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Original Article

Activity of polymyxin B combinations against genetically well-characterised *Klebsiella pneumoniae* producing NDM-1 and OXA-48-like carbapenemases

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ABSTRACT

Background: Combination therapy can enhance the activity of available antibiotics against multidrug-resistant Gram-negative bacteria. This study assessed the effects of polymyxin B combinations against carbapenemase-producing *Klebsiella pneumoniae* (*K. pneumoniae*).

Methods: Twenty clinical *K. pneumoniae* strains producing NDM-1 (n = 8), OXA-48-like (n = 10), or both NDM-1 and OXA-48-like (n = 2) carbapenemases were used. Whole-genome sequencing was applied to detect resistance genes (e.g. encoding antibiotic-degrading enzymes) and sequence alterations influencing permeability or efflux. The activity of polymyxin B in combination with aztreonam, fosfomycin, meropenem, minocycline, or rifampicin was investigated in 24-hour time-lapse microscopy experiments. Endpoint samples were spotted on plates with and without polymyxin B at 4 x MIC to assess resistance development. Finally, associations between synergy and bacterial genetic traits were explored.

Results: Synergistic and bactericidal effects were observed with polymyxin B in combination with all other antibiotics: aztreonam (11 of 20 strains), fosfomycin (16 of 20), meropenem (10 of 20), minocycline (18 of 20), and rifampicin (15 of 20). Synergy was found with polymyxin B in combination with fosfomycin, minocycline, or rifampicin against all nine polymyxin-resistant strains. Wildtype *mgrB* was associated with polymyxin B and aztreonam synergy (P = 0.0499). An absence of *arr-2* and *arr-3* was associated with synergy of polymyxin B and rifampicin (P = 0.0260). Emergence of populations with reduced polymyxin B susceptibility was most frequently observed with aztreonam and meropenem.

Conclusion: Combinations of polymyxin B and minocycline or rifampicin were most active against the tested NDM-1 and OXA-48-like-producing *K. pneumoniae*. Biologically plausible genotype-phenotype associations were found. Such information might accelerate the search for promising combinations and guide individualised treatment.

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) producing carbapenemases (e.g. KPC, NDM, and OXA-48) pose a major medical threat [1,2]. These pathogens are normally multidrug resistant and associated with high mortality [3,4]. Polymyxin B and E (colistin) remain last-resort agents against carbapenem-resistant isolates when recently introduced antibiotics (e.g. cefiderocol and β-lactam/β-lactamase inhibitor combinations) are unavailable, too costly, or inappropriate due to resistance or intolerance. However, polymyxins should always be used in combination with a second active antibiotic [3–5].

Polymyxins target the lipopolysaccharide structure [5] and induce membrane disruption, which may increase permeability of a second antibiotic. Polymyxins have shown synergistic potential against carbapenem-resistant isolates in combination with several other antibiotics [3,4], also against isolates with resistance to both antibiotics used in combination [6–8]. However, data are still limited and there is great variability in results, probably largely due to differences in the genetic background. A better understanding

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of which genetic characteristics determine the synergistic potential of combinations may help explain some of the observed variability and provide new insights regarding the mechanisms of synergy.

This study evaluated polymyxin B in combination with aztreonam, fosfomycin, meropenem, minocycline, and rifampicin against 20 clinical *K. pneumoniae* isolates producing NDM-1 or OXA-48-like carbapenemases in 24-hour time-lapse microscopy experiments [9]. Moreover, it explored potential associations between bacterial genetics and combination effects.

2. Methods

2.1. Antibiotics and media

Cation-adjusted Mueller-Hinton (MH-II) (BD Diagnostics, Sparks, MD, USA) broth and agar plates were used for all experiments. Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of 10 000 mg/L were prepared by dissolving polymyxin B, fosfomycin, and meropenem in sterile water and aztreonam, minocycline, and rifampicin in DMSO. Glucose-6-phosphate (25 mg/L) was added to experiments including fosfomycin.

2.2. Strains and antibiotic susceptibility testing

Twenty carbapenemase-producing *K. pneumoniae* strains collected from seven hospitals in Oman during 2015 were used. A maximum likelihood SNP-based phylogenetic tree was constructed in CLC Microbial Genomics Module 22.1.1 (CLC Genomics Workbench 22.0.2) using *K. pneumoniae* (GeneBankID: CP095557.1) as reference. Susceptibilities were determined with broth microdilution for polymyxin B, meropenem, minocycline, and rifampicin, and with agar dilution for fosfomycin. Aztreonam MICs were determined using the Sensititre Antimicrobial Susceptibility Testing System (Trek Diagnostic Systems, Cleveland, OH, USA). Susceptibilities were interpreted according to CLSI M100-ED32:2022.

