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Original article

Evaluation of polymyxin B in combination with 13 other antibiotics against carbapenemase-producing *Klebsiella pneumoniae* in time-lapse microscopy and time-kill experiments

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ABSTRACT

Objectives: This study aimed to explore the interactions of polymyxin B in combination with 13 other antibiotics against carbapenemase-producing *Klebsiella pneumoniae*.
Methods: Five clinical isolates of multidrug-resistant *K. pneumoniae* producing KPC-2, KPC-3, NDM-1, OXA-48 and VIM-1 carbapenemases were used. Polymyxin B was tested alone and in combination with amikacin, aztreonam, cefepime, chloramphenicol, ciprofloxacin, fosfomycin, linezolid, meropenem, minocycline, rifampicin, temocillin, thiamphenicol and trimethoprim. Inhibition of growth during antibiotic exposure was evaluated in 24-hr automated time-lapse microscopy experiments. Combinations that showed positive interactions were subsequently evaluated in static time-kill experiments.
Results: All strains carried multiple (≥9) resistance genes as determined by whole-genome sequencing. In the initial screening the combination of polymyxin B and minocycline was most active with enhanced activity compared with the single antibiotics detected against all strains. Positive interactions were also observed with polymyxin B in combination with rifampicin and fosfomycin against four of five strains and less frequently with other antibiotics. Time-kill experiments demonstrated an additive or synergistic activity (1–2 $\log_{10}$ or ≥2 $\log_{10}$ CFU/mL reduction, respectively, compared with the most potent single antibiotic) with 21 of 23 tested combinations. However, because of regrowth, only 13 combinations were synergistic at 24 hr. Combinations with minocycline or rifampicin were most active, each showing synergy and bacteriostatic or bactericidal effects resulting in 1.93–3.97 and 2.55–5.91 $\log_{10}$ CFU/mL reductions, respectively, after 24 hr against four strains.
Discussion: Polymyxin B in combination with minocycline, rifampicin or fosfomycin could be of potential clinical interest. Time-lapse microscopy showed some discrepancy in results compared with the time-kill data but was useful for screening purposes. **P. Wistrand-Yuen, Clin Microbiol Infect 2020;26:1214**

Introduction

Infections caused by carbapenemase-producing *Klebsiella pneumoniae* are associated with high mortality in critically ill patients [1,2]. Because of frequent antibiotic co-resistance in these bacteria, few or no effective treatment options for monotherapy exist and combination therapy is often used despite a lack of clinical evidence [1–3]. The polymyxins, polymyxin B and polymyxin E (colistin) [4], have become cornerstone antibiotics in the treatment of multidrug-resistant Gram-negative bacteria, including carbapenem-resistant *K. pneumoniae*.

The polymyxins act by destabilizing the outer membrane of Gram-negative bacteria, which also facilitates the entry of other antibiotics into the cell [5]. *In vitro* synergy against carbapenemase-producing *K. pneumoniae* has been demonstrated with polymyxins in combination with several other antibiotics [6]. However, more research is needed to determine which antibiotic combinations are most active against strains with different genotypes. Few studies

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have evaluated the activity of polymyxin B combinations against *K. pneumoniae* producing carbapenemases other than KPC. Automated time-lapse microscopy has shown potential as a screening tool for effective antibiotic combination regimens [7,8] but further investigation is required to establish the applicability of this method.

In this study, the antibacterial activities of polymyxin B in combination with 13 other antibiotics against five clinical isolates of *K. pneumoniae* producing KPC-2, KPC-3, NDM-1, OXA-48 and VIM-1 carbapenemases were evaluated. Automated time-lapse microscopy was used to identify combinations showing positive interactions during 24-hr antibiotic exposure and promising regimens were subjected to 24-hr static time-kill experiments to assess synergistic and bactericidal effects and enable a comparison between the two methods.

Materials and methods

Strains and growth media

Five clinical isolates of carbapenem-resistant *K. pneumoniae* were provided by the Public Health Agency of Sweden. Mueller-Hinton II (BD Diagnostics, Sparks, MD, USA) broth and agar were used in all experiments. For experiments including fosfomycin, the broth was supplemented with 25 mg/L glucose 6-phosphate.

Antibiotics

All antibiotics were purchased from Merck KGaA (Darmstadt, Germany), except temocillin, which was kindly provided by Eumedica (Eumedica S.A., Manage, Belgium). A wide range of drug concentrations were used to reduce the risk of overlooking potentially useful combinations. In the time-lapse microscopy experiments, antibiotics were added to the following concentrations: polymyxin B (0.25, 0.5, 1.0, 2.0 mg/L), amikacin (4, 16, 128 mg/L), aztreonam (2, 8, 64 mg/L), cefepime (2, 8, 64 mg/L), chloramphenicol (1, 8, 32 mg/L), ciprofloxacin (0.25, 2, 8 mg/L), fosfomycin (8, 32, 128 mg/L), linezolid (2, 8, 16 mg/L), meropenem (2, 16, 64 mg/L), minocycline (0.5, 4, 16 mg/L), rifampicin (1, 8, 32 mg/L), temocillin (4, 16, 64 mg/L), thiamphenicol (2, 8, 32 mg/L) and trimethoprim (1, 4, 8 mg/L).

