Interactions of Polymyxin B in Combination with Aztreonam, Minocycline, Meropenem, and Rifampin against Escherichia coli Producing NDM and OXA-48-Group Carbapenemases

Anna Olsson,a Marcus Hong,a Hissa Al-Farsi,b,c Christian G. Giske,b,c Pernilla Lagerbäck,a Thomas Tängdén*a

aDepartment of Medical Sciences, Uppsala University, Uppsala, Sweden
bDepartment of Laboratory Medicine, Division of Clinical Microbiology, Karolinska Institute, Stockholm, Sweden
cDepartment of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden

Marcus Hong and Hissa Al-Farsi contributed equally to the study; author order was based on when each joined the project.

ABSTRACT Carbapenemase-producing Enterobacterales pose an increasing medical threat. Combination therapy is often used for severe infections; however, there is little evidence supporting the optimal selection of drugs. This study aimed to determine the in vitro effects of polymyxin B combinations against carbapenemase-producing Escherichia coli. The interactions of polymyxin B in combination with aztreonam, meropenem, minocycline or rifampin against 20 clinical isolates of NDM and OXA-48-group-producing E. coli were evaluated using time-lapse microscopy; 24-h samples were spotted on plates with and without 4×MIC polymyxin B for viable counts. Whole-genome sequencing was applied to identify resistance genes and mutations. Finally, potential associations between combination effects and bacterial genotypes were assessed using Fisher’s exact test. Synergistic and bactericidal effects were observed with polymyxin B and minocycline against 11/20 strains and with polymyxin B and rifampin against 9/20 strains. The combinations of polymyxin B and aztreonam or meropenem showed synergy against 2/20 strains. Negligible resistance development against polymyxin B was detected. Synergy with polymyxin B and minocycline was associated with genes involved in efflux (presence of tet[B], wild-type soxR, and the marB mutation H44Q) and lipopolysaccharide synthesis (epiA C27Y, lpxB mutations, and lpxK L323S). Synergy with polymyxin B and rifampin was associated with sequence variations in arnT, which plays a role in lipid A modification. Polymyxin B in combination with minocycline or rifampin frequently showed positive interactions against NDM- and OXA-48-group-producing E. coli. Synergy was associated with genes encoding efflux and components of the bacterial outer membrane.

KEYWORDS carbapenem resistance, Gram-negative bacteria, combination therapy, synergy, polymyxins

The increasing prevalence of carbapenemase-producing Enterobacterales is an emerging threat worldwide. These bacteria are common causes of severe infections, such as sepsis, urinary tract infections, and hospital-acquired pneumonia, and are difficult to treat due to their multidrug-resistant phenotypes (1–3). The last resort antibiotics polymyxin B and E (colistin) remain active against most isolates and have been widely used for these infections (4, 5). Although combination therapy is always recommended based on observational clinical data (6), evidence is still scarce on the optimal selection of companion drug.

In vitro synergy against carbapenemase-producing Enterobacterales has been shown with polymyxins in combination with multiple other antibiotics (e.g., β-lactams, minocycline, rifampin) (7–10). Most studies have addressed Klebsiella pneumoniae, and data are...
limited for *Escherichia coli*. The prevailing theory for the observed synergistic interactions is that the polymyxin-induced membrane disruption increases the membrane permeability, thereby facilitating entry of the second antibiotic (11, 12). Polymyxins may also act by counteracting the function of membrane-associated efflux pumps (11). However, the mechanisms of synergistic interaction remain largely unknown. Therefore, to date, the activity of antibiotic combinations cannot be predicted based on antibiotic susceptibility testing of single drugs or genetic characterization.

We previously evaluated automated time-lapse microscopy (the oCelloScope, BioSense Solutions Aps, Farum, Denmark) as a screening tool for antibiotic combinations (13) and reported synergy with several polymyxin B combinations against multidrug-resistant *K. pneumoniae* and *Pseudomonas aeruginosa* (9, 14). In the present study, we evaluated the effects of polymyxin B in combination with aztreonam, meropenem, minocycline and rifampin against 20 NDM- and OXA-48-producing *E. coli* in 24-h time-lapse microscopy experiments. A spot assay in which 24-h samples were placed on plates with and without polymyxin B at 4×MIC was added to provide viability data and detect emerging subpopulations with reduced susceptibility. All isolates were subjected to whole-genome sequencing to map genes known to impact the susceptibility to the tested antibiotics. Finally, we explored potential associations between the observed combination effects and bacterial genetics.

**RESULTS**

**Antibiotic susceptibilities.** All strains were intermediate to polymyxin B with MICs of 0.5 mg/liter (Table 1). Only three strains were susceptible to aztreonam. Strains carrying *blaNDM* (*blaNDM-1*, *blaNDM-5* and *blaNDM-7*) were resistant to meropenem, whereas those carrying only *blaOXA-48* ~group carbapenemase genes (*blaOXA-48* and *blaOXA-181*) were classified as susceptible. Minocycline MICs varied greatly between the strains (range 1–64 mg/liter) and rifampin MICs were mostly high (8 to 32 mg/liter).