2.3. Genetic characterisation

DNA was extracted using the MagNA Pure 96 System (F. Hoffmann-La Roche, Basel, Switzerland) followed by whole genome sequencing (WGS) using HiSeq 2500 (Illumina, San Diego, CA, USA). Reads were assembled de novo using CLC Genomics Workbench (version 20). ResFinder 4.1 identified acquired resistance genes [10]. Non-identical hits were aligned against the ResFinder entry sequence in CLC Main Workbench (version 20). ResFinder identified *mcr-1* to *-10* and further analyses of polymyxin B resistance genes (*pmrA, pmrB, pmrD, phoP, phoQ, crrA, crrB, mgrB, eptA, arnT, lpxM*) were performed in CLC Genomics Workbench (version 20) using *K. pneumoniae* MGH78578 (GeneBankID:NC_009648) as reference. The same reference was used to identify variations in genes associated with AcrAB-TolC efflux (*acrA, acrB, acrR, tolC, marR, marA, marB, soxS, soxR, rob*), membrane permeability (*ompC, ompF, ompR, envZ, uhpT, glpT*), and rifampicin resistance (*rpoB*).

2.4. Time-lapse microscopy experiments

Screening was performed using the oCelloScope (BioSense Solutions ApS, Farum, Denmark) as previously described [6]. Log phase bacterial cultures of ca. 10^6 CFU/mL were added to a total volume of 200 µL per well in flat bottom 96-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany). The following concentrations were used: polymyxin B (0.25, 0.5, 1, and 2 mg/L), aztreonam (2, 8, and 64 mg/L), fosfomycin (8, 32, and 128 mg/L), meropenem (2, 16, and 64 mg/L), minocycline (0.5, 4, and 16 mg/L), and rifampicin (1, 8, and 32 mg/L). A lower range was applied if all tested concentrations prevented bacterial growth: polymyxin B (0.125, 0.25, 0.5, and 1 mg/L), fosfomycin (2, 8, and 32 mg/L), and meropenem (0.125, 0.5, and 2 mg/L). Quality control strains (*Escherichia coli* ATCC 25922 for polymyxin B, aztreonam, fosfomycin, and meropenem, and *Staphylococcus aureus* ATCC 29213 for minocycline and rifampicin) were included in all experiments. Images were generated every 15 minutes for 24 hours during incubation at 37 °C using the bottom search function, illumination level 150, and image distance 4.9 µm.

2.5. Automated readout using background corrected absorption and segmentation extracted surface area

The background corrected absorption (BCA) and segmentation extracted surface area (SESA) algorithms of the UniExplorer software version 6.0.0 (Philips BioCell A/S, Allerød, Denmark) were used. BCA > 8 and maximum SESA ($SESA_{max}$) > 5.8 indicate bacterial densities > 10^6 CFU/mL at 24 hours, and the lower limit of detection (LOD) is ca. 1×10^4 CFU/mL [6]. If BCA and $SESA_{max}$ were below the cut-off values at 24 hours with the combination but not with any of the single antibiotics at the same concentration, the combination was considered to exhibit an enhanced effect. A combination was considered to exhibit a reduced effect if both BCA and $SESA_{max}$ were above the cut-off values with the combination, but not with one or both single antibiotics.

2.6. Viable counts and population analysis

Endpoint 24-hour samples from the time-lapse microscopy experiments were serially diluted in PBS and 10 µL aliquots were spotted on MH-II agar plates without and with polymyxin B at a concentration of 4 x MIC. Viable counts were performed after overnight incubation at 37 °C. No visible growth was recorded as 1 $\log_{10}$ CFU/mL (LOD 2 $\log_{10}$ CFU/mL). Synergy was defined as a $\geq$ 2-$\log_{10}$ CFU/mL reduction with the combination at 24 hours compared with the most effective single antibiotic. A bactericidal effect was defined as a $\geq$ 3-$\log_{10}$ reduction in CFU/mL compared with the starting inoculum. Antagonism was defined as a $\geq$ 2-$\log_{10}$ CFU/mL increase with the combination compared with either of the single antibiotics. Potential antibiotic carry-over effects were assessed by comparing a subset of spot test results with regular plating, allowing a 100 µL sample to sink into the agar before spreading with glass beads.