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) were determined at least in duplicate. Broth microdilution (BMD) was used for polymyxin B and thiamphenicol and agar dilution for fosfomycin according to Clinical and Laboratory Standards Institute guidelines [9], with one modification for polymyxin B for which direct dilution rather than serial dilution was performed to reduce plastic binding [10]. For all other antibiotics, the gradient method (Etest, bioMérieux, Marcy-l'Étoile, France) was applied according to the manufacturer's instructions. Antibiotic susceptibility was classified in accordance with EUCAST clinical breakpoints [11].

Genetic characterization

Whole-genome sequencing was performed using Illumina HiSeq X (2 × 150 bp). Reads were assembled into contigs by CLC Genomics Workbench version 11 (Qiagen Bioinformatics, Aarhus, Denmark) using default settings. Resistance genes were identified with ResFinder [12]. CLC Main Workbench 8.0.1 (Qiagen Bioinformatics, Aarhus, Denmark) was employed to detect sequence variations between polymyxin B susceptible and resistant strains in *mgrB*, *pmrCAB*, *phoPQ* and *crrB* associated with colistin resistance [13,14]. Alterations in genes encoding the outer membrane porins OmpK35 and OmpK36, the efflux pumps AcrAB-TolC and OqxAB, and their regulators, were analysed using *K. pneumoniae* MGH78578 (NCBI Ref. Seq. NC_009648.1) as a reference.

Time-lapse microscopy experiments

Time-lapse microscopy experiments were performed as previously described [7,8]. Bacteria in exponential growth were added to a pre-warmed 96-well microtitre plate to obtain a starting inoculum of 10^6 CFU/mL in a total volume of 200 μL. The microtitre plate was placed in an oCelloScope (BioSense Solutions ApS, Farum, Denmark) [15] in a 37°C incubator. The focus for each well was set in the software UniExplorer (version 6.0.0.5419) using the bottom search function. Five images with an image distance of 4.9 μm were acquired for each well every 15 min over 24 hr. Mean values of background-corrected absorption (BCA) and maximum segmentation and extraction of surface area ($SESA_{max}$) values from duplicate experiments were used in the data analysis. BCA >8 and $SESA_{max}$ >5.8 were designated as cut-off values indicating bacterial concentrations $>10^6$ CFU/mL [7,8]. If both the BCA and $SESA_{max}$ values were above the cut-off values after the 24 hr experiments with the single antibiotics but not with the combination, the interaction was classified as positive and the combination was selected for further evaluation in time-kill experiments.

Time-kill experiments

Time-kill experiments were performed with polymyxin B at 0.5 × MIC (0.25 mg/L) for the susceptible strains. For the two resistant strains, the targeted clinical steady state total drug concentration of 2 mg/L [16] was applied. When the time-lapse microscopy experiment indicated a positive interaction with more than one concentration of the non-polymyxin antibiotic, the higher concentration was used in the time-kill experiments. Precultures were prepared to achieve starting inocula of 10^6 CFU/mL in a total volume of 2.5 mL. The tubes were incubated on a shaker (190 rpm) at 37°C. Samples for viable counts were taken at 0 (before the addition of antibiotics), 1, 3, 6 and 24 hr. All experiments were performed at least in duplicate and mean CFU/mL values were used in the analysis. Data points below the lower limit of detection (10 CFU/mL) were set to 1 $\log_{10}$ CFU/mL. A combination was classified as synergistic if the bacterial concentration was ≥2 $\log_{10}$ CFU/mL lower with the combination than with the most potent single antibiotic and as additive if the bacterial reduction was 1–2 $\log_{10}$ CFU/mL. A bactericidal effect was defined as a ≥3 $\log_{10}$ CFU/mL reduction in bacterial concentrations compared with the starting inoculum and a bacteriostatic effect as a <3 $\log_{10}$ CFU/mL bacterial reduction.

Results

Antibiotic susceptibilities and resistance genes

All strains carried genes encoding several beta-lactamases, including NDM-1, VIM-1, KPC-3, OXA-48 or KPC-2 carbapenemases and were phenotypically resistant to meropenem with MICs ≥16 mg/L (Table 1). ARU613 and ARU616 were resistant to polymyxin B, probably due to an amino acid substitution in *crrB* and an IS element insertion in the promotor region of *mgrB*, respectively [14,17]. All strains were resistant to chloramphenicol, trimethoprim and ciprofloxacin. ARU613 harboured the tetracycline resistance genes *tet(A)* and *tet(D)* encoding tetracycline efflux pumps [18]. ARU601 carried the *arr-2* gene that results in inactivation of rifampicin by ADP-ribosylation [19]. The fosfomycin resistance gene *fosA* [20] was detected in all five bacterial strains. Several

Table 1
MIC values and resistance genes For polymyxin B, non-silent modifications in genes commonly involved in polymyxin resistance are also shown. Classifications into the susceptible (S), intermediate (I) or resistant (R) category are based on EUCAST clinical breakpoints version 9.0. For some of the tested antibiotics, there are no clinical breakpoints for Enterobacterales (-).

