

# Evaluation of Double- and Triple-Antibiotic Combinations for VIM- and NDM-Producing *Klebsiella pneumoniae* by *In Vitro* Time-Kill Experiments

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Combination therapy is recommended for infections with carbapenemase-producing *Klebsiella pneumoniae*. However, limited data exist on which antibiotic combinations are the most effective. The aim of this study was to find effective antibiotic combinations against metallo-beta-lactamase-producing *K. pneumoniae* (MBL-KP). Two VIM- and two NDM-producing *K. pneumoniae* strains, all susceptible to colistin, were exposed to antibiotics at clinically relevant static concentrations during 24-h time-kill experiments. Double- and triple-antibiotic combinations of aztreonam, ciprofloxacin, colistin, daptomycin, fosfomycin, meropenem, rifampin, telavancin, tigecycline, and vancomycin were used. Synergy was defined as a  $\geq 2 \log_{10}$  decrease in CFU/ml between the combination and its most active drug after 24 h, and bactericidal effect was defined as a  $\geq 3 \log_{10}$  decrease in CFU/ml after 24 h compared with the starting inoculum. Synergistic or bactericidal activity was demonstrated for aztreonam, fosfomycin, meropenem, and rifampin in double-antibiotic combinations with colistin and also for aztreonam, fosfomycin, and rifampin in triple-antibiotic combinations with meropenem and colistin. Overall, the combination of rifampin-meropenem-colistin was the most effective regimen, demonstrating synergistic and bactericidal effects against all four strains. Meropenem-colistin, meropenem-fosfomycin, and tigecycline-colistin combinations were not bactericidal against the strains used. The findings of this and other studies indicate that there is great potential of antibiotic combinations against carbapenemase-producing *K. pneumoniae*. However, our results deviate to some extent from those of previous studies, which might be because most studies to date have included KPC-producing rather than MBL-producing strains. More studies addressing MBL-KP are needed.

Carbapenem resistance in *Enterobacteriaceae* has increased globally during the past decade, particularly in *Klebsiella pneumoniae*, and is typically caused by carbapenemase production. However, there have been marked geographic differences in the epidemiology of carbapenemases. Serine KPC-type carbapenemases are predominant in the United States and frequently reported in Southern Europe (1). OXA-48, which was first detected in Turkey, has increased particularly in Europe, the Mediterranean, and North Africa (1, 2). VIM-type metallo-beta-lactamases (MBLs) are endemic in some European countries, whereas IMP-type MBLs are predominant in Asia (3–5). In recent years, *Enterobacteriaceae* producing NDM-1-type MBLs have disseminated rapidly on the Indian subcontinent (1).

Carbapenemase-producing *K. pneumoniae* strains are of great clinical concern because of frequent co-resistance to multiple antibiotic classes (6, 7). Clinical studies have concluded that combination antibiotic therapy is associated with a better outcome than monotherapy for the treatment of severe infections with these strains, even if the isolated bacteria are susceptible *in vitro* to the individual drugs (8–10). However, inadequate data exist on which antibiotic combinations are the most effective. To date, recommendations are based on a few retrospective clinical studies and *in vitro* studies with time-kill or checkerboard methods. Clinical studies have reported favorable outcomes for patients treated with combinations of colistin and a carbapenem, tigecycline, fosfomycin, or an aminoglycoside (10–12). Retrospective analyses have supported the use of combinations including a carbapenem for infections with KPC-producing *K. pneumoniae* when the carbapenem MIC of the causative bacteria is  $\leq 4$  mg/liter (11). These combinations have been recommended and used in clinical prac-

tice (13). Double- and triple-antibiotic combinations that include colistin, a carbapenem, rifampin, tigecycline, and fosfomycin have proven effective *in vitro* and been proposed for clinical use (14–18).

Notably, most studies have addressed KPC-producing *K. pneumoniae*, and antibiotic combinations considered effective in these studies have not been readily evaluated against MBL-producing strains. Because MBL-producing *K. pneumoniae* are increasing globally, there is an urgent need to evaluate antibiotic regimens for the treatment of infections caused by these strains. In the absence of clinical studies, which are difficult to perform, *in vitro* studies may give some insight into this issue. The aim of the present study was to find antibiotic combinations with synergistic and bactericidal activity against VIM- and NDM-producing *K. pneumoniae* isolates *in vitro*.