2.7. Statistical analysis

Associations between synergistic effects, resistance genes, and susceptibility to the single antibiotics were assessed by Fisher's exact test using R (version 3.6.3). No clinical breakpoints were available for fosfomycin or rifampicin; instead, MIC > 128 mg/L was used as a cut-off in the assessment of phenotypic susceptibility effects. Associations with $P < 0.05$ were considered statistically significant. Resistance genes that showed a significant association with synergy were further explored at the mutation level.

2.8. Data availability

The WGS raw data were uploaded to the Sequence Read Archive as project PRJNA544438 (accession numbers SRR9122670-SRR9122671, SRR9122674, SRR9122677, SRR9122683-SRR9122685, SRR9122688, SRR9122691-SRR9122692, SRR9122696, SRR10294874, SRR10294886, SRR10294920, SRR10294947- SRR10294948, SRR10295015, SRR10295028, SRR10295037, and SRR10295071).

Table 1
Carbapenemase genes, MIC values (mg/L), and classification of antibiotic susceptibility according to CLSI breakpoint tables, CLSI M100-ED32:2022.

ARU strain	Carbapenemase genes	Aztreonam	Fosfomycin	Meropenem	Minocycline	Polymyxin B	Rifampicin
805	bla_{NDM-1}	> 32 (R)	8 (NA)	32 (R)	4 (S)	32 (R)	32 (NA)
806	$bla_{OXA-232}$	> 32 (R)	16 (NA)	32 (R)	1 (S)	8 (R)	128 (NA)
807	bla_{OXA-48}	> 32 (R)	16 (NA)	32 (R)	2 (S)	8 (R)	16 (NA)
808	bla_{OXA-48}	> 32 (R)	8 (NA)	1 (S)	4 (S)	0.5 (I)	8 (NA)
809	bla_{NDM-1}	> 32 (R)	> 64 (NA)	> 32 (R)	8 (I)	8 (R)	128 (NA)
810	bla_{NDM-1}	> 32 (R)	32 (NA)	> 32 (R)	4 (S)	32 (R)	16 (NA)
811	bla_{NDM-1}	> 32 (R)	64 (NA)	32 (R)	1 (S)	0.5 (I)	32 (NA)
812	bla_{OXA-48}	> 32 (R)	16 (NA)	16 (R)	2 (S)	16 (R)	16 (NA)
813	$bla_{OXA-232}$	> 32 (R)	64 (NA)	32 (R)	4 (S)	0.5 (I)	256 (NA)
814	$bla_{OXA-232}$	> 32 (R)	16 (NA)	1 (S)	2 (S)	0.25 (I)	128 (NA)
815	bla_{NDM-1}	> 32 (R)	32 (NA)	> 32 (R)	1 (S)	0.25 (I)	32 (NA)
816	$bla_{OXA-232}$	> 32 (R)	32 (NA)	8 (R)	64 (R)	8 (R)	128 (NA)
817	$bla_{OXA-232}$	> 32 (R)	64 (NA)	16 (R)	1 (S)	4 (R)	256 (NA)
818	bla_{NDM-1}	> 32 (R)	8 (NA)	> 32 (R)	4 (S)	0.5 (I)	32 (NA)
819	bla_{NDM-1} $bla_{OXA-232}$	> 32	64	> 32	2	8	8
820	bla_{NDM-1}	> 32 (R)	32 (NA)	32 (R)	4 (S)	0.5 (I)	16 (NA)
821	bla_{OXA-48}	4 (S)	64 (NA)	1 (S)	8 (I)	0.25 (I)	256 (NA)
822	$bla_{OXA-232}$	> 32 (R)	64 (NA)	16 (R)	4 (S)	0.25 (I)	128 (NA)
823	bla_{NDM-1} bla_{OXA-48}	> 32	64	32	2	1	256
824	bla_{NDM-1}	> 32 (R)	32 (NA)	32 (R)	1 (S)	0.25 (I)	128 (NA)

Abbreviations: S, susceptible; I, intermediate; R, resistant; NA, not applicable.