Antibiotic class (bacterial target)	Antibiotic	MIC (mg/L) and resistance genotype				
		ARU601	ARU602	ARU604	ARU613	ARU616
Cationic peptides (outer membrane)	Polymyxin B[a]	0.5 (S) ND	0.5 (S) ND	0.5 (S) ND	16 (R) Mutation in *crr*B (N311T)	32 (R) IS-element in promotor region of *mgr*B
β-Lactams (cell wall synthesis)	Meropenem Aztreonam Cefepime Temocillin	>32 (R) >256 (R) >256 (R) 1024 (−) *blaNDM-1, blaSHV-155, blaOXA-9, blaCMY-4, blaTEM-1B, blaOXA-1, blaCTX-M-15*	32 (R) 0.25 (S) 64 (R) >1024 (−) *blaVIM-1*	>32 (R) >256 (R) 128 (R) 32 (−) *blaKPC-3, blaSHV-11, blaTEM-!A*	32 (R) 128 (R) 256 (R) >1024 (−) *blaOXA-48, blaSHV-27, blaCTX-M-15, blaOXA-9, blaCTX-M-14b, blaOXA-1, blaTEM-199*	16 (R) >256 (R) 16 (S) 32 (−) *blaKPC-2, blaTEM-1A*
Aminoglycosides (protein synthesis)	Amikacin	>256 (R) *aac(3)-IId, armA, aph(3″)-Ib, aph(6)-Id, aac(6′)-Ib3, aadA1*	8 (S) *aadA3, aph(3″)-Ib, aph(6)-Id, aph(3′)-Ia, aadA1, aadA3, aac(6′)-Il*	64 (R) *aadA3, aac(6′)-Ib, aph(3′)-Ia*	128 (R) *aph(3″)-Ib,aph(3′)- Vib, aac(3)- IIa, aph(6)- Id, aadA1, aadA3*	16 (I) *aac(6′)-Ib3, aph(3′)-Ia, aadA1*
Fosfomycin (cell wall synthesis)	Fosfomycin	32 (S) *fosA*	32 (S) *fosA*	64 (R) *fosA*	256 (R) *fosA*	64 (R) *fosA*
Oxazolidinone (protein synthesis)	Linezolid	>256 (-) ND	>256 (-) ND	>256 (-) ND	>256 (-) ND	>256 (-) ND
Phenicols (protein synthesis)	Chloramphenicol Thiamphenicol	>256 (R) >128 (−) *catA1, cmlA1*	64 (R) >128 (−) ND	>256 (R) >128 (−) *catA1*	>256 (R) >128 (−) *floR, cmlA1*	32 (R) >128 (−) ND
Pyrimidines (nucleic acid synthesis)	Trimethoprim	>32 (R) *dfrA1*	>32 (R) *dfrA1, dfrA12*	>32 (R) *dfrA12*	>32 (R) *dfrA14*	>32 (R) *dfrA1*
Quinolones (nucleic acid synthesis)	Ciprofloxacin	>32 (R) *oqxA, oqxB*	>32 (R) *oqxA, oqxB*	>32 (R) *oqxA, oqxB*	>32 (R) *oqxA, oqxB, qnrB1, aac(6′)-Ib-cr*	>32 (R) *oqxA, oqxB*
Rifampicins (nucleic acid synthesis)	Rifampicin	>32 (-) *arr-2*	>32 (-) ND	>32 (-) ND	>32 (-) ND	>32 (-) ND
Tetracyclines (protein synthesis)	Minocycline	16 (-) ND	16 (-) ND	8 (-) ND	16 (-) *tet(A), tet(D)*	4 (-) ND

ND, no detected resistance genes.
[a] Breakpoints for colistin are applied for polymyxin B.

Table 2
Genotypic characterization of regulators and subunits of efflux pumps and porins commonly involved in resistance in *K. pneumoniae*

Efflux pump/outer membrane porin	AcrAB-TolC								OmpK35	OmpK36	OqxAB			
Gene	*acrA*	*acrB*	*tolC*	*ramA*	*marA*	*robA*	*ramR*	*acrR*	*ompK35*	*ompK36*	*oqxA*	*oqxB*	*rarA*	*oqxR*
Function	S	S	S	A	A	A	R	R	S	S	S	S	A	R
ARU601	—	—	N73T, I82V, E203G, N251S, S271N, S276T, I278V, S282R, S284N, H289Del, N291T, Q293L, Q294A, Q296N, N298S, A300N	—	—	—	K9I	—	—	—	—	K148N, G540S, Y783F, P1049L	Q99K	—
ARU602	—	—	T480N	E13D	—	—	T69Del, M70Del, D152Y	—	—	—	—	K148N, R341S, I960M	Q49H	—
ARU604	A188T	—	N73T, I82V, E203G, N251S, S271N, S276T, I278V, S282R, S284N, H289Del, N291T, Q293L, Q294A, Q296N, N298S, A300N	—	—	—	Ins194K (195Stop)	—	R42E[a]	134InsGD, P178V, R349H	—	K148G, E749D	—	V130A
ARU613	D142E	—	T480N	—	—	—	—	—	—	N276D, D306N, 308Ins QNNFTGVN, D350E, S352D, N356K	T341I	K148N, G540S	—	—
ARU616	—	—	—	—	—	—	N84D	—	L14V	T86V, S88G, S89T, S90D, D91K, A93S, P178V, G182D, A183M, Del pos. 184–186, T192G, L194Q, Y201F, L205V, Y210W, N221H, L225T, G226D, S230N, K231Del, A233V, N239D, T258S, G309R	—	K148N	I108T	Ins81L, Ins82S

NCBI Ref. Seq. NC_009648.1 was used as a reference sequence. S, subunit; A, activator; R, repressor.
[a] Frameshift.

sequence variations were found in porin and efflux genes, especially the *ompK36* and *tolC* genes (Table 2).

