated in 10,000 virtual patients. MIC and MPC values in monotherapy (red), two- (blue), and three-drug combination (green) are shown by dashed lines.

provided plasma concentrations sufficient to achieve the MIC against all strains ($\geq 128 \mu\text{g/mL}$) (Fig. 2). On the other hand, when used in the triple combination, fosfomycin concentrations remained above the MPC of $64 \mu\text{g/mL}$ for more than 70% of the dosing interval at the high dose of 8 g q8h for all strains, except Ac-PMBs/Mr for which a T_{MSW} up to 57% of the dosing interval was observed.

The meropenem regimens evaluated as monotherapy provided reasonable $T_{>\text{MPC}}$ only for the ATCC 19606 strain that exhibited MPC of $0.25 \mu\text{g/mL}$; the regimen of 2 g q8h resulted in a $T_{>\text{MPC}}$ of 85% (Table 3 and Fig. 2). Meropenem as double combination achieved 75% $T_{>\text{MPC}}$ against Ac-PMBs/Mi and Ac-PMBs/Mr isolates at the same dosing regimen, whereas a T_{MSW} of 25% was observed within the concentration range between MIC of $2 \mu\text{g/mL}$ and MPC of $16 \mu\text{g/mL}$. The triple combination provided the $T_{>\text{MPC}}$ of 98% against Ac-PMBr/Mr at $4 \mu\text{g/mL}$ MPC for the meropenem regimen of 2 g q8h, which was also observed for the Ac-PMBs/Mi isolate. Only ATCC 19606 strain presented T_{MSW} of 0%, while meropenem concentrations remained above the MPC over the entire dosing interval in both simulated regimens. Ac-PMBs/Mr isolate did not show an increase in $T_{>\text{MPC}}$ of meropenem in the triple combination.

Discussion

Polymyxin B is one of the few remaining options for the treatment of CR-Ab, but its use in monotherapy has led to the emergence of resistant strains^{9–11}. To the best of our knowledge, this study is the first to report the MPC and MSW results for polymyxin B against *A. baumannii* strains and to evaluate the duration of time that polymyxin B levels remain above the MPC during monotherapy or combination therapy regimens, as well as whether combination therapy reduces the duration of MSW. Our results showed that the combination of polymyxin B with meropenem and fosfomycin inhibited the in vitro growth of polymyxin B-resistant subpopulation and significantly lowered the MPC of polymyxin B ($0.25 \mu\text{g/mL}$).