**Resistance genes and mutations.** Polymyxin resistance genes *mcr-1*–*10* were not found in the strains. All strains harbored genes encoding carbapenemases: NDM (*n* = 13), OXA-48-group enzymes (*n* = 5) or both (*n* = 2) (Table 2). In addition, other β-lactamase genes were present in all strains, most frequently *blaTEM-16* (*n* = 15), *blaCTX-M-15* (*n* = 14) and *blaOXA-1* (*n* = 11). Tetracycline efflux genes *tet(A) (n* = 8), *tet(B) (n* = 8) or *tet(D) (n* = 2) were found in 18/20 strains. All eight strains harboring *tet(B)* and eight of nine strains with wild type *soxR* (Table 3) had increased minocycline MICs (≥8 mg/liter). An amino acid

**TABLE 1** MIC values (mg/liter) and classification of antibiotic susceptibilities according to CLSI breakpoint tables M100-ED30:2020

| Strain       | Carbapenemase | Polymyxins | β-lactams | Tetracyclines | Rifamycins |
|--------------|---------------|------------|-----------|---------------|------------|
|              |               | PMB        | ATM       | MEM           | MIN        | RIF       |
| ARU770       | NDM-1         | 0.5 (I)    | >16 (R)   | >64 (R)       | 32 (R)     | 16 (NA)   |
| ARU771       | NDM-1         | 0.5 (I)    | >16 (R)   | 64 (R)        | 32 (R)     | 16 (NA)   |
| ARU772       | NDM-7         | 0.5 (I)    | >16 (R)   | 32 (R)        | 4 (S)      | 16 (NA)   |
| ARU773       | NDM-5         | 0.5 (I)    | 1 (S)     | 64 (R)        | 16 (R)     | 16 (NA)   |
| ARU774       | NDM-1         | 0.5 (I)    | >16 (R)   | >64 (R)       | 16 (R)     | 32 (NA)   |
| ARU775       | NDM-5         | 0.5 (I)    | >16 (R)   | >64 (R)       | 4 (S)      | 16 (NA)   |
| ARU776       | NDM-1         | 0.5 (I)    | >16 (R)   | >64 (R)       | 4 (S)      | 16 (NA)   |
| ARU777       | NDM-5         | 0.5 (I)    | >16 (R)   | 16 (R)        | 16 (R)     | 16 (NA)   |
| ARU778       | NDM-1         | 0.5 (I)    | >16 (R)   | 16 (R)        | 16 (R)     | 32 (NA)   |
| ARU779       | NDM-5         | 0.5 (I)    | >16 (R)   | >64 (R)       | 8 (I)      | 16 (NA)   |
| ARU780       | NDM-5         | 0.5 (I)    | 8 (I)     | 64 (R)        | 8 (I)      | 16 (NA)   |
| ARU781       | NDM-5         | 0.5 (I)    | >16 (R)   | >64 (R)       | 8 (I)      | 16 (NA)   |
| ARU782       | OXA-48        | 0.5 (I)    | >16 (R)   | 64 (R)        | 4 (S)      | 32 (NA)   |
| ARU783       | OXA-48        | 0.5 (I)    | ≤0.5 (S)  | 0.5 (S)       | 2 (S)      | 8 (NA)    |
| ARU785       | OXA-48        | 0.5 (I)    | >16 (R)   | 2 (S)         | 1 (S)      | 8 (NA)    |
| ARU786       | OXA-48        | 0.5 (I)    | ≤0.5 (S)  | 1 (S)         | 8 (I)      | 16 (NA)   |
| ARU787       | OXA-181       | 0.5 (I)    | >16 (R)   | 1 (S)         | 8 (I)      | 32 (NA)   |
| ARU788       | OXA-181       | 0.5 (I)    | >16 (R)   | 0.5 (S)       | 16 (R)     | 16 (NA)   |
| ARU790       | NDM-5, OXA-181| 0.5 (I)    | >16 (R)   | 16 (R)        | 32 (R)     | 32 (NA)   |
| ARU791       | NDM-1, OXA-48 | 0.5 (I)    | >16 (R)   | 32 (R)        | 64 (R)     | 32 (NA)   |

*Abbreviations: S, susceptible; I, intermediate; R, resistant; NA, not available; ATM, aztreonam; MEM, meropenem; MIN, minocycline; PMB, polymyxin B; RIF, rifampin*
| Antibiotic class | Resistance gene | Strain |
|------------------|-----------------|--------|
| β-lactams        |                 | ARU770 | ARU771 | ARU772 | ARU773 | ARU774 | ARU775 | ARU776 | ARU777 | ARU778 | ARU779 | ARU780 | ARU781 | ARU782 | ARU783 | ARU785 | ARU786 | ARU787 | ARU788 | ARU790 | ARU791 |
|                  | blbxxXX-2       | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-6       | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-42      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-15      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-1       | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-A        | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-9       | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-8       | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-61      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-81      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | blbxxXX-101     | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| Tetracyclines    | tet(A)          | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | tet(B)          | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
|                  | tet(D)          | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| Rifamycins       | rpoD*           | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |

Abbreviations: *, full-length gene present and without amino acid variations compared to the reference in the ResFinder database; †, gene sequence not complete due to scaffold or contig breaks after assembly.