Time-lapse microscopy

Thirteen antibiotic combinations were evaluated against the five bacterial strains, resulting in 65 antibiotic combination/strain setups, of which 24 showed positive interactions after 24 hr at one or more concentrations (Fig. 1). However, the positive interaction of polymyxin B 0.25 mg/L and aztreonam 64 mg/L against ARU613 was discarded as bacterial growth occurred in experiments with higher concentrations of the two drugs. The combination of polymyxin B and minocycline showed the most promise, with enhanced activity compared with the single antibiotics at the same concentration against all five strains. Polymyxin B and rifampicin demonstrated positive interactions against all strains except ARU601. Polymyxin B and fosfomycin also showed positive interactions against four of the five strains.

The combination of polymyxin B and meropenem demonstrated positive interactions at a single concentration against ARU602, ARU604 and ARU616, carrying bla_{VIM-1}, bla_{KPC-3} and bla_{KPC-2}, respectively (Fig. 1). Amikacin and chloramphenicol showed an enhanced effect in combination with polymyxin B against two of the five bacterial strains, whereas aztreonam (Fig. S1), linezolid and temocillin combinations showed enhanced effects against one strain each. No interactions were detected with cefepime, ciprofloxacin, thiamphenicol or trimethoprim combinations.

Static time-kill experiments

The activity of polymyxin B alone was limited against all five strains, with continuous growth or <1.5 $\log_{10}$ CFU/mL bacterial reductions observed during the first 3 hr of the experiments; regrowth invariably occurred (Table 3). With the other antibiotics, early bacterial killing was frequently observed during the first 6 hr of the experiments. However, except for aztreonam against ARU602 and temocillin against ARU616, 24-hr bacterial concentrations were always higher than the starting inocula.

Of the 23 combinations evaluated in the time-kill experiments, 21 showed additive or synergistic effects at one or more time points (Table 3). At 24 hr, 13 combinations were synergistic while two combinations showed an additive effect. Of the combinations showing synergy at 24 hr, six were bactericidal and seven bacteriostatic. Synergy or additive effects were most frequently found against the polymyxin-resistant strains and when polymyxin B was combined with minocycline, rifampicin or fosfomycin.

Polymyxin B and minocycline demonstrated synergy after 24 hr against four of the five strains, with a bactericidal effect against two strains and a bacteriostatic effect against two others. The combination of polymyxin B and rifampicin was synergistic at 24 hr against all four tested strains (bactericidal against three strains and bacteriostatic against one). With polymyxin B and fosfomycin, a bactericidal activity during the first 3 hr of the experiments and a synergistic activity at one or more time points were noted against all four strains. However, regrowth occurred in all experiments and 24-hr synergy and bacteriostatic activity was

