Fosfomycin-meropenem synergistic combination against NDM carbapenemase-producing
*Klebsiella pneumoniae* strains

Maria Teresa Della Rocca¹*, Giovanni Di Caprio²*, Francesca Colucci², Filomena Merola¹, Vittorio Panetta¹, Emanuele Cordua², Rita Greco¹

¹UOSD Microbiology - AORN Sant’Anna and San Sebastiano, Caserta, Italy; ²Infectious and Tropical Diseases Clinic, AORN Sant’Anna and San Sebastiano, Caserta, Italy; *Share the first authorship

**SUMMARY**

Carbapenemase-producing Enterobacteriaceae (CPE) are an increasing threat to global public health. Treatment of CPE isolates, like New Delhi metallo-β-lactamase (NDM), is limited and often necessitates combination therapies. The aim of this study was to evaluate the synergistic meropenem/fosfomycin combination against *K.pneumoniae*-producing NDM isolates. Fosfomycin/meropenem, fosfomycin/colistin and meropenem/colistin were tested alone and in combination, using e-test and time-kill assay against 20 clinical carbapenemase-producing *K. pneumonia* (CPKP NDM) isolates collected from September 2022 to December 2022. *K. pneumoniae* strains were resistant to meropenem, ceftazidime/avibactam and ceftolozano/tazobactam, 75% and 80% of isolates were susceptible for cefiderocol and for colistin respectively. Fosfomycin/meropenem combination was synergic in 95% (n=19) strains. Fosfomycin/colistin and colistin/meropenem combination showed only 10% synergistic combination strains. In 16 isolates (80%) indifference action for fosfomycin/colistin and colistin/meropenem was reported. For 0.8% of CpKP NDM isolates colistin/meropenem and fosfomycin/colistin combinations found to be antagonistic. In this study, time kill assay showed combination therapies action versus *K.pneumoniae* metallo-β-lactamase producing (NDM) strains and confirmed the synergistic action of fosfomycin/meropenem combination. *In vitro* synergy testing should be routinely performed in multidrug resistance infections and combo therapies can be used as a possible alternative in targeted patients with the goal of reducing overall antibiotic costs.

**INTRODUCTION**

Antimicrobial resistance (AMR) has been increasing dramatically in recent years and threatens global public health. In 2019 there were estimated 4.95 million deaths associated with AMR, of which 1.7 million were directly attributable to drug resistance (Murray et al., 2022). In Europe, antibiotic-resistant bacteria infections increased from 685,433 cases in 2016 to 801,517 cases in 2020 (EARS-Net, 2021). Since 2015, Italy has had more antibiotic-resistant bacteria than other EU and EEA countries, with a large proportion of the total burden due to carbapenem-resistant or colistin resistant bacteria. It is notable that about one third of deaths due to these infections in the EU and EEA were in Italy (Falcone et al., 2023; Cassini et al., 2019). The COVID-19 pandemic has impacted on multi-drug resistant infections. Patient admissions to hospitals have contributed to and continue to increase the risk of health-care-associated infections and the transmission of multidrug-resistant (MDR) organisms (Greco et al., 2022). In 2017, the World Health Organization published a list of antibiotic-resistant priority pathogens. The most critical group includes Acinetobacter, Pseudomonas and Enterobacteriales (WHO, 2017). In particular, carbapenem-resistant *K. pneumoniae* (CRE) cases reported in Europe increased 31% in 2020 and 20% in 2021. In Italy, 2,225 CRE bacteremia cases were documented in 2020, almost all caused by *K.pneumoniae* (96.7%) (Iacchini et al., 2020). CRE resistance is commonly mediated by carbapenemase enzymes production: carbapenemase-producing *Enterobacteriales* (CPE) (Queenan et al., 2007). The most commonly detected belong to three classes of β-lactamases defined by the Ambler classification system: Class A (Klebsiella pneumoniae carbapenemases,
KPC), Class B (metallo-β-lactamases, MBL, including New Delhi metallo-β-lactamase (NDM), imipenemase (IMP), Verona-integron encoded metallo-β-lactamase (VIM)), and Class D (OXA-48-like carbapenemases) (Pitou et al., 2015).