Strain	Antimicrobial dosage regimen ^a	Monotherapy		Combination therapy			
		%T _{MSW} [†]	%T _{>MPC} [†]	%T _{MSW}	%T _{>MPC}	Triple-combination with 8 g q8h fosfomicin/LD plus 1 mg/kg q12h polymyxin B	
	Meropenem			Double-combination with LD plus 1 mg/kg q12h polymyxin B ^b			
ATCC 19606	1 g q8h	55.6 ± 16	40.5 ± 16.7	2.3 ± 3.6	97.3 ± 3.6	0	100 ± 0
	2 g q8h	14.6 ± 12.9	84.9 ± 12.9	0.7 ± 0.6	99.0 ± 0.6	0	100 ± 0
Ac-PMBs/Mi	1 g q8h	93.4 ± 8.9	0.10 ± 0.99	68.7 ± 16.6	29.7 ± 17.4	5.9 ± 8.6	93.6 ± 8.9
	2 g q8h	68.7 ± 16.6	29.7 ± 17.4	24.8 ± 16.8	74.6 ± 17.0	1.3 ± 3.0	98.5 ± 3.1
Ac-PMBs/Mr	1 g q8h	29.7 ± 17.4	0	68.7 ± 16.6	29.7 ± 17.4	70.2 ± 17.4	29.7 ± 17.4
	2 g q8h	74.5 ± 17.0	0.10 ± 0.99	24.8 ± 16.8	74.6 ± 17.0	25.3 ± 17.0	74.6 ± 17.0
Ac-PMBr/Mr	1 g q8h	NC	NC	29.6 ± 17.2	0.10 ± 0.99	6.2 ± 9.5	93.6 ± 8.9
	2 g q8h	NC	NC	44.9 ± 6.1	29.7 ± 17.4	1.5 ± 3.1	98.5 ± 3.1
	Fosfomicin					Triple-combination with 2 g q8h meropenem/LD plus 1 mg/kg q12h polymyxin B	
ATCC 19606	4 g q8h	NC	NC	ND	ND	54.8 ± 44.8	43.2 ± 45.9
	6 g q8h	NC	NC	ND	ND	38.4 ± 42.7	60.4 ± 43.9
	8 g q8h	NC	NC	ND	ND	27.8 ± 37.8	71.3 ± 39.0
Ac-PMBs/Mi	4 g q8h	NC	NC	ND	ND	24.2 ± 32.8	71.3 ± 39.0
	6 g q8h	NC	NC	ND	ND	14.4 ± 24.4	82.9 ± 30.1
	8 g q8h	NC	NC	ND	ND	9.5 ± 19.0	88.6 ± 24.0
Ac-PMBs/Mr	4 g q8h	NC	NC	ND	ND	42.6 ± 45.5	0.62 ± 6.3
	6 g q8h	NC	NC	ND	ND	54.6 ± 43.8	5.8 ± 21.2
	8 g q8h	NC	NC	ND	ND	57.1 ± 42.1	14.2 ± 32.6
Ac-PMBr/Mr	4 g q8h	NC	NC	ND	ND	28.0 ± 33.9	43.2 ± 45.9
	6 g q8h	NC	NC	ND	ND	22.5 ± 25.5	60.4 ± 43.9
	8 g q8h	NC	NC	ND	ND	17.3 ± 24.0	71.3 ± 39.0

Table 3. MSW-derived PD indices of meropenem and fosfomicin as monotherapy and in combination against *A. baumannii* strains. MSW mutant selection window, MPC mutant prevention concentration, LD loading dose of 3.0 mg/kg, NC not computable because polymyxin B MIC is higher than maximum. ND not tested. Values reported as mean ± SD. ^aMeropenem and fosfomicin regimens were administered as 3-h prolonged infusions. ^bPolymyxin B regimen were 1-h infusions. ^cT_{MSW} or T_{>MPC} was calculated as the percentage of the first 24 h.

Three clinical isolates of *A. baumannii* with different sequence types and susceptibility profiles to polymyxin B and meropenem were selected in order to investigate the emergence of polymyxin resistance in MDR clonal lineages, or international clones, that are spreading around the world^{27,28}. Several in vitro studies using colistin have shown that its MSW varied between 0.5 and ≥ 128 µg/mL for colistin-susceptible MDR *A. baumannii* isolates^{29–31}. Our findings demonstrated that the MSW of polymyxin B against these isolates is not similar to that of colistin. Polymyxin is the treatment of choice for these isolates but its use as monotherapy should be avoided due to the resistant mutants that may be selectively amplified.

Interestingly, metallo-β-lactamase-producing carbapenem-resistant isolate (Ac-PMBs/Mr) had the widest MSW of polymyxin B (2 and 16 µg/mL). This phenomenon can be partially attributed to the presence of mobile genetic elements such as transposons contained in certain regions of the genome which favor modifications of the lipopolysaccharide and can lead to polymyxin resistance^{32,33}. VIM-type metallo-β-lactamase is often encoded on mobile gene cassettes inserted into class 1 integron, which can be found as part of transposons³⁴. In the case of oxacillinase-producing isolate (Ac-PMBr/Mr), although the bacterial population was composed predominantly of polymyxin B-resistant cells (MIC 32 µg/mL), a subpopulation with a higher degree of resistance was detected at concentrations up to 64 µg/mL, as indicated by MPC testing. This may be associated with virulence factors such as its remarkable ability to adhere and survive in hospital surfaces already identified in a previous study³⁵. Both carbapenemase-producing CR-Ab had STs that belonged to clonal complex 1 and international clone I, which presents a broad international distribution among several countries, including Brazil (Fig. 1)^{27,36}. Conversely, Ac-PMBs/Mi isolate is an ancient lineage assigned to ST374, belonging to clonal complex 3 and international clone III, that was reported in few countries to date²⁸ (PubMLST.org databases) and shown similar behavior to the ATCC 19606 strain.