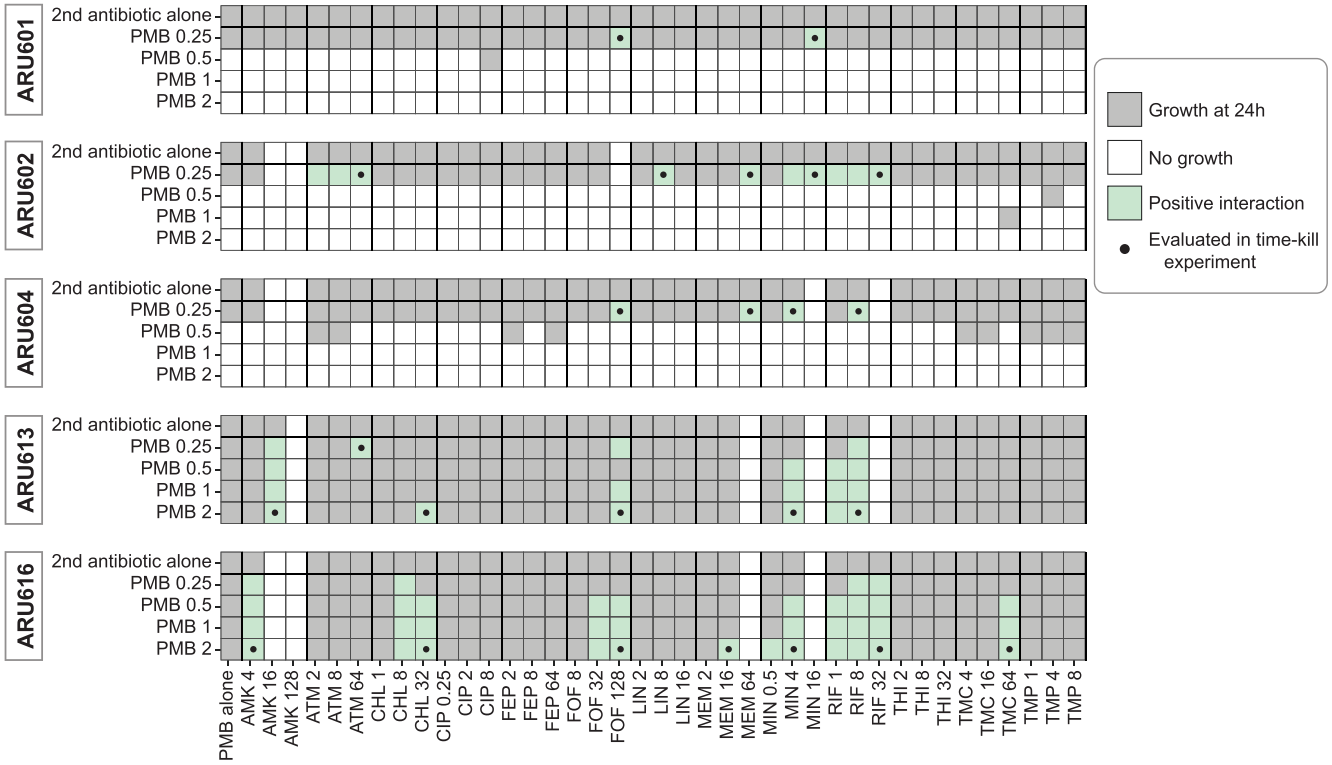

Fig. 1. Overview of the screening results from the time-lapse microscopy experiments using polymyxin B in combination with 13 other antibiotics. Growth is defined as BCA and $SESA_{max}$ values above the cut-off values (BCA >8 and $SESA_{max}$ >5.8). When growth at 24 hr was not detected with the combination but with both single antibiotics, the interaction was determined positive. Antibiotic concentrations are presented in mg/L.