## MATERIALS AND METHODS

**Bacteria and media.** Mueller-Hinton II broth (MHBII) and Mueller-Hinton II agar plates were used for all experiments (Becton, Dickinson & Co., Sparks, MD, USA). Clinical isolates of *K. pneumoniae* producing VIM-1 and NDM-1 carbapenemases were obtained from the Department

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**TABLE 1** Antibiotic susceptibilities of VIM- and NDM-producing *K. pneumoniae* strains expressed as MIC values and classification according to the clinical breakpoints defined by EUCAST<sup>a</sup>

Antibiotic	VIM-KP T14789		VIM-KP ÖN-2211		NDM-KP IR8		NDM-KP IR62E	
	MIC (mg/liter)	SIR	MIC (mg/liter)	SIR	MIC (mg/liter)	SIR	MIC (mg/liter)	SIR
Aztreonam	2	I	4	I	>256	R	>256	R
Ciprofloxacin	>32	R	4	R	>32	R	>32	R
Colistin	0.125	S	0.125	S	0.125	S	0.125	S
Daptomycin	>256	–	>256	–	>256	–	>256	–
Fosfomycin	32	S	4	S	256	R	64	R
Meropenem	>32	R	2	S	4	I	>32	R
Rifampin	>32	–	>32	–	32	–	>32	–
Telavancin	256	–	256	–	256	–	256	–
Tigecycline	2	I	1	S	2	I	3	R
Vancomycin	>256	–	>256	–	>256	–	>256	–

<sup>a</sup> SIR, classification according to EUCAST definitions; S, susceptible; I, intermediate; R, resistant; –, not defined.

of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden. The VIM-1-producing *K. pneumoniae* (VIM-KP) strains T14789 and ÖN-2211 were originally isolated in Scandinavia but derived from patients recently transferred from Greek hospitals, while the NDM-1-producing *K. pneumoniae* (NDM-KP) isolates IR8 and IR62E originated from Chennai, India. All strains have been described in previous publications (7, 19).

**Antibiotic susceptibility testing.** MICs were determined in triplicate with the Etest method according to the manufacturer's instructions (bioMérieux, Marcy l'Étoile, France).

**Detection of coproduced beta-lactamases.** The Check-MDR CT101 array (Check-Points, Wageningen, The Netherlands) was used as recommended by the manufacturer to identify the beta-lactamase genes of extended-spectrum beta-lactamases (ESBLs) and plasmid-mediated AmpCs (pAmpCs). Carbapenemase genes had been detected earlier (7, 19).

**Antibiotic concentrations.** Antibiotic concentrations used during time-kill experiments represent mean steady-state concentrations of non-protein-bound drug in humans calculated from literature data (based on the area under the antibiotic concentration-time curve in serum or plasma over 24 h divided by 24 h [AUC<sub>0–24</sub>/24 h]). For rifampin, the mean concentration [(C<sub>max</sub> + C<sub>min</sub>)/2] was used instead due to a lack of other data. The following concentrations were used: 17 mg/liter for aztreonam (20), 1.0 mg/liter for ciprofloxacin (21), 4.0 mg/liter for colistin (22), 1.7 mg/liter for daptomycin (23), 83 mg/liter for fosfomycin (24), 6.8 mg/liter for meropenem (25), 1.7 mg/liter for rifampin (26), 3.3 mg/liter for telavancin (27), 0.1 mg/liter for tigecycline (28), and 9.1 mg/liter for vancomycin (29).

**Time-kill experiments.** A single bacterial colony was added to 2 ml MHBII and grown for 15 to 18 h in a 37°C rocking water bath (150 rpm). A total of 20 µl of the overnight culture was added to 2 ml of prewarmed MHBII, further incubated for 1.5 h, and then inoculated in prewarmed MHBII to obtain a starting inoculum of approximately 5 × 10<sup>6</sup> CFU/ml. Antibiotics were added to the concentrations described above. Samples were taken at 0, 1, 2, 4, 6, and 24 h, serially diluted, spread on plates, and incubated at 37°C. Bacterial colonies were counted after 24 h.