Although there are new and effective antibiotics against KPC-producing bacteria, therapeutic options currently available for NDM-producing microorganisms are still limited. The aztreonam and ceftazidime-avibactam combination has been given with promising responses to patients with severe infections (Falcone et al., 2021; Alghoribi et al., 2021; Mauri et al., 2021); however, aztreonam is not available in Italy and there have been shortages in supplies. Cefiderocol, a siderophore cephalosporin recently approved and targeted for activity against carbapenem and Multi Drug Resistance (MDR) Gram-negative species, is active in vitro against >90% of Enterobacterales isolates, including MBL. (Jacobs et al., 2018). However, cefiderocol exhibited reduced activity against NDM-producing isolates compared to other carbapenemase (Yamano et al., 2019). Furthermore, a reduction in the supply of cefiderocol has been reported in Italy since September 2022, due to an unexpected increase in demand resulting from large fluctuations in the occurrence of MDR Gram-negative infections. This situation has prompted a search for synergistic combinations against carbapenemase-producing K. pneumoniae (CPKp) infections until new antibiotics are developed.

From September 2022 to November 2022, a K. pneumoniae-producing NDM outbreak occurred in our Intensive Care Unit (ICU). This situation prompted a search for synergistic combinations against CPKp infections until new antibiotics are developed. The presumed source was a Pakistani patient, in which NDM was isolated for the first time on rectal swab in September 2022. After this date, nine patients tested positive on rectal swabs, of which five remained asymptomatic while four developed a bloodstream infection. The only available therapeutic option was cefiderocol until October, when supply shortages forced the use of rescue therapies. Fosfomycin was reported as having good action against CPKp infections when used in combinations with other agents because of monotherapy lead resistance (Karageorgopoulos et al., 2012). Combo therapies may provide benefits and broadening of the antimicrobial spectrum to combat emerging resistance (Souli et al., 2011). However, there is no consensus on effective combinations against CPKp strains. Several in vitro studies have been performed on KPC-producing strains in synergic combinations, while studies on other carbapenemase-producing organisms are limited. The aim of this study was to evaluate in vitro fosfomycin (FOS) synergistic activity with meropenem (MEM) and colistin (COL) against K. pneumoniae-producing NDM isolated during an ICU outbreak documented in Italy.

**MATERIAL AND METHODS**

**Bacterial strains and antimicrobial susceptibility testing**

This study was conducted at the Microbiology Unit of Sant’Anna and San Sebastiano Hospital in Caserta, Italy. A total of 20 clinical isolates of carbapenemase-producing K. pneumonia (CPKp NDM) were collected from patients admitted to the Intensive Care Unit (ICU) from the second half of September 2022 to December 2022. Isolates identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Biomerieux, Marcy l’Étoile, France) and susceptibility antimicrobial agents were carried out with broth microdilution (Thermo Scientific™ Sensititre™, USA) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023). Fosfomycin susceptibility was evaluated with agar dilution (Liofilchem, Inc, USA). Carbapenemase resistance genes detection was performed with real time PCR methods (Xpert CARBA-R, Cepheid, Sunnyvale, CA, USA).

**Antimicrobial Synergy Testing: e-test assay**

Combinations of fosfomycin-meropenem (FOS-MEM), fosfomycin-colistin (FOS-COL) and meropenem-colistin (MEM-COL) were tested using e-test assay. The interactions between antimicrobial agents were determined by calculating Fractional Inhibitory Concentration Index (FICI), where FICI=FIC A (MIC of drug A in combination/MIC of drug A alone) + FIC B (MIC of drug B in combination/MIC of drug B alone). The FICIs were interpreted as follows: 0.5=synergy; 0.5-1=additivity (partial synergism); 1-4=no interaction (indifference); >4=antagonism (Meletiadis et al., 2021).

**Time-kill assay (TKA)**

Representative CPKp NDM strains isolated from rectal and blood cultures were tested by the time-kill method to confirm synergistic or additive response obtained by e-test assay. Overnight cultures (baseline inoculum of 10^5-10^6 CFU/mL) were performed with MEM and FOS alone as well as in combinations. FOS and MEM, with concentrations (µg/mL) 40 and 10 respectively, were used in all experiments. In vitro activity was assessed at 0, 3, 6, 21, and 24 hours. The bacterial cell counts for growth control, individual antibiotic, and antimicrobial combination were plotted over time to create time-kill curves. In time-kill assay, synergism and antagonism were defined as a 2 log10 CFU/mL decrease and increase, respectively (Foster et al., 2016). A 3-log10 CFU/mL decrease in bacterial counts in antimicrobial combination compared with counts in the growth control indicated an adequate bactericidal response.
**Statistical analysis**

Statistical analysis was conducted with IBM SPSS software (version 22.0; IBM SPSS Inc., New York, NY, USA). Descriptive statistics of the categorical variables are expressed as counts and percentages. P values were calculated using a chi-square test with significance level at ≤0.5.