Table 3
Time-kill results and interaction classification

Strain	Antibiotic regimen and conc. (mg/L)	Bacterial concentration (log_{10} CFU/mL)								
		0 hr	1 hr	Δ1 hr	3 hr	Δ3 hr	6 hr	Δ6 hr	24 hr	Δ24 hr
ARU601	Control	6.67 (0.15)	7.11 (0.15)		8.50 (0.12)		8.97 (0.16)		9.53 (0.12)	
	PMB 0.25	6.67 (0.06)	5.58 (1.03)		7.06 (1.07)		8.08 (1.11)		9.59 (0.09)	
	FOF 128	6.63 (0.08)	3.80 (0.40)		**2.54 (0.24)**		4.41 (0.98)		8.59 (0.16)	
	PMB 0.25 + FOF 128	6.65 (0.09)	4.25 (1.02)	0.46	**2.68 (1.17)**	0.14	**2.45 (1.53)**	-1.96	5.73 (1.99)	-2.86
	MIN 16	6.65 (0.05)	6.38 (0.09)		6.44 (0.08)		6.22 (0.25)		9.24 (0.13)	
	PMB 0.25 + MIN 16	6.61 (0.12)	4.13 (0.42)	0.33	3.83 (0.93)	1.29	**3.26 (0.92)**	-1.15	**2.64 (2.58)**	-5.95
ARU602	Control	6.64 (0.15)	7.41 (0.17)		8.64 (0.10)		8.92 (0.09)		9.26 (0.12)	
	PMB 0.25	6.67 (0.15)	5.25 (1.34)		6.67 (1.47)		7.34 (1.99)		9.25 (0.17)	
	ATM 64	6.47 (0.10)	5.68 (0.46)		3.95 (0.00)		**1.30 (0.30)**		**1.00 (0.00)**	
	PMB 0.25 + ATM 64	6.50 (0.06)	**2.08 (1.08)**	-3.18	**1.30 (0.30)**	-2.65	**1.00 (0.00)**	-0.3	**1.00 (0.00)**	0.00
	LIN 8	6.67 (0.01)	7.25 (0.01)		8.55 (0.07)		8.92 (0.05)		9.33 (0.03)	
	PMB 0.25 + LIN 8	6.70 (0.04)	6.54 (0.32)	1.28	8.29 (0.10)	1.62	8.91 (0.04)	1.57	9.36 (0.09)	0.11
	MEM 64	6.67 (0.16)	**3.42 (0.37)**		4.08 (0.78)		6.40 (1.39)		9.40 (0.13)	
	PMB 0.25 + MEM 64	6.63 (0.14)	4.52 (1.07)	1.10	**3.19 (1.80)**	-0.88	4.30 (2.95)	-2.11	4.84 (3.48)	-4.42
	MIN 16	6.79 (0.06)	6.68 (0.01)		6.71 (0.03)		6.76 (0.37)		8.44 (0.09)	
	PMB 0.25 + MIN 16	6.80 (0.09)	3.97 (0.01)	-1.29	**2.65 (0.65)**	-4.02	**3.50 (1.35)**	-3.26	**2.83 (1.83)**	-5.61
	RIF 32	6.78 (0.06)	7.42 (0.05)		7.78 (0.09)		6.70 (0.00)		9.22 (0.04)	
	PMB 0.25 + RIF 32	6.87 (0.05)	**3.70 (0.01)**	-1.55	**1.00 (0.00)**	-5.67	**2.34 (1.34)**	-4.37	**1.24 (0.24)**	-7.98
ARU604	Control	6.84 (0.13)	7.53 (0.08)		8.71 (0.08)		9.15 (0.17)		9.54 (0.10)	
	PMB 0.25	6.80 (0.13)	5.74 (1.24)		6.27 (2.17)		6.39 (2.86)		8.17 (2.00)	
	FOF 128	6.85 (0.16)	4.52 (0.37)		**2.93 (0.21)**		6.09 (1.11)		8.78 (0.25)	
	PMB 0.25 + FOF 128	6.83 (0.08)	4.50 (0.97)	-0.02	**2.86 (1.86)**	-0.07	**2.62 (1.62)**	-3.46	7.08 (1.38)	-1.09
	MEM 64	6.85 (0.02)	6.99 (0.06)		5.28 (0.28)		7.93 (0.16)		9.37 (0.04)	
	PMB 0.25 + MEM 64	6.81 (0.06)	5.05 (1.01)	-0.69	**3.53 (1.73)**	-1.75	5.12 (3.11)	-1.27	8.39 (1.38)	0.22
	MIN 4	6.79 (0.07)	6.38 (0.18)		7.02 (0.02)		7.26 (0.30)		9.29 (0.11)	
	PMB 0.25 + MIN 4	6.83 (0.07)	5.29 (0.86)	-0.45	5.36 (0.61)	-0.91	5.95 (0.90)	-0.44	8.54 (0.49)	0.37
	RIF 8	6.90 (0.01)	7.56 (0.02)		7.88 (0.00)		8.33 (0.01)		8.91 (0.10)	
	PMB 0.25 + RIF 8	6.91 (0.03)	**3.52 (1.22)**	-2.21	**1.33 (0.29)**	-4.94	**1.00 (0.00)**	-5.39	**1.00 (0.00)**	-7.17
ARU613	Control	6.82 (0.16)	7.57 (0.24)		8.64 (0.12)		9.16 (0.06)		9.60 (0.09)	
	PMB 2	6.80 (0.16)	6.71 (0.47)		8.04 (0.69)		8.99 (0.51)		9.46 (0.43)	
	AMK 16	6.83 (0.21)	6.70 (0.63)		7.05 (1.11)		7.81 (1.13)		9.30 (0.08)	
	PMB 2 + AMK 16	6.93 (0.00)	6.24 (0.65)	-0.46	6.04 (0.90)	-1.01	6.47 (0.69)	-1.34	8.15 (0.65)	-1.15
	CHL 32	6.75 (0.06)	6.95 (0.11)		7.67 (0.23)		8.52 (0.14)		9.24 (0.06)	
	PMB 2 + CHL 32	6.72 (0.06)	6.41 (0.16)	-0.30	6.33 (0.09)	-1.35	6.44 (0.41)	-2.07	8.72 (0.34)	-0.51
	FOF 128	6.74 (0.20)	4.10 (0.57)		**3.03 (0.16)**		4.47 (0.44)		9.30 (0.16)	
	PMB 2 + FOF 128	6.84 (0.18)	**2.43 (1.43)**	-1.67	**2.59 (0.31)**	-0.44	**2.01 (0.11)**	-2.46	5.01 (0.78)	-4.29
	MIN 4	6.77 (0.10)	6.76 (0.01)		7.67 (0.20)		8.36 (0.04)		9.62 (0.03)	
	PMB 2 + MIN 4	6.81 (0.00)	6.57 (0.25)	-0.14	5.90 (0.21)	-1.77	5.24 (0.29)	-3.11	4.58 (0.79)	-4.88
	RIF 8	6.79 (0.15)	7.41 (0.32)		7.77 (0.00)		6.91 (0.40)		8.57 (0.93)	
	PMB 2 + RIF 8	6.93 (0.32)	4.38 (1.11)	-2.32	**1.92 (0.31)**	-5.86	**2.26 (1.78)**	-4.65	**1.36 (0.51)**	-7.21
ARU616	Control	6.73 (0.14)	7.56 (0.13)		8.71 (0.11)		9.15 (0.17)		9.54 (0.08)	
	PMB 2	6.75 (0.15)	7.34 (0.11)		8.64 (0.13)		9.33 (0.09)		9.50 (0.13)	
	AMK 4	6.92 (0.01)	7.24 (0.01)		6.22 (0.03)		6.54 (0.12)		9.12 (0.11)	
	PMB 2 + AMK 4	6.85 (0.08)	6.93 (0.01)	-0.31	5.75 (0.18)	-0.47	3.90 (0.13)	-2.64	**1.00 (0.00)**	-8.12
	CHL 32	6.87 (0.04)	7.10 (0.02)		7.16 (0.04)		7.16 (0.08)		8.49 (0.39)	
	PMB 2 + CHL 32	6.85 (0.05)	6.50 (0.02)	-0.60	6.34 (0.11)	-0.82	6.35 (0.06)	-0.81	5.34 (0.25)	-3.15
	FOF 128	6.84 (0.03)	4.87 (0.25)		5.57 (0.48)		6.57 (0.12)		9.54 (0.02)	
	PMB 2 + FOF 128	6.68 (0.06)	4.93 (0.33)	0.06	**3.20 (0.17)**	-2.37	5.74 (1.20)	-0.84	9.63 (0.08)	0.12
	MEM 16	6.91 (0.09)	5.78 (0.27)		5.34 (0.53)		7.76 (0.49)		9.44 (0.08)	
	PMB 2 + MEM 16	6.85 (0.05)	6.60 (0.12)	0.83	5.02 (0.49)	-0.32	6.62 (0.58)	-1.13	9.53 (0.00)	0.09
	MIN 4	6.67 (0.01)	6.36 (0.18)		7.08 (0.22)		7.64 (0.23)		9.20 (0.04)	
	PMB 2 + MIN 4	6.77 (0.06)	6.13 (0.04)	-0.22	6.17 (0.29)	-0.90	5.95 (0.29)	-1.69	4.84 (0.18)	-4.36
	RIF 32	6.79 (0.05)	7.49 (0.02)		7.62 (0.02)		7.62 (0.10)		9.12 (0.16)	
	PMB 2 + RIF 32	6.90 (0.05)	6.93 (0.02)	-0.41	6.73 (0.04)	-0.89	6.44 (0.02)	-1.18	4.35 (0.97)	-4.76
	TMC 64	6.78 (0.01)	6.76 (0.03)		5.69 (0.00)		4.67 (0.03)		6.66 (0.11)	
	PMB 2 + TMC 64	6.82 (0.03)	6.51 (0.07)	-0.25	4.67 (0.25)	-1.01	**2.76 (0.40)**	-1.91	5.94 (0.99)	-0.71

