# Synergistic activity of colistin in combination with clofoctol against colistin resistant Gram-negative pathogens

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## SUPPLEMENTAL MATERIAL

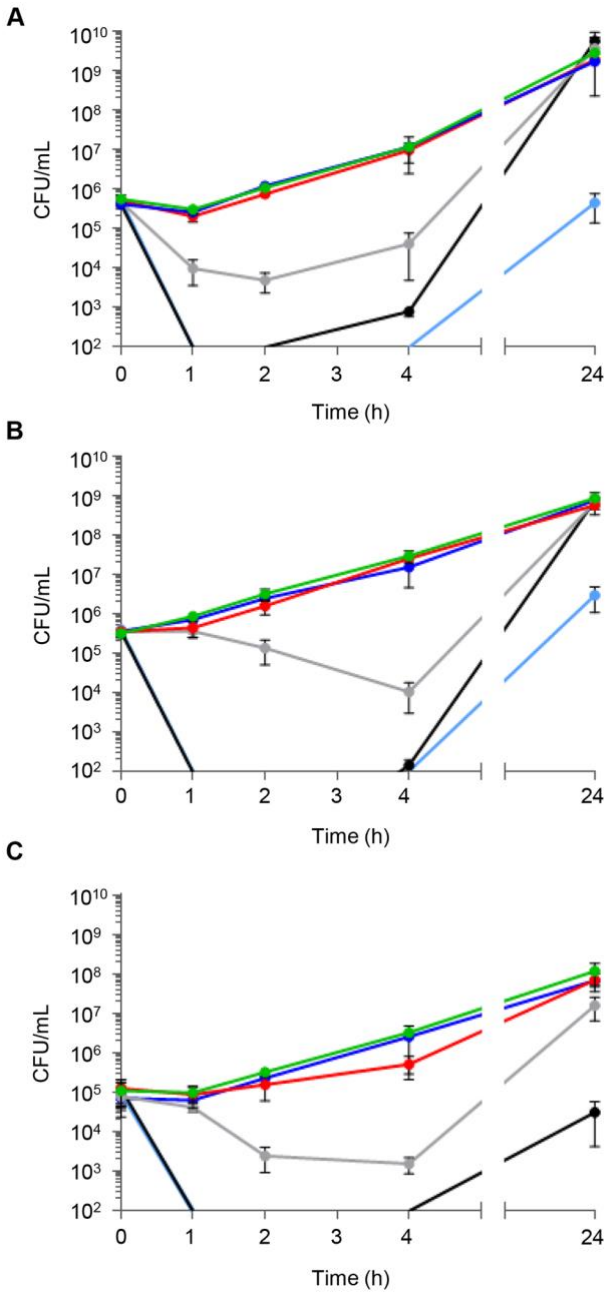


**FIG S1**. Time-kill curves of **(A)** *P. aeruginosa* PAO1, **(B)** *K. pneumoniae* KP-MO-27, **(C)** *A. baumannii* ATCC19606 treated with 1X MIC colistin (gray lines), 0.5X MIC colistin (red lines), 73 µg/mL clofoctol (blue lines), or the same concentrations of colistin and clofoctol in combination (black lines, 0.5X MIC colistin in combination with clofoctol; light blue lines, 1X MIC colistin in combination with clofoctol). The untreated controls are shown with green lines. **(A, B)** 1X MIC = 1 µg/mL colistin; 0.5X MIC = 0.5 µg/mL colistin. **(C)** 1X MIC = 0.25 µg/mL colistin; 0.5X MIC = 0.125 µg/mL colistin. Data are

mean values from three independent experiments and the error bars represent standard deviations

The detection limit of the assay was $10^2$ CFU/mL.