The PK/PD evaluation shows that a high daily dose of polymyxin B results in an average steady-state plasma drug concentration of ≥ 2 µg/mL, which is expected to be efficacious against an organism with a MIC ≤ 2 µg/mL³⁷; however, this exposure of polymyxin B alone may not be sufficient to achieve MPC, as shown in our study. In order to optimize dosages with respect to minimizing resistance development, we estimated two PK/PD indices, T_{>MPC} and T_{MSW}, based on the MPC concept and the emergence of resistance. The results of the simulation

showed that higher polymyxin B exposures in monotherapy increased the percentage of time during which polymyxin B stays within the MSW (T_{MSW}) and the opportunity for resistant subpopulation to be selectively enriched in polymyxin B-susceptible strains based on MIC. In a recent in vitro hollow-fiber infection model of *A. baumannii*, Tsuji et al. also demonstrated that increasing the dose of polymyxin B in monotherapy amplified polymyxin B-resistant subpopulation¹¹. Thus, increasing polymyxin B dose is not beneficial in suppressing resistance development and maximizing therapeutic effect, especially because polymyxins present a very narrow therapeutic window¹⁴.

Combination therapy is generally an effective strategy to prevent antimicrobial resistance, although clinical data regarding the advantage of combination therapy over polymyxin monotherapy are limited³⁸. We investigated whether combining polymyxin B with a second or third bactericidal antibiotic could reduce the duration that polymyxin B levels stay in MSW while maximizing the duration that polymyxin B levels remain above the MPC. Among the polymyxin B-susceptible strains, with the exception of Ac-PMBs/Mr, the combination of polymyxin B plus meropenem resulted in the reduction of MIC and MPC values of polymyxin B to 0.25 µg/mL, closing the MSWs, while MIC of meropenem decreased to values close to those of the susceptible phenotypes (≤ 2 µg/mL). For carbapenemase-producing isolates, there were two new MSWs of the combination polymyxin B plus meropenem; the resistant mutant subpopulation is likely to become enriched when the concentrations of polymyxin B and meropenem fall within their MSW in the combination therapy. The complete suppression of resistance occurs when the combination closes all the original MSW of each antimicrobial. However, it is difficult to find a combination that can achieve this. When fosfomycin was added to the combination, the MIC and MPC of polymyxin B decreased to 0.25 µg/mL, closing the MSW and allowing that a lower dose of polymyxin B to be used in the therapy. The triple combination also improved the activity of meropenem and fosfomycin against carbapenemase-producing bacteria, with the exception of Ac-PMBs/Mr for which the MPC of the carbapenem was 16 µg/mL either in the double or triple combination.

A. baumannii is naturally resistant to a number of antimicrobials commonly used in the clinical practice against GNB, including fosfomycin²⁶. However, fosfomycin presents a characteristic that makes it an attractive option as a partner drug for combinations, such as negligible plasma protein binding, very low toxicity and excellent distribution throughout the fluid and tissues in the body²². In our study, fosfomycin concentrations needed to close the MSW of polymyxin B ranged from 64 to 512 µg/mL. Previous in vivo studies showed that, in general, the serum concentration of fosfomycin was high, reaching a peak of up to 606 mg/L after intravenous administration²², which would probably prevent the emergence of polymyxin B-resistant mutants during treatment. Furthermore, other factors need to be taken into account in the antimicrobial activity and the suppression of resistance including specific characteristics of patient and pathogen.