**Experimental design.** The VIM-KP strains were exposed to colistin alone and aztreonam, fosfomycin, meropenem, rifampin, ciprofloxacin, tigecycline, daptomycin, telavancin, and vancomycin alone and in combination with colistin in 24-h time-kill experiments. Based on the synergistic and bactericidal effects detected in these experiments and results from previously published studies, we decided to perform further experiments with double- and triple-antibiotic combinations of aztreonam, fosfomycin, meropenem, rifampin, and colistin. Antibiotics that demonstrated synergistic or bactericidal effects when used in combination with other drugs against the VIM-KP strains were selected for further experiments against the NDM-KP strains. Experiments were performed at least

in triplicate for the VIM-KP strains and at least in duplicate for the NDM-KP strains. In total, we performed more than 200 time-kill experiments with 24 antibiotic regimens, including 14 antibiotic combinations.

**Analysis of time-kill experiments.** The lower limit of bacterial detection was 1.0 log<sub>10</sub> CFU/ml. Bacterial concentrations of <1.0 log<sub>10</sub> CFU/ml were counted as 1.0 log<sub>10</sub> CFU/ml. Synergy was defined as a ≥2 log<sub>10</sub> decrease in CFU/ml between the combination and its most active constituent after 24 h. Bactericidal effect was defined as a ≥3 log<sub>10</sub> decrease in CFU/ml after 24 h compared with the starting inoculum.

## RESULTS

All strains were susceptible to colistin according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints ([www.eucast.org](http://www.eucast.org)), whereas susceptibilities to the other antibiotics varied substantially (Table 1). The following genes encoding ESBLs and pAmpCs were detected: VIM-KP T14789, *bla*<sub>CMY-I</sub> and *bla*<sub>MOX</sub>; VIM-KP ÖN-2211, *bla*<sub>CTX-M-1</sub> group; NDM-KP IR8, *bla*<sub>CTX-M-1</sub> group; NDM-KP IR62E, *bla*<sub>CMY-II</sub>, *bla*<sub>DHA</sub>, and *bla*<sub>CTX-M-1</sub> group.

Colistin, alone and in any combination with other antibiotics, was associated with a >3 log<sub>10</sub> decrease in CFU/ml during the first hour of the experiments. However, considerable regrowth occurred for most colistin combinations and during experiments with colistin alone. Synergistic and bactericidal effects after 24 h were found for combinations that included aztreonam, fosfomycin, meropenem, rifampin, and colistin (Tables 2, 3, 4, 5, and 6; see also Fig. S1 to S4 in the supplemental material).

Aztreonam-colistin and aztreonam-meropenem-colistin were bactericidal against the VIM-KP strains, both of which displayed intermediate susceptibility to aztreonam. These combinations resulted in more rapid killing than aztreonam alone and less regrowth than colistin alone and proved a synergistic effect against VIM-KP ÖN-2211 (Tables 3 and 4; see also Fig. S1 and S2a in the supplemental material). Against the NDM-KP strains, aztreonam-meropenem-colistin demonstrated a synergistic effect despite high-level resistance to aztreonam and reduced susceptibility to meropenem. However, the effect was not bactericidal (Tables 5 and 6; see also Fig. S3 and S4a in the supplemental material). Meropenem-colistin had a synergistic effect against NDM-KP IR62E, but the combination was not bactericidal and showed no synergistic or bactericidal effects against the other strains (Tables 3 to 6; see also Fig. S1 to S4d in the supplemental material).

The combination fosfomycin-colistin demonstrated synergy

**TABLE 2** Compiled results from time-kill experiments with combinations that include meropenem, colistin, aztreonam, fosfomycin, and rifampin against VIM- and NDM-producing *K. pneumoniae*

Antibiotic combination	Result <sup>a</sup>			
	VIM-KP T14789	VIM-KP ÖN-2211	NDM-KP IR8	NDM-KP IR62E
MEM-CST				+
ATM-CST	+			
ATM-MEM				
ATM-MEM-CST		+	+	+
FOF-CST	+		+	+
FOF-MEM				
FOF-MEM-CST	+	+	+	+
RIF-CST			+	+
RIF-MEM-CST	+	+	+	+

<sup>a</sup> +, synergy; MEM, meropenem; CST, colistin; ATM, aztreonam; FOF, fosfomycin; RIF, rifampin. Synergy ( $\geq 2 \log_{10}$  reduction in CFU/ml after 24 h compared with the most effective individual drug) and bactericidal effect ( $\geq 3 \log_{10}$  decrease in CFU/ml after 24 h compared with the starting inoculum) is depicted. Bactericidal effect is highlighted in gray.

and a bactericidal effect against one of the VIM-KP strains and both NDM-KP strains despite that the latter strains were resistant to fosfomycin (Tables 3 to 6; see also Fig. S1 to S4b in the supplemental material). Fosfomycin-meropenem-colistin showed a synergistic effect against all strains and a bactericidal effect against three of the four strains.