3. Results

3.1. Antibiotic susceptibility

Nine strains were resistant to polymyxin B (MICs 4–32 mg/L) and 11 were classified intermediate (MICs 0.25–1 mg/L) (Table 1). Susceptibility rates were high for minocycline (17 of 20), while most strains were resistant to meropenem (17 of 20) and aztreonam (19 of 20). For fosfomycin, MIC values ranged from 8 to > 64 mg/L (clinical breakpoints do not apply to *K. pneumoniae*). Due to intrinsic resistance, there are no clinical breakpoints for rifampicin, and MICs were high (8–256 mg/L).

3.2. Genetic characterisation

All strains harboured carbapenemase genes: bla_{NDM-1} ($n = 8$), $bla_{OXA-232}$ ($n = 6$), bla_{OXA-48} ($n = 4$), bla_{NDM-1} and $bla_{OXA-232}$ ($n = 1$), and bla_{NDM-1} and bla_{OXA-48} ($n = 1$) (Table 1). Additional β-lactamase genes were identified in all strains, most frequently $bla_{CTX-M-15}$ ($n = 19$), bla_{TEM-1B} ($n = 11$), and $bla_{SHV-106}$-alike ($n = 9$) (Table S1). Other relevant resistance genes included: fosfomycin, *fosA* ($n = 20$), minocycline, *tet(A)* ($n = 3$), *tet(D)* ($n = 1$), rifampicin, *arr-2* ($n = 6$), and *arr-3* ($n = 3$). Sequence variations were found in the polymyxin B resistance genes *pmrB, mgrB*, and *lpxM*, of which several were previously detected in clinical isolates (Table S2). One of the *mgrB* mutations (W20S) has been shown to increase polymyxin B resistance alone [11]. None of the strains carried *mcr-1* to *-10*.

Multiple variations were found in *ompK36* and all strains carried a disrupted *ompK35* sequence (Table S3), which have been associated with increased resistance against β-lactam antibiotics, fosfomycin, and minocycline [12–14]. Moreover, frameshifts were identified in *acrR* (ARU815 and ARU816) and *ramR* (ARU820), which encode negative regulators of AcrAB-TolC, suggesting increased efflux of β-lactam antibiotics, minocycline, and rifampicin [15]. ARU816 carried an *rpoB* mutation (I572M) causing rifampicin resistance, and three strains carried a previously unreported mutation (N596D) [16]. Genes and mutations associated with resistance to antibiotics not included in this study and phylogenetic data are provided as supplementary material (Table S4, Figure S1).

3.3. Time-lapse microscopy experiments: background corrected absorption and segmentation extracted surface $area_{max}$ readout

An enhanced effect based on the BCA and $SESA_{max}$ readout was frequently observed with polymyxin B in combination with fosfomycin (15 of 20 strains), minocycline (20 of 20), and rifampicin (17 of 20) (Figure S2). Combinations including β-lactam antibiotics were less successful; an enhanced effect was observed against 12 of 20 strains with polymyxin B and aztreonam, and against nine of 20 strains with polymyxin B and meropenem. A reduced effect was detected for polymyxin B with meropenem against 13 of 20 strains, and less frequently with aztreonam (six of 20), fosfomycin (five of 20), minocycline (two of 20), and rifampicin (two of 20).

3.4. Time-lapse microscopy experiments: viable counts

The automated readout and viable counts showed high agreement; synergy was found for 70 of 73 (96%) combinations that showed an enhanced effect according to BCA and $SESA_{max}$ (Figure 1, Figure S2). Synergistic effects that were also bactericidal were frequently detected for polymyxin B in combination with fosfomycin (16 of 20), minocycline (18 of 20), and rifampicin (15 of 20). Of note, synergy was found against all nine polymyxin B-resistant strains. Synergistic and bactericidal effects were shown for polymyxin B and aztreonam against 11 of 20 strains and for polymyxin B and meropenem against 10 of 20 strains. Antagonism was most frequently detected with polymyxin B in combination with meropenem (14 of 20 strains), aztreonam (eight of 20), and fosfomycin (seven of 20). No antibiotic carry-over effects were observed.

