Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Apr 05; 10(B):1308-1314.
https://doi.org/10.3889/oamjms.2022.8934
eISSN: 1857-9655
Category: *B - Clinical Sciences*
Section: *Microbiology*

In vitro Investigation of Antibiotic Combinations against Multi- and Extensively Drug-Resistant *Klebsiella pneumoniae*

Elina Dobreva[1]*, Ivan Ivanov[1], Deyan Donchev[1], Krasimira Ivanova[1], Rumyana Hristova[1], Veselin Dobrinov[1], Stefana Sabtcheva[2], Todor Kantardjiev[1]

[1]National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria; [2]Specialized Hospital of Active Treatment of Oncology (National Oncology Center), Sofia, Bulgaria

Edited by: Slavica Hristomanova-Mitkovska
Citation: Dobreva E, Ivanov I, Donchev D, Ivanova K, Hristova R, Dobrinov V, Sabtcheva S, Kantardjiev T. *In vitro* Investigation of Antibiotic Combinations against Multi- and Extensively Drug-Resistant *Klebsiella pneumoniae*. Open Access Maced J Med Sci. 2022 Apr 05; 10(B):1308-1314. https://doi.org/10.3889/oamjms.2022.8934
Keywords: Antibiotic combinations; Interaction effect; Synergy
***Correspondence:** Elina Dobreva, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria. E-mail: elina_g@abv.bg
Received: 08-Feb-2022
Revised: 23-Mar-2022
Accepted: 24-Mar-2022

Funding: This study was funded by National Science Fund in Competition for financial support for the project of junior basic researchers and postdoc– 2018, contract: КП-06-М23/5, 19.12.2018, "*In vitro* investigations of synergism or antagonism of new antibiotic combinations in multi-, extensive and pan drug-resistant Gram-negative bacteria" and by the European Regional Development Fund through Operational Program Science and Education for Smart Growth 2014–2020, Grant BG05M2OP001-1.002-0001 "Fundamental Translational and Clinical Investigations on Infections and Immunity"
Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: Community and hospital-acquired *Klebsiella pneumoniae* infections have become a ubiquitous medical issue due to the limited treatment options and high mortality rate; therefore, the aims of this study are *in vitro* investigation of double antimicrobial combinations against multidrug-resistant and extensively drug-resistant isolates.

MATERIALS AND METHODS: Antimicrobial interaction effect against 12 *K. pneumoniae* isolates from eight Bulgarian hospitals was determined to nine antimicrobial combinations: meropenem-colistin (MER-COL), MER-fosfomycin (FOS), MER-gentamicin (GEN), MER-rifampicin (RIF), MER-tigecycline (TGC), COL-FOS, COL-GEN, COL-RIF, and COL-TGC through the fractional inhibitory concentration method. The isolates were subjected to genotyping by multi-locus sequence typing and detection of carbapenemase genes by multiplex polymerase chain reaction. The results were assessed by groups of either NDM- or KPC-carbapenemase.

RESULTS: The 12 *K. pneumoniae* producing either KPC-2 (KPC-KP, 41.7%, 5/12) or NDM-1 (NDM-KP, 58.3%, 7/12) was distributed in ST11 (58.3%, 7/12), ST15 (25%, 3/12), and ST258 (16.7%, 2/12). All KPC-KP strains (ST258 and ST15) originated from three hospitals. The rest were NDM-1 carriers isolated from six hospitals and belonged to ST11. The highest synergistic effect was determined for MER-GEN (83.3%, 10/12) and COL-RIF (83.3%, 10/12). The MER-FOS combination was most efficient against NDM-KP, as opposed to the KPC strains. Antagonism was not observed for any combinations.

CONCLUSIONS: The evaluated joint synergistic effect of the MER-GEN and COL-RIF may facilitate the treatment options for patients infected with NDM- and KPC-KP, whereas MER-FOS is highly synergetic against NDM-KP.

Introduction

Klebsiella pneumoniae is an opportunistic Gram-negative pathogen that causes community-acquired and healthcare-associated infections [1]. Life-threatening urinary tract infections (UTIs), pneumonia, surgical-site infections (SSIs), bloodstream infections, especially in critically ill patients, newborns, and immunocompromised individuals are among the most common nosocomial *K. pneumoniae* infections and are related to increased morbidity and prolonged hospital stay [1], [2], [3].

In cases of severe infections with extended-spectrum–β-lactamases, *K. pneumoniae* carbapenems have been regularly used as a treatment option [4], [5], [6]. However, in the past decade, the number of emerging carbapenem-resistant *K. pneumoniae* (CR-KP) has increased significantly as a result of the acquisition of various carbapenemases such as KPC (Class A) and NDM (Class B) [1]. According to the Annual Epidemiological Report for 2019 for antimicrobial resistance (AMR) in the European Union (EU)/EEA (EARS-Net), CR has increased remarkably in Bulgaria as well [7]. The mean percentage of the CR in *K. pneumoniae* averaged 27.0%, whereas in EU/EEA it is sitting at 7.9%. The highest percentage of CR-KP observed in south and south-eastern Europe has also been reflected with data from the second point prevalence survey of healthcare-associated infections and antimicrobial use in acute care hospitals (ECDC, PPS II, 2016-2017) [8].