**TABLE S1.** Bacterial strains used in this study.

| Bacterial strains | Characteristics | Reference |
|---|---|---|
| ***Escherichia coli*** | | |
| S17.1λ*pir* | Conjugative strain for suicide plasmids | (1) |
| ***P. aeruginosa*** | | |
| PAO1 (ATCC15692) | Wild type strain, colistin-sensitive | American type culture collection |
| PAO1 Δ*pqsR* | PAO1-derived strain carrying an in frame clear deletion of the *pqsR* gene | This study |
| PAO1 $col^R1$ | PAO1 colistin-resistant strain obtained from *in vitro* evolution experiments | (2) |
| KK27 $col^R7$ | KK27 colistin-resistant strain obtained from *in vitro* evolution experiments | (3) |
| BG98 | MDR cystic fibrosis isolate, colistin-resistant | (4) |
| ***K. pneumoniae*** | | |
| KP-MO-5 | Colistin-resistant clinical isolate | (5) |
| KP-MO-6 | Colistin-resistant clinical isolate | (5) |
| KP-MO-25 | Colistin-resistant clinical isolate | (5) |
| KP-MO-27 | Colistin-sensitive clinical isolate | (5) |
| ***A. baumannii*** | | |
| ATCC19606 | Wild type strain, colistin-sensitive | (6) |
| Ab249 | Colistin-resistant clinical isolate | (7) |
| Ab347 | Colistin-resistant clinical isolate | (7) |
| Ab4452 | Colistin-resistant clinical isolate | (8) |

## MATERIALS AND METHODS

**MIC assays.** MIC of colistin and clofoctol was determined with the standard broth microdilution method using Mueller-Hinton cation-adjusted broth (MH II), according to the Clinical and Laboratory Standards Institute (9). MIC was visually defined as the lowest concentration of antibiotic able to inhibit bacterial growth after 20 h incubation at 37°C under static conditions and interpreted accordingly to the EUCAST clinical breakpoints. All strains were tested in at least three independent experiments.

**Checkerboard assays.** Broth microdilution method was performed according to clinical laboratory standards institute (9). Briefly, colistin was 2-fold diluted in MH II along the abscissa (x-axis) and clofoctol was 2-fold diluted in MH II along the ordinate (y-axis) to test all possible combinations of the two compounds. 100 µL of a bacterial suspension containing about $5x10^5$ cells of the tested strain was added to each well. After 20 h of incubation in static conditions at 37°C, the MIC was defined as the lowest concentration of the antibiotic-clofoctol combination showing no visible growth of bacteria. All strains were tested in at least three independent experiments.

**MBC assays.** The Minimum Bactericidal Concentration (MBC) was obtained as follows: from the checkerboard assays, after MIC determination aliquots of 10 µL from wells where growth was inhibited were spotted on MH II agar plates and incubated for 18 h at 37°C. MBC was defined as the lowest concentration of an antibiotic required to kill 99.9% (3 $\log_{10}$) of the starting bacterial population, *i.e.*, about $5x10^5$ cells (10). All strains were tested in at least three independent experiments.

**Time-kill assays.** Overnight cultures in MH II were adjusted to about $5x10^5$ cells in the same medium in the presence of colistin alone, clofoctol alone, or a combination of both (details of concentrations used are described in Fig. 1 and Fig. S1). Bacterial cultures were incubated at 37°C with shaking (200 rpm); at different time points (0 h, 1 h, 2 h, 4 h, 24 h) 100-µL aliquots were diluted serially in MH II, plated on MH II agar plates, and incubated for 18 h at room temperature before counting the number of CFU/mL. Strains were tested in at least three independent experiments.

**Generation of the PAO1 Δ*pqsR* deletion mutant.** The strain PAO1 Δ*pqsR* was generated by standard stepwise allelic exchange using the pDM4-derivative plasmid as previously described (11, 12). Briefly, about 500 base pairs (bp) upstream and downstream of the *pqsR* gene were PCR amplified from *P. aeruginosa* PAO1 genome with the following primer pairs: FW*pqsR*UP (5'-TGCTCTAGAACAAAAGACATAGGTTTCGGT-3') and RV*pqsR*UP (5'-CCGGAATTCAATAGGCATCCCTTATTCCTTT-3'), FW*pqsR*DOWN (5'-

CCGGAATTCGCCTGGCAACCGAGCATC-3') and RV*pqsR*DOWN (5'-ACGCGTCGACCCGTTGCCGACGATCCAG-3') (restriction sites are underlined). Resulting amplicons were cloned together in pDM4 and then conjugated from *E. coli* to *P. aeruginosa* PAO1. *P. aeruginosa* clones with a chromosomal insertion of the pDM4-derivative plasmid were selected on LB agar plates supplemented with 375 μg/mL chloramphenicol and 15 μg/mL nalidixic acid. Plasmid excision from the chromosome was subsequently selected on LB agar plates supplemented with 10% (w/v) sucrose. Deletion was checked by PCR analysis.

**References (additional to those included in the main text)**


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