Highlights

- We studied the *in vitro* and *in vivo* activities of mefloquine in combination with colistin against 114 antibiotic-resistant Enterobacterales including strains producing $bla_{NDM,,}$ ESBL or containing *mcr-1* plasmids.
- The combination of mefloquine and colistin showed synergistic activities against the test strains and revived the therapeutic potencies of the drugs *in vivo.*

# Mefloquine enhances the activity of colistin against antibiotic-resistant Enterobacterales *in vitro* and *in vivo* animal studies

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ABSTRACT

Infections caused by carbapenem-resistant Enterobacterales are difficult to treat. Colistin is the last resort drug for the treatment of these infections, but colistin resistance has emerged in animals and humans. This study investigated the *in vitro* efficacy of mefloquine in combination with colistin against 114 antibiotic-resistant Enterobacterales isolates including NDM-1, ESBL and *mcr*-1 containing strains from a broad range of origins. The effect of the mefloquine and colistin combination was examined using chequerboard method, time-kill analysis and a murine peritoneal infection model. The fractional inhibitory concentration index of the combination indicated that synergy was detected for all NDM-1 and *mcr*-1 strains, 87.5% of ESBL *E. coli* and 97.9% of ESBL *K. pneumoniae* strains. Time kill curves demonstrated significant synergistic activity with low concentrations of colistin which were boosted by mefloquine. The combination showed enhanced activity against infection with NDM-1 or *mcr*-1 Enterobacteriaceae in mice at 4 and 6 hours after treatment. The findings suggest the combination of mefloquine and colistin has the potential for rejuvenating the activity of colistin against multidrug resistant Enterobacterales.

**Keywords:** Enterobacterales, colistin, mefloquine, NDM-1, ESBL, *mcr*-1

## 1. Introduction

Antimicrobial resistance (AMR) remains a major cause of mortality worldwide (1). In particular, there is an urgent global threat with an increasing prevalence of multidrug resistant Gram-negative bacteria. Enterobacterales, especially carbapenem-resistant strains such as those produce New Delhi metallo-β-lactamase-1 (NMD-1) (2) is extremely resistant to almost all antibiotics (3). Unfortunately, the development of new chemical single drug entities (SDE) cannot control AMR (4) as resistance develops quickly, within a few years after market release of any SDE. Furthermore, the drug discovery process takes too long and costs too much to provide investors with a return. The result is an alarming rise of carbapenem-resistant Enterobacterales (CRE). An alternative approach to cope with this growing global crisis is to resuscitate existing legacy antibiotics with repurposed antibiotic enhancers.

As the rapid emergence of CRE sweeps the world, many countries have turned to colistin which has become an important treatment option. However, the increased use of colistin has led to colistin resistance in patients with *Klebsiella pneumoniae* carbapenemase - producing strains (5). A recent study has found that about 10% of NDM-1 producing CRE are colistin resistant in the UK (6) and that plasmid-born colistin resistance is present in both animals and humans (7). Therefore, it is crucial to enhance and preserve the effectiveness of colistin against both colistin sensitive and resistant Enterobacterales. Treatment with colistin is associated with both nephrotoxic and neurotoxic side effects. It is likely that these undesirable side-effects might be reduced with a lower dose of colistin, which could be achieved by boosting its efficacy with a combination that includes a non-antibiotic drug for the treatment of multidrug resistant Enterobacterales (8).

Mefloquine is an antimalarial drug used for the prophylaxis of malaria. It is administered once a week due to its long half-life (between 2 and 4 weeks). Mefloquine exhibited bactericidal activities against Gram-positive bacteria and Mycobacterial species (9, 10) and a low activity

against Gram-negative bacteria (11). The combination of mefloquine with antimicrobials has not been tested against Gram-negative bacteria.

Here we performed the first investigation to test the *in vitro* activities of mefloquine in combination with colistin against 114 antibiotic-resistant Enterobacterales including NDM-1 and ESBL producers and *mcr*-1 containing strains. Additionally, the therapeutic effect of the combination was tested using a mouse peritoneal infection model.