K. pneumoniae is resistant to most antibiotic classes and has been classified as one of "ESKAPE pathogens" (incl. *Enterococcus faecium, Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [9]. The World Health Organization has also recently listed

K. pneumoniae in the list of 12 bacteria in "ESKAPE group" for which new antibiotics are urgently needed [10]. Highly resistant *K. pneumonia* strains are a significant burden to the healthcare systems and have an important global economic impact. Effects include high mortality and morbidity rates, and recent data on the impact of AMR in the European Union (EU) have shown that >33,000 deaths and 874,541 disability-adjusted life-years [9], [11]. The most recent emergence of extensive-drug resistant (XDR, resistant to all drugs except cefepime, tigecycline (TGC), and ceftazidime-avibactam) and pan-drug resistant (PDR, resistant to all drugs) *K. pneumoniae* is a wake-up call for us to contemplate more strategic measures to control their spread.

Multifactorial dissemination processes through mobile genetic elements play a major role in the spread of multidrug-resistant (MDR) *K. pneumoniae* [12]. Understanding the mechanisms of action of antimicrobial agents and resistance mechanisms is crucial for the development of novel antibiotics in the future. Unfortunately, the availability of novel antimicrobials is limited due to the lengthy process of drug discovery and certification [13]. Ultimately, the increase of AMR has led to the urge in discovering alternative therapeutic plans. At present, some of the treatment options for infections caused by CR-KP include optimization of dosing regimens, various antimicrobial combinations, and application of new drugs such as ceftazidime-avibactam, ceftolozane-tazobactam, meropenem (MER)-vaborbactam, and plazomicin [4], [14], [15].

Antibiotic combinations are often used to treat serious and life-threatening hospital *K. pneumoniae* infections and to prevent the emergence of resistant strains. The selection of antibiotic combination against MDR *K. pneumoniae* should be dependent upon current susceptibility pattern, site of infection, patient clinical status, clinicians' experience, and cost. Of note, a potentially viable combination of two antibiotics that otherwise show non-susceptibility to the current strain could also be used [16].

In addition, the application of new therapeutic regimes and dosage for infections with MDR and XDR *Enterobacteriales* must be further validated. For instance, TGC and colistin (COL) exhibit good antimicrobial activity against resistant isolates [17]. Fosfomycin (FOS) is primarily used for uncomplicated UTIs treatment and is effective against Gram-negative isolates [17]. Rifampicin (RIF) is considered a viable option in combinations due to its ability to penetrate into many tissues and biofilms [18]. Altogether, these drugs may have synergistic effects if combined properly, hence the continuous *in vitro* assays. Finally, antibiotic combinations such as carbapenem-COL, carbapenem-TGC, carbapenem-gentamicin (GEN), carbapenem-FOS, COL-TGC, and COL-RIF have already been reviewed and are recommended for the treatment of CR-KP [19], [20], [21], [22], [23], [24]. The most appropriate antibiotic treatment regimens for the treatment of CP-KP infections are not well defined and some authors demonstrate that polymyxin is being utilized as the common "backbone" antibiotic in combination therapy [25], and is regarded as a key component in combinations against CR-KP infections [26], [27].

The objective of the present study was to investigate the most effective double antimicrobial combinations against MDR and XDR *K. pneumoniae in vitro,* and whether there is a difference between the behavior of KPC- and NDM- producers.

Materials and Methods

The selection of the strains was carried out based on the current prevalence and dominating sequence types in hospital settings for the 5 years between 2014 and 2018 as reported by Markovska *et al*. [26]. In total, 12 KPC-2 and NDM-1 carrying *K. pneumoniae* strains were subjected for *in vitro* investigation of their susceptibility profile against different drug combinations. The isolates were acquired from urine (4/12), blood (3/12), wound (2/12), cerebrospinal fluid (1/12), tracheobronchial aspirate (1/12), and rectal swab (1/12) from eight Bulgarian hospitals within the same period and were sent to the National Reference Laboratory for Control and Monitoring of Antimicrobial Resistance, National Center of Infectious and Parasitic Diseases. Identification was carried out by Matrix-assisted Laser Desorption Ionization-time of flight Mass Spectrometry (Bruker Daltonics Inc., Billerica, MA, United States) with a high confidence score (>2). Antimicrobial susceptibility testing (AST) was performed for MER; TGC; FOS; COL; GEN; and RIF by individually minimal inhibitory concentrations (MIC) gradient strips (0.016–256 mg/L) and results were interpreted according to European Committee on AST (EUCAST, 2021).

Next, nine double antimicrobial combinations MER-COL, MER-FOS, MER-GEN, MER-RIF, MER-TGC, COL-FOS, COL-GEN, COL-RIF, and COL-TGC were selected during the study design. The strains were cultured for 24 h and diluted to starting inoculums of approximately 5×10^8 CFU/ml (0.5 McFarland) and streaked into Muller-Hinton II media. The MIC strips were placed at 90º at the point of intersection using an applicator system (MTS-SAS, Liofilchem) and incubated at 35°C for 18 h. Afterward, the individual MICs of antibiotics were applied in calculating fractional inhibitory concentrations (FICs), according to the following formula displayed in Figure 1 [20], [21]. The obtained interaction effect was defined following the accepted criteria: Synergy (FIC ≤0.5); additive (FIC >0.5 to ≤1.0); indifference (FIC >1 to ≤4.0); and antagonism (FIC > 4) [28], [29], [30], [31] as seen on the tested strains as shown in Figure 2.