*Escherichia coli K12 MG1655 used as reference (NCBI accession number: NC_000913.3).
| Porin, efflux pump or regulator | Function | Gene | Strain |
|---------------------------------|----------|------|--------|
| **OmpC**                        | S        | **ompC**<sup>a</sup> | ARU770 | ARU771 | ARU772 | ARU773 | ARU774 | ARU775 | ARU776 | ARU777 | ARU778 | ARU779 |
|                                 |          |      | Q54K   | Q54K   | L296V  | Q54K   | Q54K   | Q54K   | Q54K   | G216A | Q54K   |        |
|                                 |          |      | N165D  | N165D  |        | N165D  | N165D  | N165D  | N165D  | I218V | N165D  |        |
|                                 |          |      | G216A  | G216A  |        | G216A  | G216A  | G216A  | G216A  | G216A | G216A  |        |
|                                 |          |      | L218V  | L218V  |        | L218V  | L218V  | L218V  | L218V  | I218V |        |        |
| **OmpF**                        | S        | **ompF**<sup>b</sup> | N31fs  | N31fs  |        |        |        |        |        |        |        |        |
| **AcrAB-To1C**                  | S        | **acrA** |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 | R        | **acrR** |        |        |        |        |        |        |        |        |        |        |
|                                 | S        | **tolC** |        |        |        |        |        |        |        |        |        |        |
| **marRAB operon**               | R        | **marR** | G103S<sup>c</sup> | G103S<sup>c</sup> | G103S<sup>c</sup> | G103S<sup>c</sup> | G103S<sup>c</sup> | G103S<sup>c</sup> | S3N<sup>c</sup> | Y120H |        |        |
| **S**                           |          |      | Y137H<sup>d</sup> | Y137H<sup>d</sup> | Y137H<sup>d</sup> | Y137H<sup>d</sup> | Y137H<sup>d</sup> | Y137H<sup>d</sup> | G103S<sup>d</sup> |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 | A        | **marA** | H44Q   | H44Q   | H44Q   | H44Q   |        |        |        |        |        |        |
|                                 | R        | **marB** |        |        |        |        |        |        |        |        |        |        |
| **SoxSR**                       | A        | **soxS** |        |        |        |        |        |        |        |        |        |        |
|                                 | A        | **soxR** |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
| **RobA**                        | A        | **rob** |        |        |        |        |        |        |        |        |        |        |
| **OmpR-EnvZ**                   | A        | **ompR** |        |        |        |        |        |        |        |        |        |        |
|                                 | A        | **EnvZ** |        |        |        |        |        |        |        |        |        |        |
| **OmpC**                        | S        | **ompC**<sup>b</sup> | G216A | I218V | L296V | Q54K | Q54K | L296V | Q54K | Q54K | L296V | G216A | Q54K |
|                                 |          |      | N165D  | G216A  | I218V  | N165D  | G216A  | I218V  | N165D  | G216A  | I218V  | G216A  | I218V |
|                                 |          |      | G216A  | L296V  |        | G216A  | L296V  |        | G216A  | L296V  |        |        |        |
|                                 |          |      | L218V  |        |        | L218V  |        |        | L218V  |        |        |        |        |
| **OmpF**                        | S        | **ompF**<sup>b</sup> | Y112F | N31fs  | N31fs  |        |        |        |        |        |        |        |
|                                 |          |      | F118I  | F118I  | F118I  |        |        |        |        |        |        |        |
|                                 |          |      | F118I  | Y204F  | Y204F  |        |        |        |        |        |        |        |
| **AcrAB-To1C**                  | S        | **acrA** | T104A<sup>d</sup> | T104A<sup>d</sup> | T104A<sup>d</sup> | T104A<sup>d</sup> | T104A<sup>d</sup> | T104A<sup>d</sup> | T104A<sup>d</sup> |        |        |        |
|                                 |          |      | A167S  | H596N<sup>d</sup> | H596N<sup>d</sup> | H596N<sup>d</sup> | H596N<sup>d</sup> | H596N<sup>d</sup> | H596N<sup>d</sup> |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
|                                 |          |      |        |        |        |        |        |        |        |        |        |        |
| **(Continued on next page)**    |          |      |        |        |        |        |        |        |        |        |        |        |
| Porin, efflux pump or regulator | Function | Gene | Strain | Strain | Strain | Strain | Strain |
|---------------------------------|----------|------|--------|--------|--------|--------|--------|
| marRAB operon                   | R        | marR| A70E   | G103S<sup>a</sup> | Y137H<sup>d</sup> | K62R<sup>d</sup> | K62R<sup>d</sup> | G103S<sup>d</sup> | X |
| A                               | marA     |      |        |        |        |        |        |        |     |
| R                               | marB     | H44Q| SSL    | SSL    | SSL    | H44Q   | H44Q   | H44Q   |     |
|                                 |          |      | T24P   | A17T   | L12F   |        |        |        |     |
|                                 |          |      | A33G   | V20I   | A17T   | A17T   |        |        |     |
|                                 |          |      | V38A   | H44Q   | V20I   | V20I   |        |        |     |
|                                 |          |      | H44Q   | H44Q   |        |        |        |        |     |
| SoxSR                           | A        | soxS|        |        |        |        |        | A12S<sup>c</sup> |     |
|                                 | A        | soxR| A111T<sup>d</sup> | T385<sup>d</sup> | T385<sup>d</sup> | T385<sup>d</sup> | A111T<sup>d</sup> |        |     |
|                                 |          |      |        | G74R<sup>d</sup> | G74R<sup>d</sup> | G74R<sup>d</sup> |        |        |     |
| RobA                            | A        | rob |        |        |        |        |        |        |     |
| OmpR-EnvZ                       | A        | ompR|        |        |        |        |        |        |     |
| A                               | EnvZ     |      | A25V   | A25V   | A25V   |        |        |        |     |
|                                 |          |      | T466A  | T466A  | T466A  |        |        |        |     |