Our simulation results showed that a dosage of 1.5 mg/kg q12h can maintain polymyxin B concentrations above the MPC during >90% of the dosing interval ($T_{>MPC} > 90\%$) given a MPC of 0.25 µg/mL for polymyxin B in combination. Given the $\%T_{MSW}$ is 0, the emergence of polymyxin B-resistant subpopulation may be restricted using triple combination therapy against CR-Ab in all dosage regimens tested. For carbapenem and polymyxin B non-resistant strains, the double combination would be sufficient. Although the original MSWs of meropenem and fosfomycin were not closed, the duration of time during which drug concentration stayed within their MSW was reduced in combination therapy. The assumption for the low dose of polymyxin B, i.e. without a loading dose, is to restrict the emergence of resistance and at the same time minimize toxicity, because loading dose is often used in serious infections when the MICs are not known prior to the initiation of the first dose. MSW-based PK/PD indices for polymyxin that relate to the suppression of resistance are largely unknown. Additional studies will be necessary to determine the variables that best predict resistance restriction in *A. baumannii*.

Most of the clinical studies for CR-Ab were associated with pneumonia infection¹. Zaccard et al. showed that lung infections due to Gram-negative bacteria can have a bacterial burden as high as 10^8 CFU³⁹. Our findings demonstrated that polymyxin B in triple combination with meropenem and fosfomycin would be able to achieve the necessary exposure required for microbiological eradication of CR-Ab from the infected lung. Lenhard et al. in an in vitro hollow fiber study using the triple combination of sulbactam, meropenem and polymyxin B found a significant bactericidal activity for a high-density burden in the MDR *A. baumannii* strain, but the bacterial inoculum used in the checkerboard study was $\sim 10^5$ CFU and did not take into account the probability of mutant subpopulation being present in the populations¹⁰. One limitation of the present study is that the bactericidal activity of polymyxin B was evaluated using culture a medium at pH 7.2, which is close to human blood. Some studies suggest that the body fluid pH, such as in the lung, may affect the therapeutic response to antibacterial agents and promote resistance^{40,41}. The other limitation of the study is that the determinations of PD parameters were based on free plasma concentrations. The extension to efficacy in the epithelial lining fluid (ELF) is limited due to the lack of information on drug concentrations in the ELF of humans for polymyxin and fosfomycin, since these two antibiotics are very old. Meropenem penetration into the ELF of patients with ventilator-associated pneumonia is approximately 25% of the plasma drug concentration⁴². Fosfomycin exposure in ELF of weaning piglets was only 13% to that in the plasma⁴³. Given the lower free drug concentrations in the ELF, the $\%T_{MSW}$ will likely be higher whereas $T_{>MPC}$ will be lower.

In conclusion, this study reported the MPC and MSW results for polymyxin B and evaluated PK/PD indices that were related to the suppression of resistance in *A. baumannii*. The triple combination of polymyxin B, meropenem, and fosfomycin can close the MSW of polymyxin B in vitro against CR-Ab and maximize the duration of time that polymyxin B levels remain above the MPC in order to prevent the emergence of resistant mutants, enabling the use of this triple combination in clinical practice against this fearsome pathogen.

Methods

Bacterial samples. The surveillance and monitoring program of antimicrobial resistance in *Acinetobacter* spp. has been established in three tertiary hospitals in the southern Brazil since 1994 by the Medical Microbiology Laboratory at State University of Maringá. Identification and antimicrobial susceptibility profiles of bacterial isolates were performed by routine automated microbiology systems, such as Phoenix[®] (BD Diagnostic Systems, Sparks, MD) and MicroScan[®] (DadeBehring, West Sacramento, CA) according to the equipment's instructions. These isolates were preserved at $-20\text{ }^{\circ}\text{C}$ in Trypticase Soy Broth (Difco Laboratories, Detroit, MI) with 30% glycerol until they were tested for their susceptibility to antibiotics.