3.5. Population analysis

Growth on plates containing polymyxin B at 4 x MIC occurred with all combinations after 24 hours, often at low bacterial concentrations of 2 $\log_{10}$ CFU/mL (Figure 1, Figure S3). Bacterial growth was most frequent after exposure to polymyxin B in combination with aztreonam or meropenem. Isolates growing on polymyxin B plates showed up to 128-fold increases in MIC compared with the parental strain. MIC elevations were more common

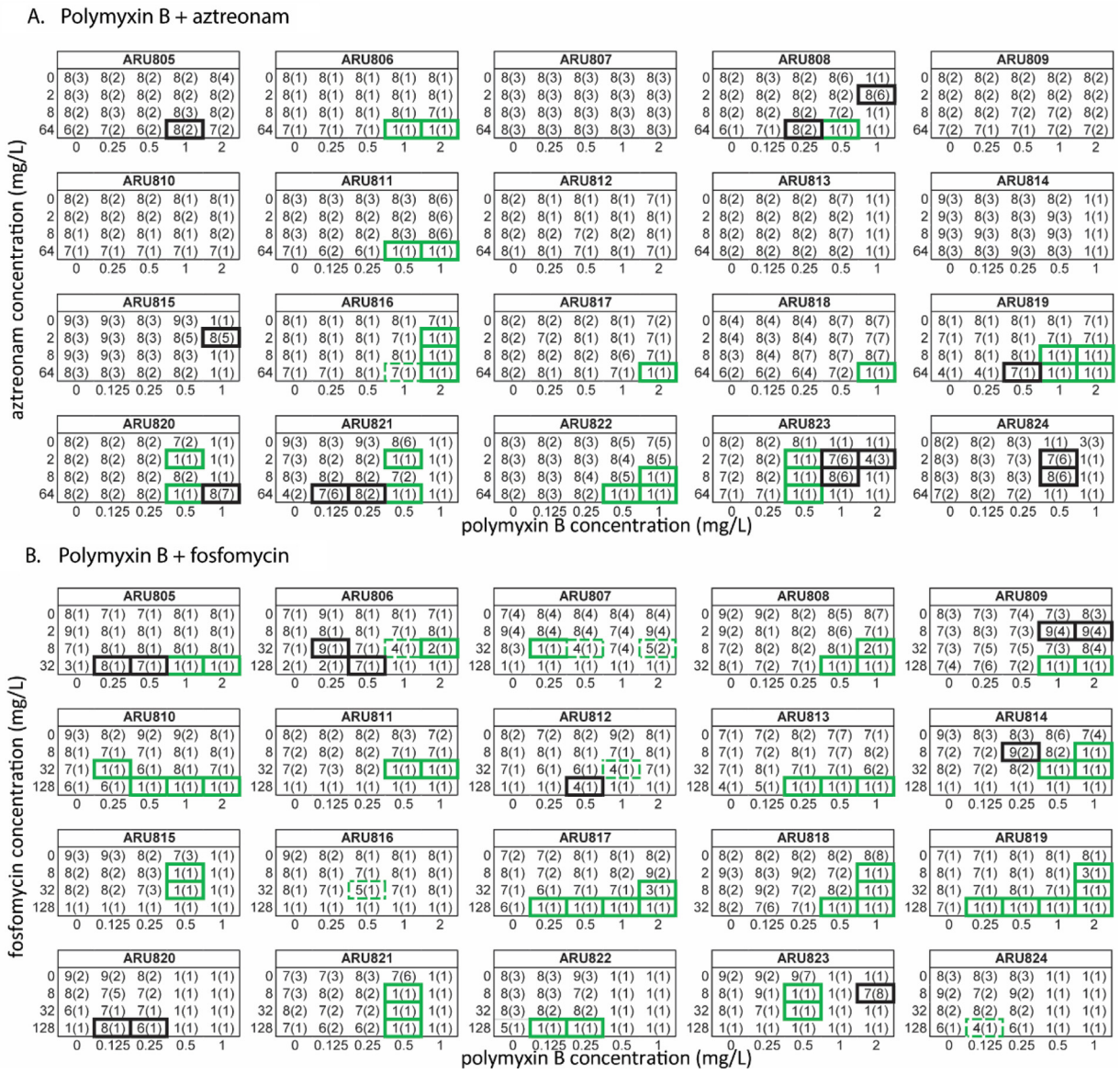

Figure 1. Bacterial concentrations ($\log_{10}$ CFU/mL) after 24 hours of antibiotic exposure in time-lapse microscopy experiments as determined by viable counts on regular plates and plates containing polymyxin B at 4 x MIC (in parentheses). No visible growth was set to 1 $\log_{10}$ CFU/mL. Solid green squares indicate a synergistic and bactericidal effect, dashed green squares indicate a synergistic but not bactericidal effect, and black squares indicate antagonism.

at higher bacterial concentrations, which indicated an inoculum effect: ≥ 4-fold MIC increases were found in 56 of 102 tested isolates growing on 4 x polymyxin B MIC at 2 $\log_{10}$ CFU/mL, 60 of 64 isolates growing at 3 $\log_{10}$ CFU/mL, and 71 of 73 isolates growing at ≥ 4 $\log_{10}$ CFU/mL.