Mean bacterial concentrations (standard deviations) of at least duplicate experiments and the difference in bacterial concentrations with the combination vs. the most effective antibiotics (Δ log_{10} CFU/mL). Synergy is highlighted in dark grey and additive effects in light grey. Bactericidal effects are marked in bold font and bacteriostatic effects are underlined. AMK, amikacin; ATM, aztreonam; CHL, chloramphenicol; FOF, fosfomycin; LIN, linezolid; MEM, meropenem; MIN, minocycline; PMB, polymyxin B; RIF, rifampicin; TMC, temocillin.

found only against two of the four strains tested. Polymyxin B and meropenem showed 24-hr synergy and a bacteriostatic effect against ARU602 expressing bla_{VIM-1}. An additive effect was detected against the two KPC-producing strains during the first 6 hr of the experiments but was followed by regrowth and no interaction was observed after 24 hr.

Discussion

This study investigated the effects of polymyxin B and 13 other antibiotics against carbapenemase-producing *K. pneumoniae* and reported positive interactions in some combinations, most frequently with minocycline, rifampicin and fosfomycin. Of note,

enhanced activity was also found with combinations of antibiotics to which the strains were highly resistant and in the presence of intrinsic or acquired resistance mechanisms.

Polymyxin B and minocycline showed enhanced activity against all five strains in the time-lapse microscopy experiments, and synergy against all strains except the KPC-3 producing ARU604 in the time-kill experiments. In a previous study, Huang et al. found synergy with polymyxin B and minocycline against six KPC-2-producing *K. pneumoniae* [21]. In that study the activity of the combination was decreased with increasing polymyxin B MICs. In our study, however, the combination was synergistic also against the OXA-48-producing ARU613 and KPC-2-producing ARU616 despite high-level polymyxin B resistance (MICs 16 and 32 mg/L, respectively). This observation indicates that the genotype and the mechanism of resistance could be more predictive than the phenotypic susceptibility regarding the synergistic capabilities of the combination.

The polymyxin B-rifampicin combination was superior to the single antibiotics against all strains, except the NDM-1-producing ARU601, which carried the *arr-2* resistance gene. Rifampicin is normally not active against Gram-negative bacteria because of its inability to penetrate the bacterial outer membrane. However, synergistic interactions, when used in combination with polymyxins, have previously been reported against KPC-producing *K. pneumoniae* [22,23] as well as NDM- or VIM-producing [24,25] *K. pneumoniae*. Considering their mechanism of action [5], polymyxins are expected to be particularly useful in combination with antibiotics that are inactive alone because of impermeability and less likely to enhance the activity of an antibiotic in the presence of target alterations or other non-membrane-associated resistance (e.g., *arr-2*). This possible scenario could in part explain the lack of synergistic activity against ARU601.

The absence of cross-resistance and remaining activity against many carbapenem-resistant strains makes fosfomycin an attractive candidate to use for combination regimens. However, clinical data are limited for parenteral fosfomycin and rapid emergence of resistance has been reported *in vitro* in ESBL- and carbapenemase-producing Enterobacterales [26]. Polymyxin and fosfomycin have previously shown synergy against KPC-producing *K. pneumoniae* in dynamic *in vitro* models but with frequent regrowth [27,28], which is in line with our findings. The observation of frequent emergence of resistance with fosfomycin implies that this antibiotic should probably be avoided at least in the treatment of severe infections.