Molecular typing was performed using ERIC-PCR assays followed by an analysis of gels in Bionumerics[®] v. 6.5 (Applied Maths, Sint-Martens-Latem, Belgium)⁴⁴. The MIC values of polymyxin B and meropenem against *A. baumannii* were additionally tested by the broth microdilution method in CAMHB (Difco Laboratories, Sparks, MD) as described in CLSI document M07-A10⁴⁵. Considering the similarity of the clusters (based on Dice correlation coefficient >0.8) and dissimilar susceptibility patterns to polymyxin B and meropenem, eight clinical isolates (data not shown) were characterized using Pasteur MLST scheme available in the *A. baumannii* MLST website (<https://pubmlst.org/organisms/acinetobacter-baumannii/>)⁴⁶.

Among these isolates, three *A. baumannii* belonging to different STs and antimicrobial susceptibility profiles were selected for this study. The MIC interpretive criteria for both polymyxin B and meropenem according to EUCAST⁶ and CLSI²⁶ guidelines were used to name the isolates as: Ac-PMBs/Mi (*A. baumannii* polymyxin B susceptible/meropenem intermediate) with no carbapenemase enzymes; Ac-PMBs/Mr (*A. baumannii* polymyxin B susceptible/meropenem resistant) carrying *bla*_{VIM-like} metallo- β -lactamase gene that was detected by the multiplex PCR assay⁴⁷; and Ac-PMBr/Mr (*A. baumannii* polymyxin B/meropenem resistant) which harbored the association of *bla*_{OXA23-like} carbapenemase gene with *ISAbal1* upstream⁴⁸, but a *mcr-1* gene was not found⁴⁹. Additionally, *A. baumannii* ATCC 19606 was included in the set of tests as a reference strain for antimicrobial quality control.

MIC and MPC determination. *Antimicrobial agents.* Fosfomicin (Sigma-Aldrich, St. Louis, MO, USA) was purchased from LabCompany (Londrina, Paraná, Brazil). Polymyxin B (Eurofarma, Itapevi, São Paulo, Brazil) and meropenem (BioChimico, Itatiaia, Rio de Janeiro, Brazil) were kindly provided by the State University of Maringá Hospital. Fosfomicin and meropenem were dissolved in water to a final concentration of 10 mg/ml and stored at $-20\text{ }^{\circ}\text{C}$ (stock solution) and polymyxin B solution was prepared at the same concentration on the day of experimentation.

Bacterial inoculum size. Two inoculum sizes of each strain were used in the experiments. Inoculum were prepared in tubes containing 3 mL of sterile Mueller–Hinton broth (MHB; Difco Laboratories, Detroit, MI) from an overnight culture on Mueller–Hinton agar (MHA; Difco Laboratories, Detroit, MI) plates at $35 \pm 2\text{ }^{\circ}\text{C}$ in ambient air.

For MIC measurements, four to five individual colonies appearing on the plate were added in MHB and the suspension adjusted to an equivalent of 0.5 McFarland standard, at a density of approximately 1.5×10^8 CFU/mL, using a nephelometer (PhoenixSpec nephelometer; BD, Sparks, MD, USA)⁴⁵. This suspension was further diluted in MHB resulting in a final inoculum of 5×10^5 CFU/mL.

For MPC measurements, a final high-density inoculum of $\geq 10^9$ CFU/mL was used to ensure the emergence of the resistant mutant subpopulation¹². In this case, the suspension was obtained from a carpet of colonies on the plate and the OD 660 nm was confirmed by absorbance reading with a spectrophotometer (Milton Roy Spectronic 21D spectrophotometer)⁵⁰. Colony counts of the inoculum suspension were previously verified by counting CFU.