3.6. Statistical analysis

Polymyxin B and aztreonam was synergistic against 10 of 14 strains with wildtype *mgrB* (P = 0.0499, OR 10.81, 95% CI 0.84–645.38). Of the six strains with *mgrB* sequence variations, polymyxin B and meropenem synergy was only seen against the strain with a W20S mutation. Absence of *arr-2* and *arr-3* genes was detected in 11 of 16 strains against which polymyxin B and rifampicin demonstrated synergy (P = 0.0260, OR 0.000, 95% CI 0–1.02); however, synergy was also found in five of nine strains carrying these genes. Wildtype *mgrB* was associated with susceptibility to polymyxin B (P = 0.0022, OR 0.000, 95% CI 0–0.42). Antibiotic susceptibility was not a determinant of synergy for any of the combinations.

4. Discussion

This study observed frequent synergistic and bactericidal effects with polymyxin B in combination with fosfomycin, minocycline, or rifampicin, against NDM- and OXA-48-like-producing *K. pneumoniae*. Polymyxin B combinations including aztreonam or meropenem were less successful. The genetic characterisation and statistical analyses revealed biologically plausible associations between bacterial genetics and the probability of synergy.

Polymyxin B in combination with 13 other antibiotics was previously screened against five *K. pneumoniae* producing NDM-1, VIM-1, KPC-3, OXA-48, and KPC-2 [6]. Polymyxin B showed 24-hour synergy in time-kill experiments in combination with fosfomycin or minocycline against the NDM-1 and OXA-48 producers, and with rifampicin against the OXA-48-producing strain. The results indicated that the genetic setup and mechanism of resistance