<sup>a</sup>Escherichia coli K12 MG1655 (NCBI accession number: NC_000913.3) used as reference. Abbreviations: A, activator; R, repressor; S, subunit; X, gene not found; *, stop codon; fs, frameshift; †, gene sequence not complete due to scaffold or contig-breaks after assembly.
<sup>b</sup>Only amino acid variations in β-strand-encoding regions of ompC and ompF are shown.
<sup>c</sup>Mutation not previously associated with increased efflux.
<sup>d</sup>Mutation previously known to cause increased resistance.
substitution in rpoB (G1261C) was identified in ARU790 but was not located in any region known to cause resistance to rifampin (15).

Eleven strains had a sequence variation (T5N, n = 2), frameshift (n = 7) or a premature stop codon (n = 2) in acrR (Table 3). These genetic variations likely result in increased expression of the AcrAB-ToIC efflux pump (16), for which aztreonam, meropenem, minocycline and rifampin are known substrates (17–19). A mutation in the AcrAB-ToIC efflux regulatory gene soxS (A125), previously reported to be associated with resistance, was found in one strain (20). We identified additional mutations commonly encountered in clinical isolates but have not been shown to increase AcrAB-ToIC efflux activity alone: acrR (T104A, A167S and N221Y [21]), marR, (S3N, K62R, G103S and Y137H) and soxR (A111T, T38S and G74R) (22). Several other mutations with unknown effects were found in marB; the most frequent mutation was H44Q which was found in 14/20 strains. In 19/20 strains, genes encoding the OmpC and OmpF porins, that facilitate entry of β-lactams (3), were associated with sequence variations in the β-sheet regions composing the porin channels (23, 24) (Table 3). Several amino acid variations were identified in genes encoding enzymes involved in the synthesis or modification of LPS, mainly in lpxB, lpxK, lpxH, arrT, and eptA (25) (Table 4). Moreover, there was large variability in core oligosaccharide types, as determined based on the waa locus (25, 26); R1 was most frequent (n = 8), followed by R4 (n = 5), R2 (n = 4) and R3 (n = 3).

**Time-lapse microscopy experiments.** The most effective combination was polymyxin B and minocycline, showing a positive interaction against 11/20 strains (Fig. 1), closely followed by polymyxin B and rifampin with 9/20 strains. For polymyxin B and meropenem a positive interaction was seen against 3/20 strains. The combination of polymyxin B and aztreonam was not superior to monotherapy at any of the tested concentrations when using the predefined cutoffs for bacterial growth (BCA > 8 at 24 h and SESₐ₉₉ₐₑ > 5.8). Negative interaction by the combination in comparison to monotherapy was observed with polymyxin B in combinations with meropenem (ARU770, ARU779, ARU781 and ARU788) and aztreonam (ARU788).

**Spot assay.** The spot assay showed synergistic and bactericidal effects with 22/23 combinations that indicated positive interactions in the time-lapse microscopy experiments (Fig. 1). In addition, synergistic and bactericidal effects were detected with polymyxin B and aztreonam against two strains (ARU780 and ARU786). No antibiotic carryover effect was observed (data not shown). Growth on polymyxin B at 4× MIC after 24 h was detected for 267 of the 504 spots (53%) that grew on nonantibiotic-containing plates (Fig. 1). However, in all but three cases, growth on 4× MIC polymyxin B was only 2 log₁₀ CFU/ml (= the lower limit of detection, LOD) and repeated susceptibility testing of 67 spots revealed no increase in polymyxin B MICs indicating an inoculum effect (data not shown).