Determination of MICs and MPCs of single agents. The MIC and MPC of all three antimicrobials against each strain were determined in duplicate based on the method reported by Dong et al., with modifications¹². In brief, the bacterial inoculum suspensions corresponding to MIC and MPC tests were added to the 96-well U-bottom plate and inoculated simultaneously onto the surface of the drug-containing agar and drug-free control agar with a Steer's replicator. MHA plates containing fosfomicin was supplemented with 25 $\mu\text{g/mL}$ of glucose-6-phosphate⁴⁵. The concentrations ranges of polymyxin B, meropenem and fosfomicin were 0.25 to 256 $\mu\text{g/mL}$, 0.25 to 512 $\mu\text{g/mL}$ and 2 to 2048 $\mu\text{g/mL}$, respectively.

MIC and MPC of antimicrobials in combinations. The antimicrobial activities of polymyxin B in combination with meropenem and fosfomicin against four *A. baumannii* strains were tested in duplicate by the checkerboard method⁵¹. For this test, the combinations analyzed included polymyxin B plus meropenem as double combination, and polymyxin B plus meropenem and fosfomicin as triple combination. The selected concentrations were $1/8 \times$, $1/4 \times$, $1/2 \times$, $1 \times$, $2 \times$, $4 \times$ and $8 \times$ MIC of both meropenem and fosfomicin, while polymyxin B concentrations ranged from 0.25 to 2 $\mu\text{g/mL}$ representing clinically achievable free or unbound plasma levels in patients with severe infections^{20,37}. Combinations containing fosfomicin were supplemented with 25 $\mu\text{g/mL}$ glucose-6-phosphate⁴⁵.

Interpretation of MIC, MPC and MSW. The MIC was defined as the lowest concentration of drug inhibiting the visible colony growth after 20 to 24 h of incubation⁴⁵. The MIC results were interpreted according to the breakpoints established by the EUCAST and CLSI guidelines for *A. baumannii* strains^{6,26}.

The MPC was defined as the lowest concentration of antimicrobial that prevented colony formation from a culture containing $\geq 10^9$ CFU bacteria. The plates were examined after the incubation period of 24 and 48 h¹².

The antimicrobial concentrations range exceeding the MIC up to the MPC was defined as the MSW, at which selective amplification of antibiotic-resistant mutants occurs⁵².

The MPC:MIC ratio of polymyxin B alone and combined was also calculated and expressed as the ability of each combination to select resistant mutants. Lower values of the ratio indicate a better ability to restrict the emergence of resistant mutants⁵³.

Population PK in critically ill patients. Polymyxin B, meropenem and fosfomycin steady-state concentration–time profiles were simulated from population PK models. The individual PK parameters were assumed to be log-normally distributed.

The model for polymyxin B in critically ill patients was a two-compartment model³⁷ parameterized with clearance of 0.0276 L/h/kg (CL; 32.4% CV), intercompartmental clearance of 0.146 L/h/kg (Q; 50.4% CV), central volume of distribution of 0.0939 L/kg (V_C ; 73.3% CV), and peripheral volume of 0.330 L/kg (V_P ; 70.1% CV). The final individual parameters were obtained by multiplying the body-weight scaled parameters with the patient's body weight. The free drug concentration was computed by multiplying the simulated plasma drug concentration by 42%, wherein the plasma protein binding of polymyxin B was 58%. Four simulated weight-based polymyxin B regimens were 1 or 1.5 mg/kg every 12 h (q12h) and 3 mg/kg first dose followed by 0.5 and 1 mg/kg q12h. These dosing regimens were based on the most common regimens used in countries in which they were registered. Additionally, the 3 mg/kg first dose followed by a fixed-dose regimen of 100 mg q12h was also evaluated as previously proposed dosing regimen⁵⁴. All regimens were administered as 1-h infusions.