Finally, genomic DNA was isolated with PureLink™ Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific, USA). Multiplex polymerase chain reaction was performed to detect carbapenemase genes (*bla*$_{KPC,}$ *bla*$_{VIM,}$ *bla*$_{IMP,}$ *bla*$_{SIM}$, *bla*$_{GIM,}$ *bla*$_{SPM,}$ *bla*$_{NDM-1,}$ bla$_{GES,}$ and *bla*$_{OXA-48}$) using previously described protocol [31]. Multi-locus sequence typing (MLST) was performed according to Protocol 2 described in the *K. pneumoniae* MLST database of Pasteur Institute [32].

Results

AST, carbapenemase detection, and ST

Isolate data and individual MICs are presented in Table 1. Isolates that are resistant to at least three classes of potentially effective antimicrobial agents were considered MDR, whereas those resistant to all except one or two classes were subcategorized as XDR [33]. Six out of the 12 *K. pneumoniae* isolates were determined as MDR, whereas 5/12 were XDR. Only one was resistant to all investigated antimicrobials in this research and therefore determined as PDR. All isolates were resistant to MER (MIC 16 ÷ >128 mg/L). Broth microdilution MICs evaluation was performed for COL in parallel to MIC gradient strips and the results correlated between the two methods (8 ÷ >16 mg/L).

Next, carbapenemase type *bla*$_{KPC}$ was found in 41.7% (5/12) and 58.3% (7/12) were *bla*$_{NDM}$ producers. All KPC producers were susceptible to ceftazidime-avibactam (MIC ≤1 mg/L ÷ 2 mg/L) and TGC (0.5 mg/L); three were susceptible to chloramphenicol (≤ 8 mg/L) and one was susceptible to trimethoprim-sulfamethoxazole (2 mg/L). NDM producers were resistant to ceftazidime-avibactam (10 ÷ >16 mg/L) and trimethoprim-sulfamethoxazole (>4 mg/L); only two were susceptible to chloramphenicol (MIC ≤8 mg/L) (Table 1). Three sequence types were discovered, that is, ST11 (58.3%, 7/12), ST15 (25%, 3/12), and ST258 (16.7%, 2/12). All NDM 1 producers were ST11 and originated from six hospitals (6/8). KPC producers belonged to ST15 (hospital B) and ST258 (hospitals A and D).

FIC index = FIC A + FIC B

Figure 1: Fractional inhibitory concentrations (FIC) index formula. The FIC index is used to define additivity results in no interactions in most combination studies. The effect ranges from 0.5 to 4.0 and is calculated as synergistic (FIC ≤0.5); additive (FIC >0.5 to ≤1.0); indifference (FIC >1 to ≤4.0) and antagonistick (FIC >4) The formula takes into account the MICs of each drug. It is a calculation of FICs A and B, where FIC A is the MIC of drug A in combination with drug B divided by the number of MIC A; and FIC B is the MIC of drug B in combination with drug A divided by MIC B, respectively.

In vitro investigation of double combinations

The results data are shown in Table 2. The highest synergistic interaction effects against NDM-1 producers were scored in the following combinations: MER-FOS (100%, 7/7); MER-GEN (85.7%, 6/7); MER-RIF (85.7%, 6/7); MER-COL (85.7%, 6/7); and COL-RIF (5/7, 71.4%), while COL-RIF (100%, 5/5) and MER-GEN (80%, 4/5) were their counterparts in respect to the KPC producers group.

Other key results are observed within the MER-FOS tests. Notably, FOS helped decrease the MIC of MER significantly when combined against FOS susceptible strains. Interestingly, synergism was found in all 7/7 of the NDM-1 strains and additive in 80% (4/5)