## 2. Materials and methods

The bacterial strains used were 114 antibiotic-resistant Enterobacterales including 6 strains harboring the $bla_{NDM}$ plasmid [BAA-2469 (*E. coli*), BAA-2470 (*K. pneumoniae*), BAA-2471 (*E. coli*), BAA-2472 (*K. pneumoniae*) and BAA-2473 (*K. pneumoniae*) and NCTC 13443 (*K. pneumoniae*)], 13 colistin resistant *E. coli* containing *mcr*-1 plasmid, 95 ESBL strains (48 *E. coli*, 47 *K. pneumoniae*) (8). The bacterial isolates were grown in nutrient broth (Oxoid, UK), on tryptone soya agar plates (Fluka, UK) or Chrome agar Orientation plates (BD, UK).

Colistin sulphate and mefloquine were obtained from Sigma, UK. Colistin methanesulfonate (CMS) (Colomycin® injection, Forrest) was used in the mouse study.

The minimum inhibitory concentrations (MIC) of colistin and mefloquine were determined using the broth microdilution method in 96-well micro-titre plates using cation-adjusted Mueller Hinton broth (CA-MHB), in accordance with the Clinical and Laboratory Standards Institute guidelines (12). The drugs were diluted with two-fold serial dilutions in triplicate followed by addition of a standard bacterial suspension of 1-2 × $10^5$ CFU/mL. After 24 hours of incubation at 37°C, the optical density (OD) readings were determined using an absorbance microplate reader (ELx800, BioTek). The $MIC_{50}$ and $MIC_{90}$ values were calculated to investigate the lowest concentrations required to inhibit growth in 50% and 90% of the strains, respectively.

Chequerboard analysis was used to determine the combination effects of mefloquine with colistin. Combinations of the two drugs were prepared using 96 well micro-titre plates with

drug concentrations starting two-fold higher than their MIC values, and were then serially diluted in a two-fold manner. The two drugs were mixed in a 96 well micro-titre plate followed by the addition of a standard bacterial suspension at 1-2 x $10^5$ CFU/mL in CA-MHB. Following 24 hours of incubation at 37°C, the OD readings were determined using the ELx800 absorbance microplate reader (BioTek). The combination effects were determined by calculating the fractional inhibitory concentration index (FICI) of each combination as follows: (MIC of Drug A, tested in combination) / (MIC of Drug A, tested alone) + (MIC of Drug B, tested in combination) / (MIC of Drug B, tested alone). Synergy was defined as a FICI ≤0.5, no interaction was identified with an FICI >0.5 – 4 and antagonism if the FICI was >4 (13).

Time-Kill analysis was performed as following**.** A range of different concentrations of colistin and mefloquine was prepared in a two-fold serial dilution and added alone or in combination with log phase bacterial culture suspension containing 1- 5 x $10^7$ CFU/mL in CA-MHB and incubated at 37°C. Viability expressed as log CFU/mL was determined at 0, 1, 2, 4, 7 and 24 hours of incubation by plating out 100 µL of serial dilutions of the cultures onto tryptone soy agar plates. The colonies on the agar plates were counted using the aCOLyte colony counter (Synbiosis) and was analyzed using the counter's software. Synergistic activity was defined as a ≥3 log10 reduction in CFU counts at 24 h between the combination and its most active single drug, colistin or mefloquine, compared with the starting CFU counts at 0 hour (14).

Female ICR mice (five to six weeks old, body weight 24 - 26 g) were used (Harlan UK Ltd) for the mouse peritoneal infection model (8). The mice were infected intraperitoneally with two hundred microliters of $10^8$ CFU counts of the bacterial strains. After 30 minutes of infection, mefloquine (20 mg/kg) and CMS (20 mg/kg) singly or in combination was given intravenously to the mice. A group of mice was treated with saline as a control. At 0, 2, 4 and 6 hours after treatment, 4 mice in each group were sacrificed and 1 ml sterile PBS was

injected intraperitoneally followed by gently massaging of the abdomen. Peritoneal fluid was sampled aseptically. The fluid was diluted and CFU counts were performed.