The intravenous infusion model for meropenem was a one-compartment model previously shown to be more suitable for the prediction of free meropenem free concentrations in critically ill patients⁵⁵. The model was parameterized with CL and V_C . CL was dependent on serum creatinine (SCr) with the following relationship: $CL(L/h) = 11.1 \times \left[\frac{SCr}{0.7} \right]^{-1}$ with a CV of 52.1%. V_C was 33.6 L. The plasma protein binding of meropenem was 2%. The dosing regimens of meropenem evaluated were 1 g and 2 g q8h as prolonged infusion of 3 h.

The intravenous infusion model for fosfomycin was a two-compartment model; creatinine clearance (CLCr) was a covariate of clearance and body weight was a covariate of central volume, as previously described⁵⁶: $CL(L/h) = 5.25 \times \left[\frac{CLCr}{90} \right]$ with a CV of 91.9%; $V_c(L) = 26.5 \times \left[\frac{WT}{90} \right]^{0.75}$ with a CV of 39%. Inter-compartmental clearance and peripheral volumes were 19.8 L/h and 22.3 L, respectively. Assuming co-administration of fosfomycin with meropenem, the dosing regimens of fosfomycin tested were 4 g, 6 g, and 8 g q8h as 3-h infusion.

Demographical characteristics of the critically ill patient population was simulated in 1:1 male:female ratio. Renal function of the population was a bimodal distribution as previously described⁵⁷. The distributions for SCr to derive CLCr were based on two normal distributions for men and women. SCr distributions were $96 \pm 14 \mu\text{mol/L}$ and $83 \pm 12 \mu\text{mol/L}$ (mean \pm SD) for male and female, respectively. These distributions were approximated from the serum creatinine distribution reported in the Hordaland Health Study⁵⁸. The units were then converted to g/dL by dividing by 0.62. Age was uniformly distributed between the age of 41 to 74 years. CLCr was computed by the Cockcroft-Gault formula⁵⁹. Body weights of patients were derived from height distributions of male and female adults as previously described⁶⁰. The simulation for height was assumed to be normally distributed: male height was $176.3 \pm 0.17\sqrt{4482}$ cm (mean \pm SD), and female height was $162.2 \pm 0.16\sqrt{4857}$ cm. The weight-height relationship was described by $WT = \exp(3.28 + 1.92 \log HT)$ and $WT = \exp(3.49 + 1.45 \log HT)$ for males and females, respectively. Simulated body weight was log-normally distributed such that $WT_i = WT \cdot \exp \eta$, wherein η is normally distributed with a mean of 0 and standard deviations (SD) of 0.14 and 0.17 for males and females, respectively, and i represents an individual.

The population PK models⁶¹ were simulated using RxODE package in R (v. 3.3.1), along with the demographical characteristics of the population.

PD assessment using Monte Carlo simulation. The PD analyses carried out for 10,000 simulated concentration–time profiles were determined by a user-defined function in the R^{62,63}. The percentage of each dosage interval in which free plasma concentration was within the MSW ($\%T_{MSW}$) and the percentage of each dosage interval in which free plasma concentration exceeds the MPC ($\%T_{>MPC}$) were estimated from the individual concentration–time profiles of polymyxin B, meropenem and fosfomycin in several dosing regimens. Summary statistics including mean and standard deviation were determined from the individual PD parameters.

The PD drug–drug interaction was taken into account in the checkerboard method^{12,51} through the reduction in MIC and MPC values in the following combinations: polymyxin B plus meropenem as a double combination; and polymyxin B plus meropenem and fosfomycin as a triple combination.

The PK/PD relationship for the three antimicrobial therapies as it relates to suppression of resistance has not yet been established. Both indices ($\%T_{MSW}$ and $\%T_{>MPC}$) of resistance prevention provided information regarding whether the polymyxin B dosing regimens in the combination (1) could reduce the time that drug concentration remains within the mutant selection window ($\%T_{MSW}$) and (2) could restrict the selection of resistant mutant when concentration above the MPC were achieved during treatment ($\%T_{>MPC}$). The schematic representation of the two PK/PD indices of resistance suppression in monotherapy and combination therapy is illustrated in Fig. 4.