**Associations between combination effects and bacterial genetics.** Statistical analysis using Fisher’s exact text showed that synergy with polymyxin B and minocycline was significantly associated with the tetracycline efflux gene tet(B); synergy was noted in 7/8 strains carrying this gene (P = 0.0281) (Table S1). In contrast, a negative association was found for tet(A); synergy was only observed in 1/8 harboring this gene (P = 0.0045). Statistically significant associations were also found when comparing wild type to any mutation(s) in marB (P = 0.0081), marR (P = 0.0499) and soxR (P = 0.0098), which are all involved in AcrAB-ToIC efflux. On the mutation level, the marB mutation H44Q was frequently associated with a synergistic effect (10/11, P = 0.04985) (Table S2). No specific marR mutation was significantly associated with synergy. Reduced susceptibility to minocycline in strains carrying tet(B) (n = 8) or wild type soxR (n = 9) was reversed in the presence of polymyxin B in 7 and 8 cases, respectively (Fig. 1C). In contrast, the soxR mutation A111T was negatively associated with synergy (1/11, P = 0.0499). Moreover, sequence alterations in the lpxB (P = 0.0499) and lpxK (P = 0.0499) genes, encoding enzymes involved in lipid A synthesis, were associated with synergy (Table S3) (25). On the mutation level, the lpxK mutation L323S (P = 0.0499) was present in 10/11 strains against which synergy was found, whereas the eptA mutation C27Y showed a negative association (1/11, P = 0.0499).
| Function | Gene | Strain | ARU770 | ARU771 | ARU772 | ARU773 | ARU774 | ARU775 | ARU776 | ARU777 | ARU778 | ARU779 | ARU780 | ARU781 | ARU782 | ARU783 | ARU784 | ARU785 | ARU786 | ARU787 | ARU788 | ARU789 | ARU790 | ARU791 |
|----------|------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Structural component | Enzymes catalyzing lipid A synthesis | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| lipo | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| lpxC | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| lpxD | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| lpxH | K58Q | K58Q | K58Q | K58Q | K58Q | P210T | R206C | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | |
| lpxB | E3K | E3K | E3K | H32R | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | H32R | V93I | |
| lpxK | A278T | L323S | A278T | L323S | N252H | R206C | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | I224V | |
| lpxL | lpxM | † | R3 | R3 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R3 | R1 | R2 | R1 | R3 | R1 | R2 | R1 | R3 | |
| Core oligosaccharide type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| Regulators of lpxC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| Modifies lipid A | arrT | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | T154A | |
| lpxA | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | D348G | |
| lpxP | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | }

Notes:
- *Escherichia coli* K-12 MG1655 (NCBI accession number: NC_000913.3) used as reference. Abbreviations: Del, deletion; †, gene sequence not complete due to scaffold or contig breaks after assembly.

**TABLE 4** Genetic differences in genes encoding enzymes involved in lipopolysaccharide synthesis and core oligosaccharide type.
A. Polymyxin B + aztreonam

| Concentration (mg/L) | ARU770 | ARU771 | ARU772 | ARU773 | ARU774 |
|----------------------|--------|--------|--------|--------|--------|
| 0                    | 8(2)  | 8(2)  | 8(2)  | 8(2)  | 8(2)  |
| 0.25                 | 8(2)  | 8(2)  | 8(2)  | 8(2)  | 8(2)  |
| 0.5                  | 1     | 1     | 1     | 1     | 1     |
| 1                    | 1     | 1     | 1     | 1     | 1     |
| 2                    | 1     | 1     | 1     | 1     | 1     |

B. Polymyxin B + meropenem

| Concentration (mg/L) | ARU770 | ARU771 | ARU772 | ARU773 | ARU774 |
|----------------------|--------|--------|--------|--------|--------|
| 0                    | 8(2)  | 9(2)  | 9(2)  | 9(2)  | 8(2)  |
| 0.25                 | 9(2)  | 9(2)  | 9(2)  | 9(2)  | 9(2)  |
| 0.5                  | 1     | 1     | 1     | 1     | 1     |
| 1                    | 1     | 1     | 1     | 1     | 1     |
| 2                    | 1     | 1     | 1     | 1     | 1     |

FIG 1 Results of time-lapse microscopy experiments and spot assay. For time-lapse microscopy experiments, wells with BCA > 8 and SEAmix > 5.8, indicating a bacterial density of >10⁶ CFU/ml at 24 h, are highlighted in gray and combinations showing positive interactions in the time-lapse microscopy experiments are marked with a square. For spot assay, bacterial growth on MH-II plates at 24 h is presented in log₁₀ CFU/ml and no visible growth is set to 1 log₁₀ CFU/ml (LOD = 2 log₁₀ CFU/ml). Growth on 4 × MIC polymyxin B is presented in parentheses. Synergistic and bactericidal effect with the combination, as determined with the spot assay, is highlighted with "*) and antagonistic effect "*".
C. Polymyxin B + minocycline