The animal husbandry guidelines and all animal experiments were performed according to the Animals Scientific Procedures Act, 1986 (an Act of the Parliament of the United Kingdom 1986 c. 14) (Home Office Project licence Number 70/7077) with approval from St George's, University of London ethics committee.

The significance of differences between experimental groups was determined by Student's t test. P values <0.05 were considered significant.

## 3. Results

### *3.1. In vitro test of mefloquine and colistin combination against 114 antibiotic-resistant Enterobacterales*

The MIC range, $MIC_{50}$ and $MIC_{90}$ for colistin are shown in Table S1. The MIC for mefloquine was between 8 and 128 mg/L with an $MIC_{50}$ and $MIC_{90}$ at 64 and 128 mg/L for the 114 strains tested.

As shown in Table 1. checkerboard analysis showed that the combination of mefloquine with colistin resulted in a FIC index of ≤0.5 against 100% of NDM-1 and *mcr*-1 *E. coli* strains, 87.5% of ESBL *E. coli*, and 97.9% of ESBL *K. pneumoniae* strains. The combined concentrations of both drugs which showed FIC index ≤0.5 for each of the strains tested are shown in Table S2.

Time kill assays were performed for mefloquine in combination with colistin for 6 NDM-1, 2 *mcr*-1 *E. coli*, 2 ESBL *E. coli* and 2 ESBL *K. pneumoniae* which showed an FICI <0.5 for each combination in the checkerboard analysis. 4 different concentrations for both drugs was used for each strain according to the FIC index (≤0.5) and tested singly and in combination. As shown in Figure 1, for BAA2470 (NDM-1 *K. pneumoniae*), colistin at 2 mg/L showed about 2 $\log_{10}$ kill at 7 hours followed by a bacterial regrowth. Colistin at 1 or 0.5 mg/L was not effective with the bacterial growth similar to the control. Similarly, mefloquine at 16 or 8 mg/L

showed no activity against the strain. However, when colistin at 2 mg/L combined with mefloquine at 16 or 8 mg/L and colistin at 1 mg/L combined with mefloquine at 16 mg/L, 6 $log_{10}$ kill (to the level of the limit detection) was seen at 7 hours post treatment (Figure 1A-C). and 99.9% reduction (3 $log_{10}$) of bacteria was achieved at 7 hours post treatment when colistin at 1 mg/L combined with 8 mg/L of mefloquine (Figure 1D); at 4 hours when colistin at 0.5 mg/L combined with 16 mg/L of mefloquine and at 13 hours when colistin at 0.5 mg/L combined with 8 mg/L of mefloquine. No bacterial regrowth was observed at 24 hours of post-treatment. The lowest concentration of mefloquine which synergized with colistin was 4 mg/L (Table S3).

The similar patterns of synergistic activities with the combination were observed against *mcr*-1 colistin resistant *E. coli* (Figure S1).

Significant synergistic activity was also demonstrated against other strains tested and lowest mefloquine concentrations which synergized with colistin are shown in Table S3.

*3.2. Combination activities of mefloquine with colistin in* a murine peritoneal infection model

A dose range study of CMS against NDM-1 or *mcr*-1 strains were performed previously (8). We used 20 mg/kg of CMS for both NDM-1 and *mcr*-1 strain infected mice. For mefloquine, the dosage of 20 mg/kg for intravenous administration was chosen which showed no toxic effect to the mice (15). The drugs were tested singly or in combination against the NDM-1 *K. pneumoniae* BAA2470 and the *mcr*-1 *E. coli* strain Af45.