**RESULTS**

*K. pneumoniae* isolates were all screened for carbapenem resistance genes and all were confirmed CPKp NDM-producing. Among the *K. pneumoniae* NDM isolates, 100% (n=20) were resistant to meropenem (MIC 8-16 mg/L), ceftazidime/avibactam (MIC 8-16 mg/L), and ceftolozano/tazobactam (MIC 16-32 mg/L), antibiotics commonly used for infection with multi-drug-resistant organisms. The majority of isolates 75% (n=15) were susceptible to cefiderocol (2 mg/L) and 80% (n=16) for colistin (0.5 µg/L) (*Table 1*).

Antimicrobial combination on all CPKp NDM strains were reported (*Table 2*). Excellent synergy with fosfomycin/meropenem combination was observed in 95% (n=19) strains. Synergy brought the MEM MIC down to a low MIC 3 mg/L in all tested isolates. In CPKp-NDM with higher initial FOS MIC (32 mg/L), FOS MIC declined to 16 mg/L and 32 mg/L for blood cultures and rectal strains, respectively. Fosfomycin/colistin and colistin/meropenem combination showed only 10% synergistic combination strains. In 16/20 isolates (80%), indifference action for fosfomycin/colistin and colistin/meropenem was reported. For 0.8% of CpKP NDM isolates colistin/meropenem and fosfomycin/colistin combinations found to be antagonistic (FICI >4). Time-kill assays (TKA) were performed for representative isolates. *Figure 1* shows the curves for single antibiotics and combination from CPKp NDM strains. Reduction in log_{10} CFU/...
ml was observed for meropenem/fosfomycin combination (Figure 1). Analyzing the time-kill curves, fosfomycin alone had an exponential logarithmic trend comparable to growth control. Therefore, in combination with meropenem it showed synergistic antibacterial activity, 2 log10 CFU/ml. Bactericidal action was more effective at the concentrations of 32 µg/ml and 8 µg/ml (Figure 1c). Bactericidal activity decreased after 21 hours of dosing for meropenem, which seemed to lose part of its activity. At 24 hours of post re-incubation, a regrowth was observed. The TKA FOS/MEM combination showed a ≥3 log10 decrease in the bacterial population after 6 h. Among combinations, better bactericidal effects were obtained with 32/8 µg/ml, 16/4 µg/ml, 8/2 µg/ml and 4/1 µg/ml (Figure 2 a-d).