Polymyxin and carbapenem combinations have previously been reported synergistic and bactericidal against KPC-producing *K. pneumoniae* in several *in vitro* studies [6]. Observational clinical data advocate that meropenem should always be used (provided that meropenem MIC is $\leq$ 8 mg/L [1]) in combination with another active antibiotic. However, both preclinical and clinical data are almost exclusively based on KPC-producing *K. pneumoniae*; hence, the validity of this recommendation for strains producing other carbapenemases remains uncertain. We have previously demonstrated limited activity with colistin and meropenem against VIM- and NDM-producing *K. pneumoniae* [24,25]. In the present study, 24-hr synergy was only observed against the VIM-producing ARU602 with meropenem MIC of 32 mg/L. The varying susceptibility to this combination could be due to differences in the enzymatic activity, permeability changes or efflux. In theory, polymyxin B can counteract porin loss. Still, we found no significant interactions with polymyxin B and meropenem against ARU604, ARU613 and ARU616 that carried mutations in the outer membrane porin gene *ompK36*. This is in line with a previous study reporting lower activity with colistin and doripenem against KPC-producing *K. pneumoniae* with an insertion in *ompK36* [29].

Further studies are needed to understand the impact of polymyxin resistance for the ability of polymyxin B or colistin to act synergistically in combination with other antibiotics. It is noteworthy that acquired resistance to polymyxins does not preclude sustained synergistic potential. In our study, two polymyxin B-resistant strains with mutations upstream *mgrB* and in *crrB* were included. Still, synergy was frequently observed against these strains at 2 mg/L polymyxin B, corresponding to 0.12 $\times$ MIC for ARU613 and 0.06 $\times$ MIC for ARU616. MacNair et al. observed synergistic activity of colistin and hydrophobic antibiotics, including rifampicin and minocycline, against *Escherichia coli* in the presence of *mcr-1*, suggesting that disruption of the outer membrane was still achieved [30].

Some variation in results between time-lapse microscopy and the time-kill method is expected, which could be attributed to several factors [8]. With aztreonam, the growth during single drug exposure was probably overestimated in some of the time-lapse microscopy experiments owing to filamentation (Fig. S1), leading to a false positive interaction. The larger working volume in the time-kill experiments implies a higher starting inoculum and an increased risk of pre-existing or emerging resistant subpopulations. Growth conditions are different between the two methods (e.g., shaking of the cultures during time-kill experiments). In addition, the time-kill method provides more comprehensive information on the bacterial concentration and enables a lower limit of detection than the time-lapse microscopy method (1 $\log_{10}$ CFU/mL vs. c. 4 $\log_{10}$ CFU/mL), which increases the likelihood of detecting combination interactions. In comparison with checkerboards, advantages with the time-lapse microscopy method include the possibility to automatically monitor bacterial growth and morphological changes of the bacteria during experiments and the lower limit of detection, as discussed in a previous publication [8].

To our knowledge, this is the first study using the oCelloScope to screen the effects of multiple antibiotic combinations against multidrug-resistant Gram-negative bacteria. The limitations of this novel method as to discrimination of bacterial concentrations due to the upper and lower limits of detection were partly overcome by assaying multiple concentrations of each antibiotic. We acknowledge that the small number of strains is a limitation of this study and our results are not sufficient to draw definitive conclusions about the general susceptibility of *K. pneumoniae* harbouring specific carbapenemases or resistance genes. Importantly, the promising combinations sometimes showed synergy only at very high antibiotic concentrations. Further study, e.g., using a dynamic model and concentrations mimicking human pharmacokinetics, is required to explore the clinical utility of these regimens provided various antibiotic susceptibilities (MICs) and dosing regimens. Genetic characterization of the strains using whole-genome sequencing and bioinformatic analyses was performed, which can facilitate future comparisons of results between studies. If similar genotype–phenotype associations are found (i.e. indicating that positive interactions by a specific combination are determined by the presence or absence of specific resistance genes or mechanisms), the search for effective combinations would be accelerated.

Conclusion

In conclusion, polymyxin B, in combination with minocycline, rifampicin or fosfomycin, could be worthwhile to investigate against a larger panel of carbapenemase-producing strains with various genotypes *in vitro* and *in vivo* to determine their potential for clinical use. Because of the scarcity of clinical data, *in vitro* studies are valuable in identifying combinations that enhance the antibacterial activity of the remaining treatment options for monotherapy. Although some discrepancy in results between methods

was observed in this study, we conclude that time-lapse microscopy is suitable for screening purposes in the search for antibiotic combinations active against multidrug-resistant Gram-negative bacteria.

Transparency declaration

All authors report no conflicts of interest relevant to this article.

Author contributions

P.W.Y., T.T., P.L., L.F. and E.N. conceptualized and designed the study. P.W.Y., A.O. and K.S. performed the experiments. P.W.Y., T.T., A.O., K.S., P.L. analysed and interpreted the data. P.W.Y., T.T., P.L. and A.O. drafted the manuscript. All authors contributed to the revision and have approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.03.007.

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