As shown in Figure 2A, for the strain BAA2470, compared with the untreated control, both colistin and mefloquine showed no activity at 2, 4 and 6 hours after treatment commenced. However, the combination inhibited the bacterial growth at 2 hours, exhibited 1.25 $log_{10}$ bacterial reduction at 4 hours and nearly 3 $log_{10}$ reduction (2.98 $log_{10}$) at 6 hours. The difference in the bacterial numbers between zero and 4 hours ($P < 0.001$, n=4) or zero and 6 hours ($P < 0.00001$, n=4) was significant. For *E. coli* strain Af45 (Figure 2B), colistin at 20 mg/kg and mefloquine at 20 mg/kg showed the same growth pattern as the control. However,

the combination exhibited 1.35 and 3.07 $log_{10}$ reduction of the bacterium at 4 and 6 hours, respectively. The difference of the bacterial numbers between zero and 4 hours or 6 hours was significant ($P < 0.002$ and 0.0002, respectively, n=4).

The same as the untreated control, the animals in the colistin and mefloquine treated groups developed mild clinical signs at 6 hours after treatment. The animals in the colistin and mefloquine combination groups showed normal and heathy behavior. All animals were sacrificed at 6 hours after treatment in adherence to the limitation of adverse effects in the project licence.

## 4. Discussion

Colistin is an old drug which was reintroduced in response to the current crisis of multidrug resistant Gram-negative bacterial infections. However, it can cause nephrotoxicity and neurotoxicity (16). In addition, there is increased evidence that colistin resistance is on the rise worldwide, especially since the discovery of plasmid born colistin resistance (7). The optimal dose of colistin for the effective treatment of patients without serious side-effects is unknown (17). However, it is generally accepted that a reduced dose of colistin is likely to lower the incidence of side-effect.

Here we show that significant synergistic activity is present when colistin is combined with mefloquine against all NDM-1 and *mcr*-1 and majority of ESBL strains. The enhanced activity of colistin that was seen after the addition of mefloquine was confirmed with time kill assays which gave rise to precise measures of bactericidal activities of the combination over time. We showed that after combination with mefloquine, colistin was able to kill 99.9% of the test bacteria at concentrations below the MIC. This is significant because enhancement of colistin by mefloquine will likely reduce the dose of colistin but remain at maximum therapeutic efficacy. This lower dose of colistin should reduce toxicity.

The therapeutic effectiveness of colistin combined with mefloquine was confirmed using a mouse peritoneal infection model. Despite 98% protein binding of mefloquine (18), its serum

concentration after 250 mg dosing in humans was about 1 µg/mL (19). It was also demonstrated that the serum peak concentration of mefloquine was about 2 µg/mL when the drug was given at 11.2 to 16.7 mg/kg (20). A further human pharmacokinetic study reported that the plasma mefloquine Cmax was 3.279 µg/mL after a dose of 200 mg in combination with another antimalarial drug (21). It is crucial that the concentrations of mefloquine achieved in the blood is able to boost the activity of colistin for clinical use. We used CMS instead of colistin sulfate because CMS is used clinically and is less toxic than colistin sulfate in mice (22). As a prodrug with a short half-life, CMS needs to convert to the active form of colistin. The conversion normally delays the activity of the drug (23). It showed that an intravenous dose of 15 and 30 mg/kg of CMS to rats produced a Cmax of colistin at 3.17 and 3.45 mg/L, respectively (24). Here we show that mefloquine or CMS both at 20 mg/kg had no activity against either NDM-1 or *mcr*-1 strains. However, when CMS was combined with mefloquine, improved therapeutic activities were seen in the mouse peritoneal cavity, with significant reduction of CFU counts for both NDM-1 and *mcr*-1 strains at 4 or 6 hours. The reduction of bacterial counts was accompanied by the complete prevention of clinical signs in the animals. However, from the in vitro studies, we showed that the lowest concentrations of mefloquine which boosted the activities of colistin varied amongst the strains but higher than Cmax achieved in humans. Therefore, it is important that human PK/PD studies of both drugs are needed to demonstrate if the concentrations reached in plasma and other body fluids are sufficiently high to show such a synergistic activity between colistin and mefloquine against MDR Enterobacterales.