Ethical approval. This study was evaluated and approved by the Human Ethics Committee of State University of Maringa (COREA/COPEP no. 0447/2017).

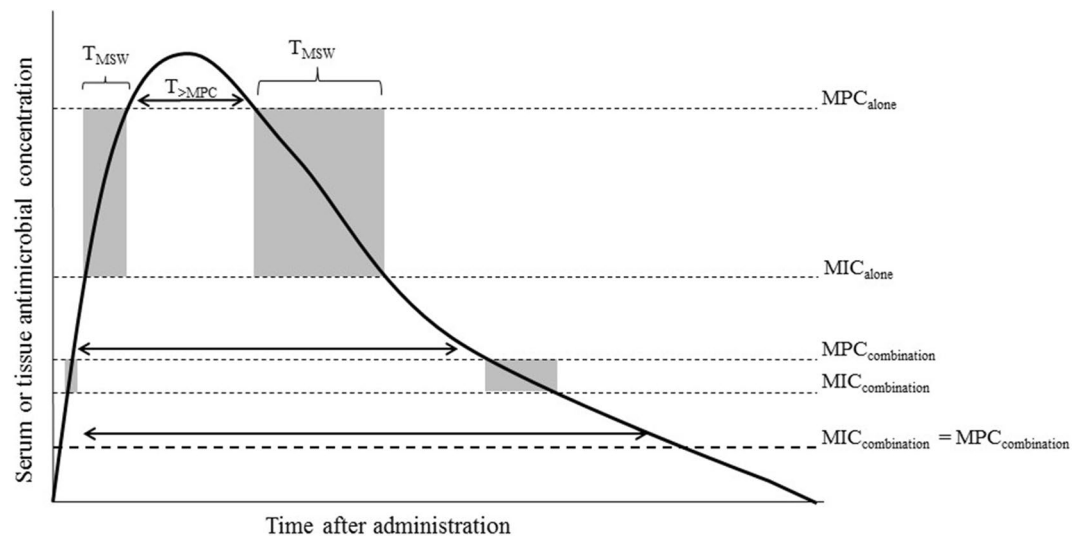


Figure 4. Schematic representation of the mutant selection window (MSW) of an antimicrobial agent alone and in combination with other drugs. As MIC and mutant prevention concentration (MPC) of the antimicrobial in the combination decrease, the time within the MSW (T_{MSW}) decreases or is even eliminated when MIC and MPC values are equal. Essentially, the longer the time spent by an antibiotic within the MSW, the greater the opportunity for resistant mutants to be selectively amplified. Dashed lines indicate the MIC and MPC; double-headed arrow indicates the time that drug concentration exceeds the MPC ($T_{>MPC}$); shaded region indicates the duration that drug concentration is within the MSW.

Received: 5 February 2021; Accepted: 13 May 2021

Published online: 31 May 2021

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Acknowledgements

This work was supported by the Brazilian government agency Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Financial Code 001. As this government fund is designed to encourage higher education training in Brazil, they only cover the cost of laboratory materials.

Author contributions

N.H.F. and D.R.S. performed the in vitro experiments and data analysis. S.K.B.S., P.Z. and J.M. developed the PK–PD model and data analysis. F.E.C.-M. provided advice regarding the in vitro experiments and assisted with data interpretation. N.H.F. and S.A.B.N. molecular typing and wrote the manuscript. F.S.M., and M.Y. contributed to the writing and editing of the manuscript. M.C.B.T. and S.K.B.S. conceived the study with N.H.F. with J.M. and assisted with manuscript preparation and data interpretation. M.C.B.T. assisted with experimental design and oversaw the writing and editing of the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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