**DISCUSSION**

The spread of *K.pneumoniae* carbapenemase-producing infection is a current challenge. The infection control program and antimicrobial stewardship action actuated in the hospital have a role in preventing Pan Drug Resistance strains diffusion (Mills *et al.*, 2021; Carrara *et al.*, 2018). The antimicrobial diagnostic stewardship program coordinated interventions designed to improve and measure the appropriate use of antimicrobials by promoting the selection of the optimal antimicrobial drug regimen, dose, duration of therapy, and route of administration (Foglia *et al.*, 2023). The main objective is reducing hospital-acquired infections, which are a major challenge for high-risk patients, especially in intensive care units (Fursova *et al.*, 2022). Recent studies reported the impact on pandemic emergence on carbapenem-resistant *Enterobacteriaceae* (CRE), extended-spectrum beta-lactamase inhibitor (ESBL)-producing *Enterobacteriaceae*, vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA) and carbapenem-resistant *Pseudomonas aeruginosa* (CRE) (Abubakar *et al.*, 2022). Our epidemiology confirms this trend with CRE incidence of 5.9% in 2020 and 20% in 2021 (Della Rocca *et al.*, 2023). An outbreak of *K. pneumoniae* carbapenemase-producing NDM (CPKp NDM) occurred in the Intensive Care Unit (ICU). During the study (September-December 2022), 20 CPKp NDM were collected from blood culture and rectal swabs. Previously, combination therapy based on carbapenem or colistin in combination with aminoglycosides, tigecycline, and fosfomycin was used as the treatment of last resort. Currently, novel approaches with ceftazidime-avibactam or meropenem-varbocactam alone are used for CRE infection. As a result, few antibiotics are currently active against carbapenem-resistant pathogens. All strains were resistant to meropenem, ceftazidime/avibactam and ceftolozano/tazobactam. These novel antibiotic agents had a favorable in vivo and in vitro action in KPC producing *K.pneumoniae* infections, but are inactive against metallo beta lactamase as CPKp NDM. The updated guidelines recommend ceftazidime-avibactam in combination with aztreonam (Karaiskos *et al.*, 2022). Colistin and ceftiderocol showed 80% and 75% susceptibility, respectively. The diminishing antimicrobial options for MDR gram negative infections is a grave concern, with a need to resort to drugs like fosfomycin, colistin, and tigecycline. AL-Quraini *et al.* reported that patients who received combination therapy survived at the same rate as patients treated by a single drug (AL-Quraini *et al.*, 2022). However, monotherapy colistin was associated with development of resistance and nephrotoxicity (Prasannan *et al.*, 2021; Yap *et al.*, 2022); then ceftiderocol was in supply shortage until October 2022. In this situation, in-vitro antimicrobial syner-
gy tests could be evaluable for management of MDR infections. Meropenem/colistin combinations were commonly prescribed for CRE infections, followed by fosfomycin/meropenem combination (Cebre-ro-Cangueiro et al., 2021). Excellent synergy (95%) of fosfomycin/meropenem combination against CPKp NDM was demonstrated (FICI 0.30). Similar synergy was reported by Flamm et al. (Flamm et al., 2019). Several studies reported double or triple combination therapy with carbapenem, polymyxins and fosfomycin for carbapenem-resistant *K. pneumoniae* (Kole et al., 2022). In particular, there was a synergistic effect of amikacin combination with meropenem (76.2%) and tigecycline (81.8%) against KPC-2 and NDM-1 carbapenemase-producing isolates (Liu et al., 2021). Fosfomycin/colistin and colistin/meropenem showed indifference action in 80% of tested strains in accordance with Bakthavatchalam et al. (Bakthavatchalam et al., 2020). Only 0.8% of isolates were antagonist (>4 FICI) to the colistin/meropenem and fosfomycin/colistin combinations, in contrast with Erdem et al. (Erdem et al., 2020). Time-kill assay confirmed synergistic action against the CPKp NDM strains. Meropenem used alone induced an antimicrobial dose-dependent bactericidal effect. Indeed, the combination of 32/8 µg/ml showed bacteria-killing activity starting from the 3rd to the 21st hour according to the study by Zhou et al. (Zhou et al., 2020). Fosfomycin/meropenem has been investigated as a treatment for complicated infections caused by multidrug-resistant bacteria (Ojdana et al., 2019). Studies have suggested that this combination therapy may be effective in treating these infections, especially when other treatment options have failed (Kulengowski et al., 2018). However, the use of the fosfomycin/meropenem combination should be carefully considered, as it may increase the risk of adverse effects and contribute to the development of antibiotic resistance. The decision to use this combination therapy should be made by an antimicrobial stewardship team member based on patient conditions and bacterial infection. The next phase of this study will focus on the distribution of isolated CPKp NDM resistance gene clusters. In conclusion, despite the treatment protocols used for NDM strains, the fosfomycin/meropenem combination could be effective in cases of ceferocol and aztreonam deficiency/lack of supply. This combo therapy can be used as a possible alternative in targeted patients, resulting in lower antibiotic costs. In *vitro* synergy tests should be routinely performed in

![Figure 2 - CPKp NDM time kill curves for fosfomycin combined with meropenem using different concentrations.](image)
multi-drug-resistance infections. Targeting all medical training in the use of antibiotics should be a strategy to reduce AMR (Di Gennaro et al., 2020). Furthermore, epidemiologic studies and global spread of antibiotic resistance could be the guidelines for new antimicrobial combination therapies.

Acknowledgements

References


European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2023). Breakpoint tables for interpretation of MICs and zone diameters.