**Acknowledgments.** We are grateful for financial support from Helperby Therapeutics Group Ltd. We would like to thank Professor Jae-Hoon Song and Professor So Hyun Kim from Asian Network for Surveillance of Resistant Pathogens and Asia Pacific Foundation for Infectious Diseases for kindly providing the *E. coli* and *K. pneumoniae* strains. We are also grateful for kindly providing of the *mcr*-1 *E. coli* strains by Professor Patrice Nordmann from

University of Fribourg and the clinical antibiotic resistant isolates by St George's Hospital, London. We thank the project funding for providing the training opportunities for students in St George's University of London.

## Declarations

**Funding:** We are grateful for financial support from Helperby Therapeutics Group Ltd.

**Competing Interests:** AC is director, chief scientific officer and shareholder of Helperby Therapeutics Ltd. YH is the director of research and shareholder of Helperby Therapeutics Ltd.

**Ethical Approval:** The animal husbandry guidelines and all animal experiments were performed according to the Animals Scientific Procedures Act, 1986 (an Act of the Parliament of the United Kingdom 1986 c. 14) (Home Office Project licence Number 70/7077) with approval from St George's, University of London ethics committee.

**Author's contributions:** All authors contributed to data analysis, drafting or revising the manuscript and gave final approval of the version to be published.

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## Figure legends

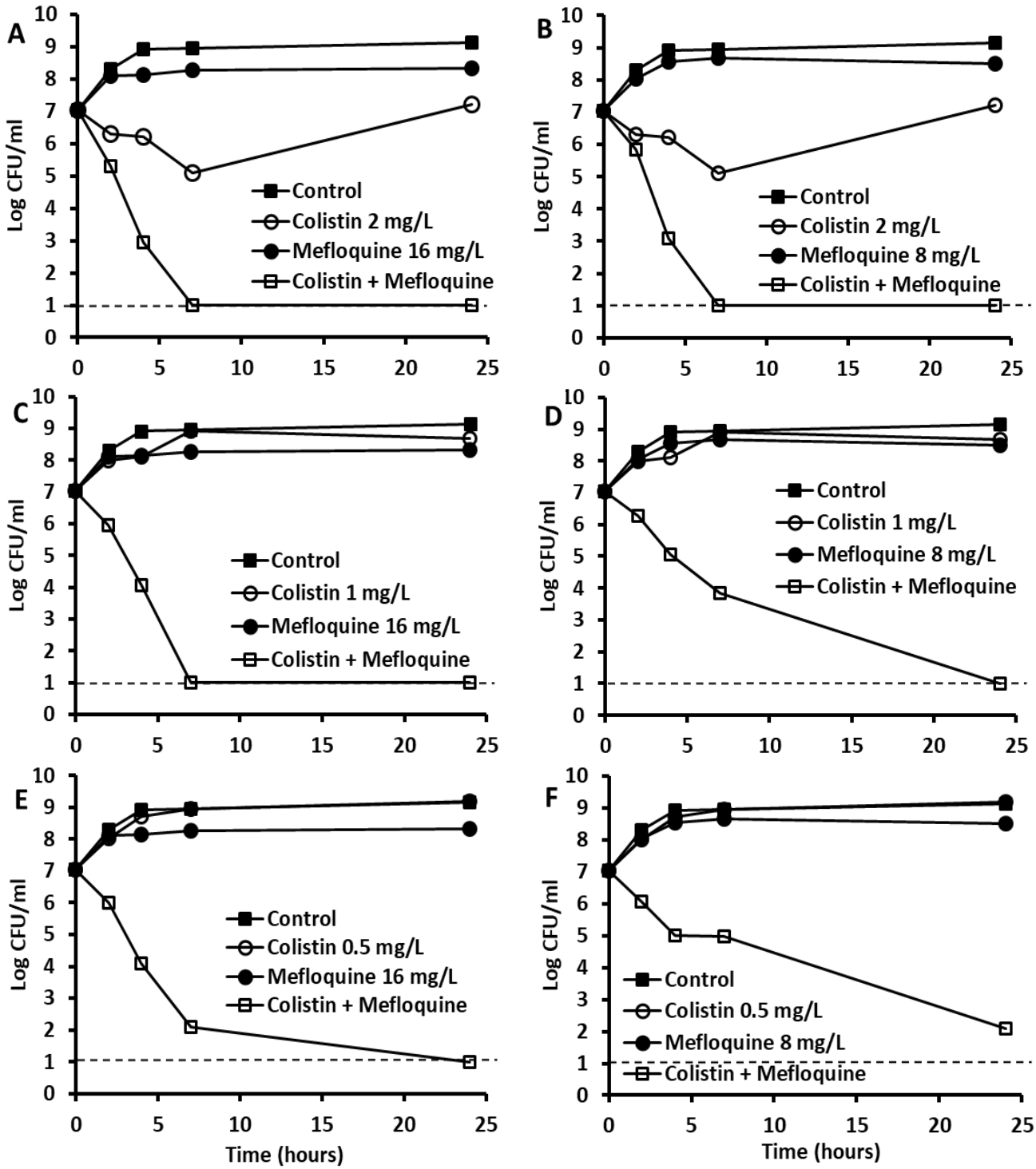
Figure 1. Time Kill analysis showing the effects of mefloquine in combination with colistin against NDM-1 *K. pneumoniae* BAA2470. Mefloquine and colistin alone or in combination were added to the log phase cultures and CFU counts were carried out at 0, 2, 4, 7 and 24 hours. Combination concentrations of colistin 2 mg/L + mefloquine 16 mg/L (A), colistin 2 mg/L + mefloquine 8 mg/L (B), colistin 1 mg/L + mefloquine 16 mg/L (C), colistin 1 mg/L + mefloquine 8 mg/L (D), colistin 0.5 mg/L + mefloquine 16 mg/L (E) and colistin 0.5 mg/L + mefloquine 8 mg/L (F). The dash line is the limit of detection in the assay (10 CFU/ml).