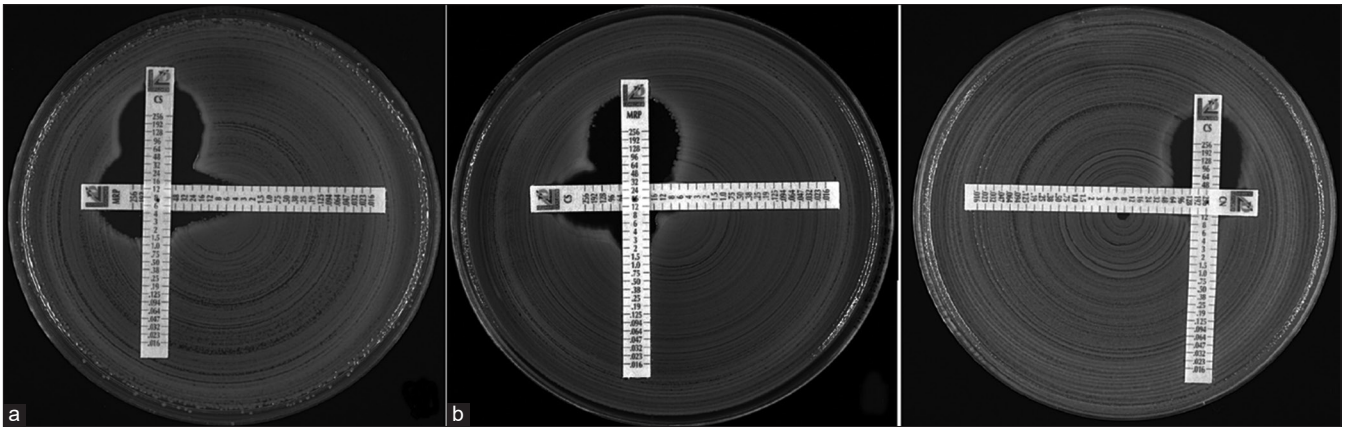

Figure 2: Mueller-Hinton media with minimal inhibitory concentrations (MIC) strips from in vitro experiments of double combination against Klebsiella pneumoniae. (a) Synergistic effect Fractional inhibitory concentrations (FIC) ≤0.5 for meropenem-colistin (MER-COL) (FIC = 0,281) against strain 3467. The MIC of COL was 8 mg/L and 1.5 mg/L, when tested alone and in combination with MER, respectively. The MIC of MER decreased from 64 mg/L to 6.0 mg/L; (b) Additive effect FIC >0.5 ÷ ≤1 for MER-COL (FIC = 0,854) against strain 2761. The MIC of COL when tested alone was 24 mg/L, but when tested in combination with MER was 16 mg/L. The MIC of MER when tested alone was 16 mg/L, but when tested with COL was 3 mg/L; (c) Indifferent effect FIC >1 ÷ ≤ 4 for COL-gentamicin (GEN) (FIC = 1.75) against strain 3473. The MIC of the two antimicrobials alone and in combination remained unchanged (16 mg/L for COL and 256 mg/L for GEN)

Table 1: Molecular characteristics and antibiotic susceptibility profile for *in vitro* combinations against multidrug-resistant and extensively drug resistant *Klebsiella pneumoniae* isolates

Strain number	Year of isolation	Hospital/ department	Clinical specimen	Antibiotic susceptibility testing (MIC, mg/L)						Susceptible to	Carbapenemase	Sequence type
				COL	MER	GEN	TGC	FOS	RIF			
2671	2013	A/ICU	Wound exudate	>16, R	32, R	1.5, S	0.5, S	≤16, S	32	CZA, GEN, TGC, FOS	KPC-2	ST258
2718	2014	B/ICU	Blood	>16, R	32, R	>256, R	0.5, S	64, R	16	CZA, TGC, CHL	KPC-2	ST15
2761	2014	B/ICU	CSF	>16, R	16, R	>256, R	0.5, S	64, R	12	CZA, TGC, CHL, SXT	KPC-2	ST15
2791	2014	B/Urology	Urine	>16, R	64, R	256, R	0.5, S	64, R	16	CZA, TGC, CHL	KPC-2	ST15
3337	2017	D/Oncology	Urine	>16, R	>128, R	1.5, S	0.5, S	64, R	16	CZA, GEN, TGC	KPC-2	ST258
3412	2017	C/ICU	TA	>16, R	>128, R	1.5, S	0.5, S	64, R	16	GEN, TGC, CHL	NDM-1	ST11
3451	2017	F/Neonatal	Rectal swab	16, R	>128, R	256, R	0.5, S	>64, R	16	TGC	NDM-1	ST11
3467	2018	C/ICU	Urine	8, R	64, R	>256, R	≤0.25, S	64, R	>256	TGC	NDM-1	ST11
3473	2017	H/Neonatal ICU	Blood	16, R	>128, R	>256, R	1, R	>64, R	16	-	NDM-1	ST11
3477	2018	D/ICU	Blood	16, R	32, R	1.5, S	1, R	32, S	48	GEN, FOS	NDM-1	ST11
3529	2018	G/Urology	Urine	>16, R	128, R	>256, R	0.5, S	>64, R	24	TGC, CHL	NDM-1	ST11
3577	2018	I/ICU	Wound exudate	16, R	128, R	2, S	1, R	≤16, S	16	GEN, FOS	NDM-1	ST11

CSF: Cerebrospinal fluid, TA: Tracheobroncheal aspirate, ICU: Intensive care unit, MIC: Minimal inhibitory concentrations (mg/L), CHL: Chloramphenicol, COL: Colistin, CZA: Ceftazidime-avibactam, FOS: Fosfomycin, GEN: Gentamicin, MER: Meropenem, RIF: Rifampicin, SXT: Trimethoprim-sulfamethoxazole, TGC: Tigecycline.

of the KPC strains. In 3/12 FOS susceptible isolates, the corresponding MIC of MER reduced from 128 to 3 mg/L, while a much smaller reduction effect was observed in the other 9/12 isolates. The MER-GEN combination obtained a similar effect. In the cases of GEN susceptible strains, the corresponding MICs of MER reduced between 5 (32 ÷ 6 mg/L) and 32-fold (128 ÷ 4 mg/L), thus resulting in a synergistic effect in 83.33% (10/12) *K. pneumoniae* isolates or 6/7 and 4/5 of both NDM and KPC groups, respectively. Moreover, a high synergy effect between MER-RIF was detected in 85.7% (6/7) NDM-1 strains, but not in the KPC group. Mixed results were yielded in the MER-TGC combination for both KPC and NDM, thus no conclusion could be drawn due to the equal distribution in the three categories (synergistic 33.3%; additive 25.0%; and indifferent 41.7%).