Rifampin-colistin demonstrated synergy and a  $>2.5 \log_{10}$  decrease in CFU/ml after 24 h compared with the starting inoculum

against both NDM strains (Tables 5 and 6; see also Fig. S3 and S4c in the supplemental material). The triple-antibiotic combination rifampin-meropenem-colistin resulted in synergistic and bactericidal effects against all strains despite high MICs for rifampin in all strains and despite that two of the strains were highly resistant to meropenem alone (Tables 3 to 6; see also Fig. S1 to S4c in the supplemental material).

The antibacterial activities of ciprofloxacin, tigecycline, daptomycin, telavancin, and vancomycin alone and in combination with colistin against the VIM-KP strains were evaluated in time-kill experiments performed in triplicate (Tables 3 and 4; see also Fig. S1 and S2e in the supplemental material). However, no synergistic or bactericidal activity was detected with these antibiotic regimens, and thus they were not further investigated against the NDM-KP strains.

## DISCUSSION

In this study, we have used 14 antibiotic combinations against four MBL-producing *K. pneumoniae* strains and found synergistic or bactericidal activity for seven of these combinations in time-kill experiments at clinically relevant static antibiotic concentrations.

Our results are in part consistent with those of previous studies. Overall, the combination of rifampin-meropenem-colistin was the most effective regimen, demonstrating synergistic and bactericidal effects against all four strains. A similar triple-antibiotic combination of rifampin-doripenem-colistin has been shown to have a bactericidal effect against KPC-producing *K. pneumoniae* strains in time-kill experiments at antibiotic concentrations of  $0.25 \times \text{MIC}$  (17). Rifampin-colistin was

**TABLE 3** Summary of mean bacterial concentrations at 0, 1, and 24 h during time-kill experiments performed against VIM-producing *K. pneumoniae* strain VIM-KP T14789<sup>a</sup>

Antibiotic(s)	Concn ( $\log_{10}$ CFU/ml) $\pm$ SD at:			$\Delta$ Concn ( $\log_{10}$ CFU/ml) after:	
	0 h	1 h	24 h	1 h	24 h
CST	6.71 $\pm$ 0.19	1.00 $\pm$ 1.10	6.71 $\pm$ 2.05	-5.71	0.00
MEM	6.91 $\pm$ 0.15	7.23 $\pm$ 0.33	9.46 $\pm$ 0.24	0.32	2.55
MEM-CST	6.82 $\pm$ 0.28	1.67 $\pm$ 0.70	7.20 $\pm$ 2.37	-5.15	0.38
ATM	6.71 $\pm$ 0.20	6.36 $\pm$ 0.51	3.35 $\pm$ 1.24	-0.35	-3.36
ATM-CST	6.61 $\pm$ 0.15	1.00 $\pm$ 0.00	2.65 $\pm$ 1.56	-5.61	-3.96
ATM-MEM	6.68 $\pm$ 0.11	5.22 $\pm$ 0.65	4.08 $\pm$ 2.16	-1.46	-2.60
ATM-MEM-CST	6.67 $\pm$ 0.10	1.00 $\pm$ 0.00	3.05 $\pm$ 2.50	-5.67	-3.62
FOF	6.65 $\pm$ 0.09	6.63 $\pm$ 0.22	9.20 $\pm$ 0.16	-0.02	2.56
FOF-CST	6.67 $\pm$ 0.10	1.00 $\pm$ 0.17	2.33 $\pm$ 1.90	-5.67	-4.34
FOF-MEM	6.67 $\pm$ 0.09	6.44 $\pm$ 0.18	8.69 $\pm$ 0.47	-0.24	2.02
FOF-MEM-CST	6.64 $\pm$ 0.17	1.00 $\pm$ 0.00	3.35 $\pm$ 2.13	-5.64	-3.29
RIF	6.80 $\pm$ 0.21	7.35 $\pm$ 0.11	9.47 $\pm$ 0.19	0.55	2.67
RIF-CST	6.92 $\pm$ 0.11	1.70 $\pm$ 1.21	4.75 $\pm$ 2.34	-5.22	-2.17
RIF-MEM-CST	6.52 $\pm$ 0.30	1.00 $\pm$ 0.17	2.14 $\pm$ 1.97	-5.52	-4.38
CIP	6.89 $\pm$ 0.08	7.31 $\pm$ 0.01	9.42 $\pm$ 0.04	0.42	2.53
CIP-CST	6.90 $\pm$ 0.19	2.41 $\pm$ 0.80	7.34 $\pm$ 2.35	-4.49	0.45
TGC	6.81 $\pm$ 0.03	7.42 $\pm$ 0.11	9.19 $\pm$ 0.16	0.61	2.37
TGC-CST	6.90 $\pm$ 0.05	1.40 $\pm$ 0.46	6.35 $\pm$ 0.86	-5.50	-0.55
DAP	6.61 $\pm$ 0.18	7.31 $\pm$ 0.19	9.49 $\pm$ 0.23	0.70	2.88
DAP-CST	6.51 $\pm$ 0.14	1.10 $\pm$ 0.17	9.13 $\pm$ 2.15	-5.41	2.63
TLV	6.65 $\pm$ 0.07	7.05 $\pm$ 0.06	9.36 $\pm$ 0.01	0.40	2.71
TLV-CST	6.56 $\pm$ 0.04	1.00 $\pm$ 0.30	8.47 $\pm$ 1.77	-5.56	1.91
VAN	6.53 $\pm$ 0.13	7.56 $\pm$ 0.51	9.32 $\pm$ 0.14	1.03	2.79
VAN-CST	6.61 $\pm$ 0.18	1.48 $\pm$ 0.83	8.68 $\pm$ 1.11	-5.13	2.07