Figure 2. Effects of mefloquine in combination with colistin against the NDM-1 *K. pneumoniae* BAA2470 (A) and the *mrc*-1 *E. coli* strain Af45 (B) in a mouse peritoneal infection model. Treatment was initiated 30 minutes after infection with mefloquine (20 mg/kg), CMS (20 mg/kg) and mefloquine plus CMS. Bacterial counts in the peritoneal cavity were determined from 4 mice for each group at 0 hour before and 2, 4- and 6-hours post-treatment.

Table 1. Combination effect of mefloquine and colistin against antibiotic resistant strains of Enterobacterales

| | | | Total numbers (%) of strains |
|---|---|---|---|
| Strains | Combination activity | FICI | Mefloquine + colistin |
| NDM-1 Strains | synergy | ≤ 0.5 | 6 (100%) |
| | no interaction | >0.5 <4 | 0 |
| | antagonism | >4 | 0 |
| *mcr*-1 *E. coli* | synergy | ≤ 0.5 | 13 (100%) |
| | no interaction | >0.5 <4 | 0 |
| | antagonism | >4 | 0 |
| ESBL *E. coli* | synergy | ≤ 0.5 | 42 (87.5%) |
| | no interaction | >0.5 <4 | 6 (12.5%) |
| | antagonism | >4 | 0 |
| ESBL *K. Pneumoniae* | synergy | ≤ 0.5 | 46 (97.9%) |
| | no interaction | >0.5 <4 | 1 (2.1%) |
| | antagonism | >4 | 0 |