Moreover, COL tested in combination with FOS, GEN, and TGC provided mostly additive and indifferent interaction effects. No significant MIC reduction or carbapenemase-specific effects were seen in any of the three variants. On the contrary, a high level of synergism (83.3%, 10/12) occurred in the group COL-RIF effectively inhibiting the growth of 5/5 KPC strains and 5/7 of the NDM by decreasing the initial COL MIC value from 12 ÷ 24 mg/L below 2 mg/L in 4/12 and to 3 mg/L in 7/12. Finally, no antagonism (defined by an FIC index >4) was noted with any of the nine double antibiotic combinations.

Discussion

Literature reviews on the effectiveness of antimicrobial combinations against carbapenemases-producing *K. pneumoniae* are scarce. In cases with MDR and XDR infections treatment with combinations of two or more antibiotics is often selected [13] due to potential benefits in lowering the sub-MICs and achieving a synergistic effect [35]. Although the *in vitro* activity of most combinations may be scored as synergistic, their *in vivo* effectiveness remains uncertain due to the complexity of the infections such as infection site, pharmacokinetic, and pharmacodynamics characteristics of the drugs [13]. The results from genome sequences and epidemiological data of more than 1700 *K. pneumoniae* samples isolated from patients in 244 hospitals in 32 countries during the European Survey of Carbapenemase-Producing Enterobacteriaceae demonstrate that carbapenemase acquisition is the main cause of CR and 477 of 682 (69.9%) carbapenemase-positive isolates are concentrated in four clonal lineages (11, 15, 101, 258/512) and their derivates [36], [37]. In this study, we focused on evaluating the interaction effect of certain combinations on twelve *K. pneumoniae* nosocomial strains belonging to the most prevalent sequence types in Bulgaria (ST11, ST15, and ST258).

Several studies reported good activity of FOS against CR-KP [17], [18], [38]. Our results showed that by combining MER with FOS the MIC of MER decreased significantly and was able to inhibit the growth of all 7/7 of the NDM-1 strains. However, it had only an additive effect on the KPC strains. Regardless of that, NDM *K. pneumoniae* UTIs could be treated with this combination. As reported by other sources, FOS has *in vitro* activity against COL and TGC resistant *K. pneumoniae* isolates [36]. It is highly recommended, however, to be used in combinations with other agents

Table 2: Results from *in vitro* investigations of nine antimicrobial combinations expressed in percent by groups of KPC and NDM producers

Antibiotic combination	FIC variations	KPC			NDM			Total isolates			
		S	Ad	I	S	Ad	I	S	Ad	I	An
MER-COL	0.13–0.85	20 (1/5)	80 (4/5)	-	85.7 (6/7)	14.3 (1/7)	-	58.3 (7/12)	41.7 (5/12)	-	-
MER-FOS	0.11–1.13	-	80 (4/5)	20 (1/5)	100 (7/7)	-	-	58.3 (7/12)	33.3 (4/12)	8.4 (1/12)	-
MER-GEN	0.19–3.17	80 (4/5)	-	20 (1/5)	85.7 (6/7)	-	14.3 (1/7)	83.3 (10/12)	-	16.7 (2/12)	-
MER-RIF	0.19–0.87	40 (2/5)	60 (3/5)	-	85.7 (6/7)	-	14.3 (1/7)	66.7 (8/12)	25.0 (3/12)	8.3 (1/12)	-
MER-TGC	0.13–2.50	40 (2/5)	40 (2/5)	20 (1/5)	28.6 (2/7)	14.3 (1/7)	57.1 (4/7)	33.3 (4/12)	25.0 (3/12)	41.7 (5/12)	-
COL-FOS	0.31–3.40	-	100 (5/5)	-	14.3 (1/7)	14.3 (2/7)	57.1 (4/7)	8.4 (1/12)	58.3 (7/12)	33.3 (4/12)	-
COL-GEN	0.23–1.75	-	60 (3/5)	40 (2/5)	28.6 (2/7)	28.6 (2/7)	42.9 (3/7)	16.6 (2/12)	41.7 (5/12)	41.7 (5/12)	-
COL-RIF	0.31–0.63	100 (5/5)	-	-	71.4 (5/7)	28.6 (2/7)	-	83.3 (10/12)	16.7 (2/12)	-	-
COL-TGC	0.21–1.16	20 (1/5)	80 (4/5)	-	14.3 (1/7)	71.4 (5/7)	14.3 (1/7)	16.7 (2/12)	75.0 (9/12)	8.3 (1/12)	-

COL: Colistin, FOS: Fosfomycin, GEN: Gentamicin, MER: Meropenem, RIF: Rifampicin, TGC: Tigecycline, FIC: Fractional inhibitory concentration, Ad: Additive, An: Antagonism, I: Indifferent, S: Synergy.

in infections with CR-KP to prevent the emergence of FOS resistance [36] as the mutation rate to FOS resistance is high compared to other drugs.

Beta-lactams and aminoglycosides are traditionally combined in infections with Gram-negative bacteria [35]. The synergistic effect lies in beta-lactam-mediated interference of the cell walls of Gram-negative bacteria, thus facilitating the passage of aminoglycosides into the cytoplasm. As expected, the duo MER-GEN achieved a synergistic effect on most of the strains regardless of KPC or NDM presence. According to numerous sources, the combination is a viable therapeutic alternative option against CR-KP [35]. Furthermore, aminoglycoside monotherapy is effective for UTIs, as they reach high peak concentrations in the kidneys renal cortex [35]. On the other hand, in systemic infections, application as a single drug results in worse clinical outcomes than in combination with β-lactams [35].