![Graph showing synergistic combinations against E. coli with Polymyxin B and minocycline concentrations.](image)

D. Polymyxin B + rifampicin

![Graph showing synergistic combinations against E. coli with Polymyxin B and rifampicin concentrations.](image)

**FIG 1 (Continued)**
No significant associations were noted for the polymyxin B and rifampin combination for genes encoding efflux, porin loss or enzymatic resistance. However, several mutations in the \textit{arnT} gene encoding a lipid A-modifying enzyme were positively associated with synergy (\textit{P} values ranging from 0.005 to 0.022) (Table S4). Because synergy was rarely observed with polymyxin B and aztreonam or meropenem, statistical analyses were not considered meaningful for these combinations.

\textbf{DISCUSSION}

In this study, positive interactions were frequently found with polymyxin B combined with minocycline or rifampin against NDM- and OXA-48-group producing \textit{E. coli}. 

\textbf{FIG 2} Changes in cell morphology during exposure to polymyxin B (PMB), aztreonam (ATM) and meropenem (MEM) against NDM-producing \textit{Escherichia coli} ARU786. Antibiotics were added to the indicated concentrations (mg/liter). Images were obtained at 0, 1, 3, 6 and 24 h. The \textit{SESA}$_{\text{max}}$ and BCA (in parentheses) values are presented below each image. Filamentation during exposure to aztreonam alone resulted in high BCA and \textit{SESA}$_{\text{max}}$ values despite low viable counts.
In contrast, according to the 24-h viable count data, combinations of polymyxin B and aztreonam or meropenem showed synergy and a bactericidal activity only against 2/20 strains. Negligible resistance development against polymyxin B was identified with all combinations. Although growth on polymyxin B at 4× MIC was often observed following antibiotic exposure, bacterial concentrations were typically low (≤2 log10 CFU/ml) and no MIC elevations were detected. Therefore, we deduce that this observation likely reflects an inoculum effect, which is of uncertain clinical relevance, rather than emergence or selection of resistant subpopulations.

Importantly, nonsusceptibility to one or both constituent antibiotics does not preclude a synergistic activity when combining the two drugs. Polymyxin B and minocycline performed well in this study despite that all strains were intermediate to polymyxin B, and most were intermediate or resistant to minocycline. To our knowledge, data on the activity of this combination against Enterobacterales are scarce. However, polymyxin B was previously reported to induce 8-fold reductions in minocycline MICs in mcr-1 positive E. coli and K. pneumoniae (8). Also, we recently reported synergy with this combination in time-kill experiments against 4/5 K. pneumoniae producing NDM, KPC or OXA-48 enzymes, including strains displaying phenotypic resistance to one or both drugs (9).

Gram-negative bacteria are intrinsically resistant to rifampin due to the inability of this molecule to penetrate the bacterial outer membrane. Yet, polymyxin B and rifampin showed synergy against 9/20 strains in this study. Our results are consistent with other studies reporting positive interactions with polymyxins and rifampin. One study observed a bactericidal activity with polymyxin B and rifampin against 2/5 KPC-producing E. coli (10) and we previously reported synergy with this combination against 4/5 NDM-, KPC- or OXA-48-producing K. pneumoniae (9). Another study showed synergy with this combination against NDM- and MCR-1-producing polymyxin-resistant E. coli (7).

Our results indicate polymyxin B and meropenem has low synergistic potential against NDM- and OXA-48-producing E. coli. Polymyxin-carbapenem combinations have been widely recommended for severe infections caused by carbapenemase-producing Enterobacterales (4, 6). Observational clinical data support the use of such combinations against KPC-producing K. pneumoniae with carbapenem MICs ≤8 mg/liter (4, 5). However, their efficacy against E. coli and strains producing non-KPC enzymes remains uncertain as illustrated in this study. As new β-lactam/β-lactamase inhibitor combinations become available, it is important to consider the bacterial genetic determinants and strain-dependent differences in antibiotic susceptibility to the single drugs and combinations. While meropenem-vaborbactam and imipenem-relebactam are normally active against KPC-producing isolates (6), their use will be limited in areas where other carbapenemases are predominant. Aztreonam is highly intriguing in this context due to its stability to metallo-β-lactamases, such as NDM-1. Still, polymyxin B and aztreonam failed to show positive interactions against most of the tested strains in this study. To our knowledge, previous data on this combination is lacking for E. coli and is scarce for K. pneumoniae (9, 27). Clearly, coadministration of polymyxin B was generally not sufficient to circumvent enzymatic resistance in these strains, e.g., mediated by CTX-M-15, which was produced by 14/20 strains and has high affinity for aztreonam (2, 28).