Figure 1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Colistin 2 mg/L
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Colistin + Mefloquine
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Colistin 0.5 mg/L
Mefloquine 16 mg/L
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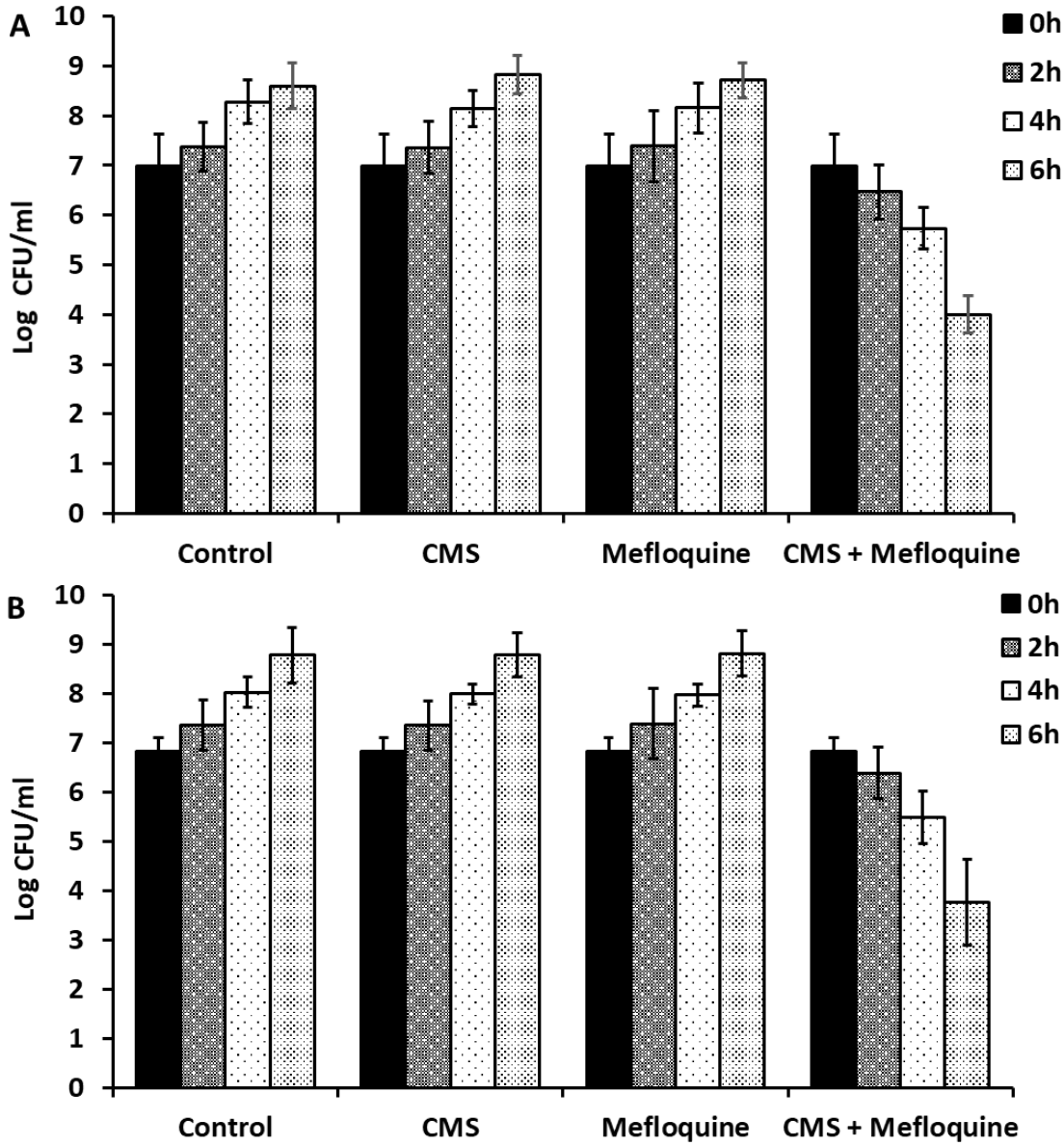
Colistin 0.5 mg/L
Mefloquine 8 mg/L
Colistin + Mefloquine
Time (hours)

Figure 2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