The interaction of COL-RIF attained the highest synergistic effect against *K. pneumoniae* isolates producing KPC-2. The joint outcome of the duo is regarded as particularly successful for MDR *A. baumannii* [13], [39]; therefore, we included it within the experiment design. Surprisingly, it occurred as a well synergistic fusion keeping high 0.31–0.63 FIC scores in comparison to other tests. Altogether, in 10/12 isolates the attained high *in vitro* synergy between the two antimicrobials is close to that of MER-GEN. Another issue is the *in vivo* applicability and whether both antimicrobials achieve high concentrations at the target sites of infection.

TGC is one of the few antimicrobial agents that remain *in vitro* susceptible to CR-KP-producing carbapenem-hydrolyzing enzymes [38]. However, the number of TGC resistant *K. pneumoniae* isolates is increasing and empirical therapy may prove unreliable [39]. In the present study, although being highly valuable drugs alone and potentially highly effective combination, the MER-TGC duo did not achieve a significant interaction effect, and only a small part of the strains were synergistically inhibited. This finding strongly corresponds with the recent recommendation from the FDA on the use of alternative drugs to TGC in the case of serious infections involving MDR and XDR *K. pneumoniae* [40]. Nevertheless, retrospective data reports and some cases suggested that TGC has to be administrated as a part of an antimicrobial combination [36], [41].

Polymixins and aminoglycosides are both cationic antibiotics and share common mechanisms of action. Although cases of successful treatment of bacteremia and endocarditis due to KPC *K. pneumoniae* with COL-GEN have been reported, herein we found synergy only in 2/12 of the strains. The rest of the FICs fluctuated between additive and indifferent.

In vitro, synergistic activity against NDM-1 producing isolates was observed in combinations of COL-FOS. Another study demonstrated a synergistic effect between COL or polymixin B with TGC against KPC-producing *K. pneumoniae*, increasing the clinical response and hopefully preventing the development of resistance to the agents [36]. Our tests with COL-FOS on NDM-KP strains seemed to provide mixed results likewise the MER-TGC. Regarding the KPC-KP group, we scored 5/5 additive effect.

Most valuable observations in the current study were scored as a high synergistic effect between FOS-MER in 7/7 NDM-KP, but as an additive in 4/5 KPC-KP, indicating the potential for application against NDM-KP. Next, COL-RIF and MER-GEN both achieved a high level of synergism against 10/12 strains regardless of the type of the carbapenemase. The combination COL-RIF is synergistic against isolates belonging to ST258 (2/2) and ST15 (3/3), whereas MER-GEN is synergistic against ST11 (6/7) and ST 258 (2/2). Interestingly, the detected synergy of MER-RIF was higher in NDM-1 strains but not in the KPC group.

Conclusion

The presented findings highlight key combinations and their corresponding interaction effects on different carbapenemase-producing *K. pneumoniae.* Although they indicate synergistic effects for some combinations against CR-KP and may provide alternative treatment algorithms in CR-KP infections, we acknowledge the small sample size of only 12 strains and the need for further validation. Nevertheless, this study provides valuable directions by narrowing the drug combination choices for when a much larger sample set is to be tested. Another major study limitation constitutes the inapplicability of the COL MIC testing method. Even though, the broth microdilution MIC (reference according to EUCAST) values results corresponded with the gradient strip values, alternative testing such as antibiotic time-kill or checkerboard assays design must be performed for verification. Consequently, there is a need for systematic reviews on the assessment of *in vitro* synergistic effect of drug combinations and their corresponding clinical outcomes.

References

1. Chung PY. The emerging problems of *Klebsiella pneumoniae* infections: Carbapenem resistance and biofilm formation. FEMS Microbiol Lett. 2016;363(20):fnw219. https://doi.org/10.1093/femsle/fnw219
PMid:27664057
2. Bi W, Liu H, Dunstan RA, Li B, Torres VV, Cao J, *et al.* Extensively drug-resistant *Klebsiella pneumoniae* causing nosocomial

bloodstream infections in China: Molecular investigation of antibiotic resistance determinants, Informing therapy, and clinical outcomes. Front Microbiol. 2017;8:1230. https://doi.org/10.3389/fmicb.2017.01230

PMid:28713357

3. Effah CY, Sun T, Liu S, Wu Y. *Klebsiella pneumoniae*: An increasing threat to public health. Ann Clin Microbiol Antimicrob. 2020;19(1):1. https://doi.org/10.1186/s12941-019-0343-8

PMid:31918737

4. Morrill HJ, Pogue JM, Kaye KS, LaPlante KL. Treatment options for carbapenem-resistant *Enterobacteriaceae* infections. Open Forum Infect Dis. 2015;2(2):ofv050. https://doi.org/10.1093/ofid/ofv050

PMid:26125030

5. Jiang W, Yang W, Zhao X, Wang N, Ren H. *Klebsiella pneumoniae* presents antimicrobial drug resistance for β-lactam through the ESBL/PBP signaling pathway. Exp Ther Med. 2020;19(4):2449-56. https://doi.org/10.3892/etm.2020.8498

PMid:32256721

6. Reyes J, Aguilar AC, Caicedo A. Carbapenem-resistant *Klebsiella pneumoniae*: Microbiology key points for clinical practice. Int J Gen Med. 2019;12:437-46. https://doi.org/10.2147/ijgm.s214305

PMid:31819594

7. European Centre for Disease Prevention. Antimicrobial Resistance in the EU/EEA (EARS-Net) Annual Epidemiological Report for 2019. Stockholm: European Centre for Disease Prevention; 2019.