We observed several biologically plausible and statistically significant associations between the interactions of polymyxin B and minocycline and bacterial genetics. For example, synergy was positively associated with genes involved in efflux, which can be counteracted by the membrane-disrupting activity of polymyxin B. Statistically significant associations were observed for mutant marB and marR. These genes regulate AcrAB-TolC efflux, for which minocycline and multiple antibiotics (e.g., meropenem, aztreonam and rifampin) are known substrates. The association with the marB mutation H44Q likely results from reduced repression of marA, which in turn increases AcrAB-TolC activity (1) (Table S2). While wild type soxR was positively associated with reduced susceptibility to minocycline and a synergistic activity with the combination, a negative association was found for soxR mutation A111T. This observation aligns with
a previous study where this mutation was not associated with resistance to tetracycline or other antibiotics (22).

Further, several sequence variations in genes involved in LPS synthesis or modification showed statistically significant associations with enhanced activity of polymyxin B and minocycline or rifampin in combination (25). These genetic variations might have altered minocycline or rifampin permeability as well as polymyxin B targets. For the minocycline combination, mutant lpxB and lpxK L323S were associated with synergy, while the C27Y mutation in eptA was negatively associated with synergy. LpxB has a role in the addition of a saccharide to the lipid A structure and LpxK catalyzes the addition of the phosphate group. The cation-linkages between phosphates of the lipid A molecules are an important feature for membrane stability and the negatively charged phosphate groups are also a target of polymyxin B (12). Interestingly, minocycline has a potent antioxidant activity and can also directly chelate Ca$^{2+}$ which could also contribute to synergy with polymyxins by displacing the cation-linkages (Ca$^{2+}$ and Mg$^{2+}$) between two lipid A molecules and increase permeability (12, 29). Synergy with polymyxin B and rifampin was positively associated with mutations in arnT. Both ArnT and EptA mediate additions of positively charged moieties to the phosphate groups, which could alter polymyxin B activity (25).

The spot assay added information on CFU/ml reductions and enabled assessment of resistance development during antibiotic exposure. The measurement of bacterial concentrations with this assay is similar to standard time-kill experiments but has lower resolution as individual colonies are not counted (only growth/no growth with a 1:10 dilution between spots) and a higher LOD of 2 log$_{10}$ versus 1 log$_{10}$ CFU/ml. Also, the time-lapse microscopy method differs from time-kill experiments in that there is no shaking during incubation and the total volume is lower (200 µl versus ca 2 ml) (13). The agreement in results between the oCelloScope readout and spot assay was excellent with the exception of aztreonam, for which filamentation complicates readout using the available SESA and BCA algorithms (Fig. 2). Filament formation is associated with β-lactam antibiotics targeting penicillin-binding protein 3 (PBP3), including aztreonam, and was previously observed in time-lapse microscopy experiments with K. pneumoniae and P. aeruginosa (9, 13, 14).

The extensive genetic characterization of resistance mechanisms and mutations, and the assessment of their potential associations with the combination effects is a strength of this study. However, we recognize that more research is needed to validate our findings and determine causality. Combination therapy will remain important in the treatment of multidrug-resistant pathogens to enhance bacterial killing and suppress emergence of resistance, and further efforts to better understand the determinants of synergistic interactions are needed. A range of clinically achievable drug concentrations was used to reduce the risk of overlooking synergistic activity. However, in some cases positive interactions were detected only at the highest drug concentrations, which may be associated with a risk of toxicity in patients. As always, translation of in vitro findings to the clinical setting must also be made with caution due to the absence of an immune system and other biological processes as well as differences in growth conditions.

In conclusion, we report positive interactions with polymyxin B combinations against E. coli producing NDM and OXA-48-group carbapenemases, most frequently with minocycline or rifampin. These combinations should be further explored in vitro and in vivo to determine their therapeutic potential. Resistance genes or mutations involved in efflux, LPS synthesis or modification and lipid A modification were associated with synergistic effect. Deciphering such associations between combination effects and bacterial genetics is a first step toward understanding the mechanisms of synergistic interactions, and may help inform individualized therapy tailored to the infecting pathogen in future patients.

**MATERIALS AND METHODS**

**Antibiotics and media.** All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of 10,000 mg/liter were prepared by dissolving polymyxin B and meropenem in sterile water and
Synergistic Combinations against *E. coli*

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aztreonam, minocycline and rifampin in DMSO. Cation-adjusted Mueller-Hinton (MH-II) (BD Diagnostics, Sparks, MD, USA) broth and agar plates were used for all experiments.

**Strains and antibiotic susceptibility testing.** Twenty carbapenemase-producing *E. coli* isolates collected from hospitalized patients in Oman during 2015 were used. The susceptibilities to polymyxin B, meropenem, minocycline, and rifampin were tested with broth microdilution according to CLSI recommendations (30). Aztreonam MICs were determined using the Sensititre Antimicrobial Susceptibility Testing System (Trek Diagnostic Systems, Cleveland, OH) according to the manufacturer’s instructions. Susceptibilities were interpreted using CLSI clinical breakpoints M100-ED30:2020 (31).