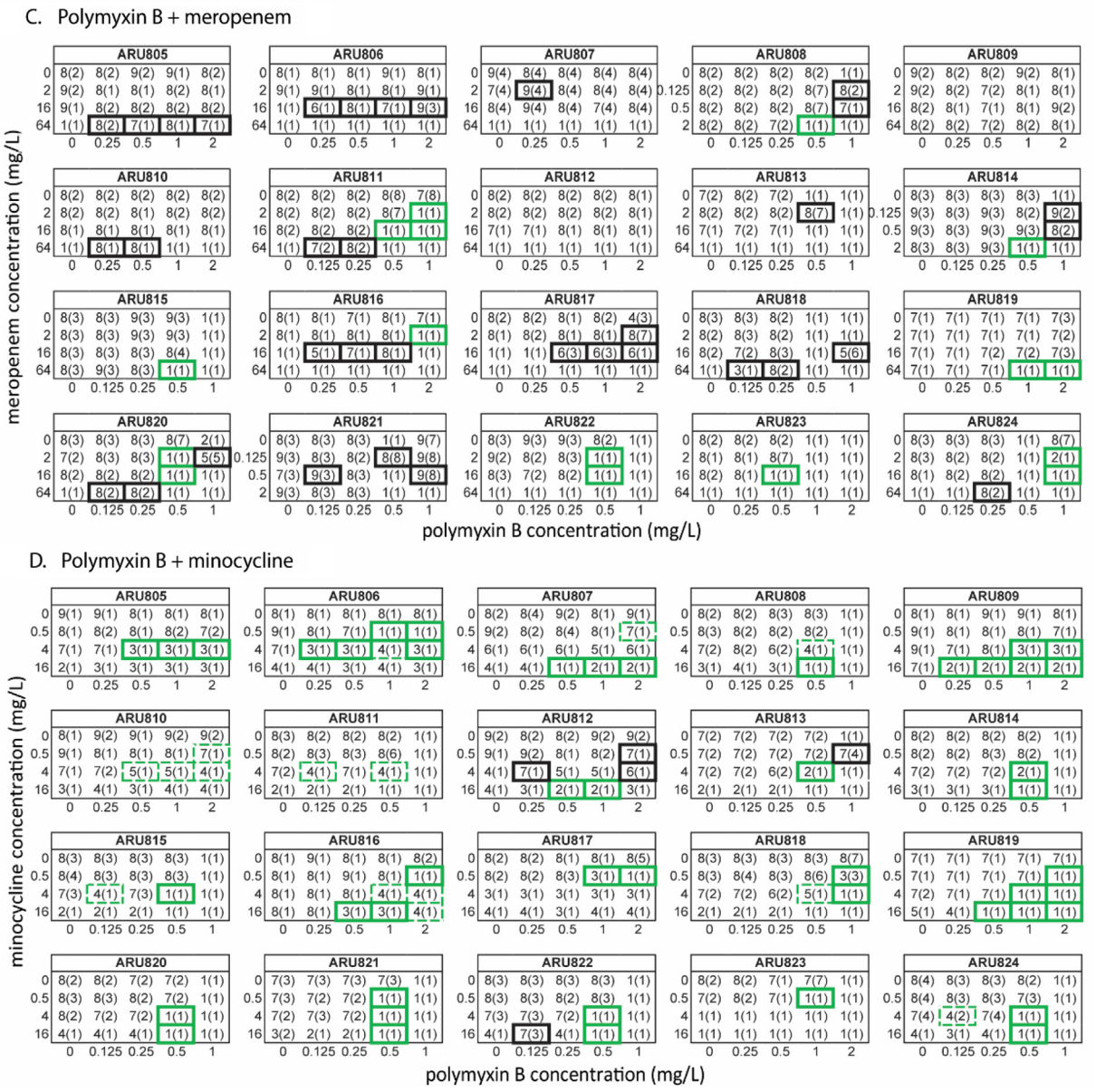

Figure 1. Continued

might be a better predictor for synergy than susceptibility to the single antibiotics. The current study explored this hypothesis in a larger strain collection.

Comparative data exist mainly for KPC-producing *K. pneumoniae*. Polymyxin B and fosfomycin showed synergy and sustained bactericidal activity in a hollow-fibre infection model [8]. Polymyxin B and minocycline demonstrated synergistic and bactericidal effects in static time-kill experiments [7], and another time-kill study reported synergy with colistin and rifampicin against a polymyxin-resistant KPC-producing *K. pneumoniae* with a high rifampicin MIC of $\geq$ 32 mg/L [17]. Unfortunately, the isolates were not subjected to WGS.

Synergy with polymyxin B and rifampicin was observed against all 11 strains not carrying *arr-2* or *arr-3*, which encode rifampicin-inactivating enzymes, while strains harbouring these genes had a low synergy rate (five of nine). This finding aligns with a previous study where synergy was not detected against the NDM producer harbouring *arr-2* [6]. In this case, polymyxin B probably allows penetration of rifampicin through the cell wall, but the synergistic potential is hampered by enzymatic resistance.

No associations between genetic background and combination effects were found for polymyxin B with minocycline or fosfomycin. However, it was hypothesised that their collective action on the bacterial cell wall increased the overall antibacterial activity. Specifically, minocycline chelates the Ca^{2+} ions, linking the lipid A molecules, and fosfomycin targets the UDP-N-acetylglucosamine enolpyruvate transferase (MurA), which has a key function in proteoglycan biosynthesis [5,18].

The combined hydrolytic activity of several β-lactamases likely explains the poor activity of polymyxin B combinations including aztreonam and meropenem. The metallo-β-lactamases (MBLs, e.g. NDM-1) have a strong affinity for meropenem, as do OXA-48-like carbapenemases, although with weaker activity [19]. Aztreonam, in contrast, is stable against MBLs and OXA-48-like enzymes but is efficiently hydrolysed by extended-spectrum β-lactamases [1,2]. In this study, 19 of 20 strains produced CTX-M-15. Previous data also suggest that the presence of several β-lactamases is negatively associated with synergy [6].

The most common cause of polymyxin resistance in *K. pneumoniae* is sequence alterations in *mgrB*, which encodes a negative