8. European Centre for Disease Prevention. Point Prevalence Survey of Healthcare-associated Infections and Antimicrobial Use in European Acute Care Hospitals, 2016-2017. Stockholm: European Centre for Disease Prevention; 2017.

9. Santajit S, Indrawattana N. Mechanisms of antimicrobial resistance in ESKAPE pathogens. Biomed Res Int 2016;2016:2475067. https://doi.org/10.1155/2016/2475067

PMid:27274985

10. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, *et al.* Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18(3):318-27.

PMid:29276051

11. Flores-Paredes W, Luque N, Albornoz R, Rojas N, Espinoza M, Pons MJ, *et al.* Evolution of antimicrobial resistance levels of ESKAPE microorganisms in a peruvian IV-level hospital. Infect Chemother. 2021;53(3):449-62. https://doi.org/10.3947/ic.2021.0015

PMid:34508324

12. Navon-Venezia S, Kondratyeva K, Carattoli A. *Klebsiella pneumoniae*: A major worldwide source and shuttle for antibiotic resistance. FEMS Microbiol Rev. 2017;41(3):252-75. https://doi.org/10.1093/femsre/fux013

PMid:28521338

13. Zhou C, Wang Q, Jin L, Wang R, Yin Y, Sun S, *et al. In vitro* synergistic activity of antimicrobial combinations against bla_{KPC} and bla_{NDM}-producing *Enterobacterales* with bla_{IMP} or mcr Genes. Front Microbiol. 2020;11:533209. https://doi.org/10.3389/fmicb.2020.533209

PMid:33193122

14. Savov E, Trifonova A, Kovachka K, Kjosseva E, Strateva T. Antimicrobial *in vitro* activities of ceftazidime-avibactam, meropenem-vaborbactam and plazomicin against multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* a pilot Bulgarian study. Infect Dis (Lond). 2019;51(11-12):870-3. https://doi.org/10.1080/23744235.2019.1653491

PMid:31433679

15. Karaiskos I, Lagou S, Pontikis K, Rapti V, Poulakou G. The "old" and the "new" antibiotics for MDR Gram-negative pathogens: For whom, when, and how. Front Public Health. 2019;7:151. https://doi.org/10.3389/fpubh.2019.00151

PMid:31245348

16. Marteva-Proevska Y, Velinov T, Markovska R, Dobrikova D, Pavlov I, Boyanova L, *et al.* Antibiotic combinations with colistin against carbapenem-resistant *Klebsiella pneumoniae in vitro* assessment. J IMAB. 2018;24(4):2258-66. https://doi.org/10.7546/crabs.2021.06.12

17. Falagas ME, Giannopoulou KP, Kokolakis GN, Rafailidis PI. Fosfomycin: Use beyond urinary tract and gastrointestinal infections. Clin Infect Dis. 2008;46(7):1069-77. https://doi.org/10.1086/527442

PMid:18444827

18. Forrest GN, Tamura K. Rifampin combination therapy for nonmycobacterial infections. Clin Microbiol Rev. 2010;23(1):14-34. https://doi.org/10.1128/cmr.00034-09

PMid:20065324

19. Benenson S, Navon-Venezia S, Carmeli Y, Adler A, Strahilevitz J, Moses AE, *et al.* Carbapenem-resistant *Klebsiella pneumoniae* endocarditis in a young adult. Successful treatment with gentamicin and colistin. Int J Infect Dis. 2009;13(5):e295-8. https://doi.org/10.1016/j.ijid.2009.01.006

PMid:19329345

20. Michalopoulos A, Virtzili S, Rafailidis P, Chalevelakis G, Damala M, Falagas ME. Intravenous fosfomycin for the treatment of nosocomial infections caused by carbapenem-resistant *Klebsiella pneumoniae* in critically ill patients: A prospective evaluation. Clin Microbiol Infect. 2010;16(2):184-6. https://doi.org/10.1111/j.1469-0691.2009.02921.x

PMid:19694767

21. Humphries RM, Kelesidis T, Bard JD, Ward KW, Bhattacharya D, Lewinski MA. Successful treatment of pan-resistant *Klebsiella pneumoniae* pneumonia and bacteraemia with a combination of high-dose tigecycline and colistin. J Med Microbiol. 2010;59(Pt 11):1383-6. https://doi.org/10.1099/jmm.0.023010-0

PMid:20688947

22. Dalfino L, Puntillo F, Mosca A, Monno R, Spada ML, Coppolecchia S, *et al.* High-dose, extended-interval colistin administration in critically ill patients: Is this the right dosing strategy? a preliminary study. Clin Infect Dis. 2012;54(12):1720-6. https://doi.org/10.1093/cid/cis286