**Genetic characterization.** DNA was extracted with the MagNA Pure96 System (F. Hoffmann-La Roche, Basel, Switzerland) followed by whole-genome sequencing using HiSeq 2500 (Illumina, San Diego, USA). De novo assembly was accomplished using CLC Genomics Workbench (version 20). ResFinder 4.1 was employed to identify acquired resistance genes, (32). Because all strains were susceptible, the search for polymyxin B resistance genes was restricted to mcr. To identify variations in genes involved in AcrAB-ToLC efflux (acrA, acrB, acrV, tolC, marR, marA, marB, sos, ssaR, ssaB), porin-specific entry (ompC, ompF, ompG, emu2), LPS synthesis (lpp, lpxA-D, lpxH, lpxM, lpxP, putH, lapB, amT, epaT, pagP and the waa locus) and rifampin resistance (rpoB) genes were aligned against *E. coli* MG1655 K-12 (NCBI Reference Sequence: NC_000913.3) and the core oligosaccharide type was determined based on the waa locus composition (25).

**Time-lapse microscopy.** Screening was performed using the oCelloScope instrument as previously described (9, 13, 14). Briefly, bacteria in exponential growth phase were added to achieve starting inocula ~10^8 CFU/ml and a total volume of 200 µl per well in a flat-bottom 96-well microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany). The following clinically achievable drug concentrations were used: polymyxin B, 0.25, 0.5, 1 and 2 mg/liter; aztreonam, 2, 8 and 64 mg/liter; meropenem, 2, 16 and 64 mg/liter; minocycline, 0.5, 4 and 16 mg/liter; and rifampin, 1, 8 and 32 mg/liter. If one of the single antibiotics of a combination prevented bacterial growth at all these concentrations, a lower concentration range was used: polymyxin B, 0.125, 0.25, 0.5 and 1 mg/liter; aztreonam, 0.125, 0.5 and 2 mg/liter; and meropenem, 0.125, 0.5 and 2 mg/liter. Quality control strains (*E. coli* ATCC 25922 for polymyxin B, aztreonam and meropenem and *Staphylococcus aureus* ATCC 29213 for minocycline and rifampin) were included in all experiments. The 96-well microtiter plate was incubated at 37°C and images of each well were generated every 15 min for 24 h by the oCelloScope. Focus was set using the bottom search function, illumination level was set to 150, and image distance to 4.9 µm.

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 to determine bacterial density. The LOD was ~1 × 10^7 CFU/ml. A BCA value >8 and a maximum SESA value (SESAmax) >5.8 were used as cutoff values to indicate a bacterial density of >10^7 CFU/ml at 24 h (13). The combination was considered to exhibit a positive interaction if BCA and SESAmax were above these cutoffs with the combination but not with any of the constituent single antibiotics at the same concentration. Conversely, the combination was considered to show a negative interaction if BCA and SESAmax were below these cutoffs with the combination but not with the single antibiotics at the same drug concentrations.

**Spot assay and population analysis.** After completing the 24-h time-lapse microscopy experiments, samples from each well were serially diluted in PBS and 10 µl aliquots were spotted on MH-II agar plates with and without 2 mg/liter polymyxin B (4× MIC) (33). Bacterial growth was recorded after overnight incubation at 37°C. The LOD was 2 log$_{10}$ CFU/ml. No visible bacterial growth was recorded as 1 log$_{10}$ CFU/ml in the microtiter plate. Synergistic and bactericidal effects. Synergy was defined as ≥2-log$_{10}$ CFU/ml reduction in bacterial concentrations with the combination at 24 h compared with the most potent single antibiotic (33). A bactericidal effect was defined as ≥3-log$_{10}$ reduction in CFU/ml at 24 h compared with the starting inoculum. A ≥1-log$_{10}$ CFU/ml increase in bacterial concentrations with the combination compared to one or both single antibiotics at the same drug concentration was classified as antagonism. Potential antibiotic carryover effects were assessed by regular plating of 100 µl undiluted and 10-fold diluted samples, allowing the sample to sink in before spreading. Two strains producing NDM-1 (ARU770) or OXA-48 (ARU783) were randomly selected for MIC determination of all spots growing on 4× MIC plates after 24 h.

**Statistical analyses.** Potential associations between synergistic effects with an antibiotic combination and the presence of resistance genes and mutations in the tested strains were assessed by Fisher’s exact test using R (version 3.6.3). Resistance genes showing statistically significant associations, defined as P < 0.05, were further explored to identify correlations between combination interactions and specific mutations in these genes.

**Data availability.** Whole-genome sequencing raw data (reads) were deposited in the Sequence Read Archive (SRA) as project PRJNA544438 (accession numbers SRR9113453, SRR9113455-SRR9113460, SRR9113462, SRR9113468, SRR9113469, SRR9113478-SRR9113487).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1,** PDF file, 1 MB.

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