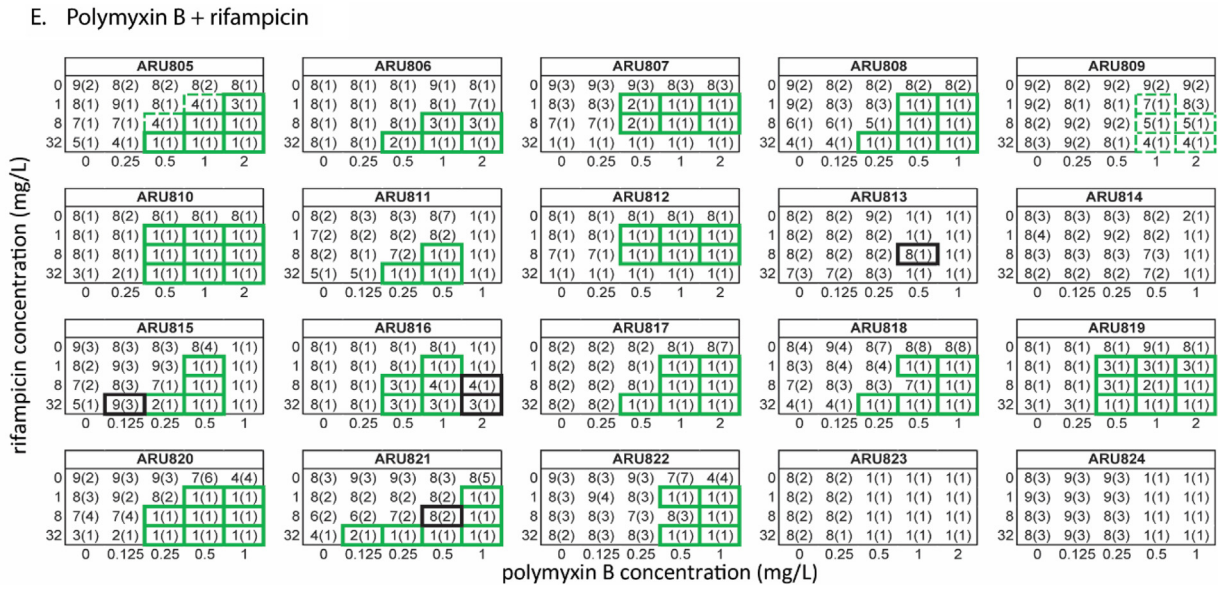

Figure 1. Continued

regulator of the PhoP/PhoQ system, resulting in lipopolysaccharide modifications [5]. In this study, wildtype *mgrB* was associated with a higher synergy rate for polymyxin B and aztreonam, which suggests that polymyxin binding to lipid A is crucial for its synergistic potential with β-lactams. Polymyxin B and meropenem did not result in synergy against any of the five strains with a deletion or frameshift in *mgrB*.

Emergence of isolates with decreased polymyxin B susceptibility was detected in experiments with polymyxin B alone and in combination. Also, an inoculum effect was often observed, resulting in growth on polymyxin B plates at low bacterial concentrations (2–3 $\log_{10}$ CFU/mL). Overall, growth on polymyxin B plates was more pronounced after exposure to polymyxin B in combination with aztreonam or meropenem.

Antagonism was most commonly found with polymyxin B and meropenem and has previously been reported [20], although the mechanisms and potential clinical implications remain unclear. The current study sometimes noted synergy and antagonism against the same strain at different antibiotic concentrations, which may have resulted from concentration-dependent effects, biological variation, or resistance development.

The genetic characterisation and the statistical analysis of combination effects are important assets of this study. However, resistance in *K. pneumoniae* is multifactorial [3,4], which aggravates the evaluation of genotype-phenotype effects. Associations may have been overlooked due to the high synergy rates with fosfomycin, minocycline, and rifampicin. Finally, we acknowledge that in vitro data cannot be directly translated to the clinical situation (e.g., due to differences in growth conditions and the lack of immune system effects).

5. Conclusion

In conclusion, this study demonstrated synergy with polymyxin B in combination with fosfomycin, minocycline, and rifampicin, also against polymyxin-resistant strains with poor permeability or active efflux of the second drug. In contrast, resistance mediated by β-lactamases or rifampicin-degrading enzymes was less likely to be counteracted by polymyxin B. Understanding the impact of bacterial genetics on combination effects can help explain differences in results between studies, provide insights into the mechanisms of synergy, and accelerate the search for effective therapies.

Conflict of Interest

All authors declare no conflicts of interest.

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Author Contributions

AO, AS, TT, and PL conceptualised and designed the study. AO and HAF determined the antimicrobial susceptibility. HAF performed the whole genome sequencing, and AO and LA completed the bioinformatics and statistical analysis. AO and AS performed the time-lapse microscopy and spot test screening. AO, AS, LA, TT, and PL analysed and interpreted the data. AO, LA, TT, and PL drafted the manuscript. All authors contributed to the revision and have approved the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2023.106967.

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