PMid:22423120

23. Oliva A, Scorzolini L, Cipolla A, Mascellino MT, Cancelli F, Castaldi D, *et al. In vitro* evaluation of different antimicrobial combinations against carbapenemase-producing *Klebsiella pneumoniae*: The activity of the double-carbapenem regimen is related to meropenem MIC value. J Antimicrob Chemother. 2017;72(7):1981-4. https://doi.org/10.1093/jac/dkx084

PMid:28369424

24. Jacobs DM, Safir MC, Huang D, Minhaj F, Parker A, Rao GG. Triple combination antibiotic therapy for carbapenemase-producing *Klebsiella pneumoniae*: A systematic review. Ann Clin Microbiol Antimicrob. 2017;16(1):76. https://doi.org/10.1186/s12941-017-0249-2

PMid:29178957

25. Lee CR, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: Epidemiology, genetic context, treatment options, and detection methods. Front Microbiol. 2016;7:895. https://doi.org/10.3389/fmicb.2016.00895

PMid:27379038

26. Markovska R, Stoeva T, Boyanova L, Stankova P, Schneider I,

Keuleyan E, *et al.* Multicentre investigation of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in Bulgarian hospitals interregional spread of ST11 NDM-1-producing *K. pneumoniae*. Infect Genet Evol. 2019;69:61-7. https://doi.org/10.1016/j.meegid.2019.01.013

PMid:30654179

27. Mathers AJ. Antibiotics in laboratory medicine. Clin Infect Dis. 2015;60(9):1446-7.

28. Sueke H, Kaye SB, Neal T, Hall A, Tuft S, Parry CM. An *in vitro* investigation of synergy or antagonism between antimicrobial combinations against isolates from bacterial keratitis. Invest Ophthalmol Vis Sci. 2010;51(8):4151-5. https://doi.org/10.1167/iovs.09-4839

PMid:20335613

29. Dundar D, Otkun M. *In-vitro* efficacy of synergistic antibiotic combinations in multidrug resistant *Pseudomonas aeruginosa* strains. Yonsei Med J. 2010;51(1):111-6. https://doi.org/10.3349/ymj.2010.51.1.111

PMid:20046523

30. Eliopoulos GM, Eliopoulos CT. Abtibiotic combinations: Should they be tested? Clin Microbiol Rev. 1988;1(2):139-56. https://doi.org/10.1128/cmr.1.2.139

PMid:3069193

31. Todorova B, Sabtcheva S, Ivanov IN, Lesseva M, Chalashkanov T, Ioneva M, *et al.* First clinical cases of NDM-1-producing *Klebsiella pneumoniae* from two hospitals in Bulgaria. J Infect Chemother. 2016;22(12):837-40. https://doi.org/10.1016/j.jiac.2016.03.014

PMid:27129373

32. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol. 2005;43(8):4178-82. https://doi.org/10.1128/jcm.43.8.4178-4182.2005

PMid:16081970

33. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, *et al.* Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268-81. https://doi.org/10.1111/j.1469-0691.2011.03570.x

PMid:21793988

34. Tamma PD, Cosgrove SE, Maragakis LL. Combination therapy for treatment of infections with gram-negative bacteria. Clin Microbiol Rev. 2012;25(3):450-70.

PMid:22763634

35. David S, Reuter S, Harris SR, Glasner C, Feltwell T, Argimon S, *et al.* Epidemic of carbapenem-resistant *Klebsiella pneumoniae* in Europe is driven by nosocomial spread. Nat Microbiol. 2019;4(11):1919-29. https://doi.org/10.1038/s41564-019-0492-8

PMid:31358985

36. Falagas ME, Kastoris AC, Kapaskelis AM, Karageorgopoulos DE. Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum β-lactamase producing, *Enterobacteriaceae* infections: A systematic review. Lancet Infect Dis. 2010;10(1):43-50. https://doi.org/10.1016/s1473-3099(09)70325-1

PMid:20129148

37. Bassetti M, Repetto E, Righi E, Boni S, Diverio M, Molinari MP, *et al.* Colistin and rifampicin in the treatment of multidrug-resistant *Acinetobacter baumannii* infections. J Antimicrob Chemother. 2008;61(2):417-20. https://doi.org/10.1093/jac/dkm509

PMid:18174197

38. Food and Drug Administration. FDA Drug Safety Communication: Increased Risk of Death with Tygacil (Tigecycline) Compared to other Antibiotics Used to Treat Similar Infections. Silver Spring, Maryland: Food and Drug Administration; 2010.

39. De Pascale G, Montini L, Pennisi MA, Bernini V, Maviglia R, Bello G, *et al.* High dose tigecycline in critically ill patients with severe infections due to multidrug-resistant bacteria. Crit Care. 2014;18(3):R90. https://doi.org/10.1186/cc13858

PMid:24887101

40. Berçot B, Poirel L, Dortet L, Nordmann P. *In vitro* evaluation of antibiotic synergy for NDM-1-producing *Enterobacteriaceae*. J Antimicrob Chemother. 2011;66(10):2295-7. https://doi.org/10.1093/jac/dkr296

PMid:21807739

41. Elemam A, Rahimian J, Doymaz M. *In vitro* evaluation of antibiotic synergy for polymyxin B-resistant carbapenemase-producing *Klebsiella pneumoniae*. J Clin Microbiol. 2010;48(10):3558-62. https://doi.org/10.1128/jcm.01106-10

PMid:20686085