<sup>a</sup> CST, colistin; MEM, meropenem; ATM, aztreonam; FOF, fosfomycin; RIF, rifampin; CIP, ciprofloxacin; TGC, tigecycline; DAP, daptomycin; TLV, telavancin; VAN, vancomycin. The standard deviation (SD) at each time point and change in bacterial concentrations in  $\log_{10}$  CFU/ml ( $\Delta$ ) at 1 and 24 h compared with the starting inoculum (0 h) is shown. Bactericidal effect ( $\geq 3 \log_{10}$  reduction in CFU/ml after 24 h) is highlighted in gray.

**TABLE 4** Summary of mean bacterial concentrations at 0, 1, and 24 h during time-kill experiments performed against VIM-producing *K. pneumoniae* strain VIM-KP ÖN-2211<sup>a</sup>

Antibiotic(s)	Concn (log <sub>10</sub> CFU/ml) ± SD at:			Δ Concn (log <sub>10</sub> CFU/ml) after:	
	0 h	1 h	24 h	1 h	24 h
CST	6.60 ± 0.17	1.44 ± 0.48	6.91 ± 1.35	-5.16	0.30
MEM	6.55 ± 0.09	5.66 ± 0.35	9.49 ± 0.21	-0.89	2.94
MEM-CST	6.67 ± 0.20	1.00 ± 0.27	6.64 ± 0.64	-5.67	-0.03
ATM	6.61 ± 0.16	6.41 ± 0.27	6.96 ± 3.43	-0.21	0.34
ATM-CST	6.58 ± 0.18	1.00 ± 0.35	1.00 ± 0.00	-5.58	-5.58
ATM-MEM	6.60 ± 0.24	4.61 ± 0.41	7.79 ± 2.89	-1.99	1.19
ATM-MEM-CST	6.65 ± 0.16	1.22 ± 1.06	2.08 ± 1.21	-5.44	-4.58
FOF	6.73 ± 0.14	6.83 ± 0.43	9.15 ± 0.10	0.10	2.41
FOF-CST	6.70 ± 0.03	2.80 ± 1.34	5.91 ± 2.27	-3.90	-0.79
FOF-MEM	6.75 ± 0.17	6.28 ± 0.44	8.91 ± 0.50	-0.47	2.16
FOF-MEM-CST	6.72 ± 0.13	2.26 ± 1.38	3.58 ± 1.81	-4.46	-3.14
RIF	6.48 ± 0.05	7.47 ± 0.25	9.53 ± 0.13	1.00	3.06
RIF-CST	6.61 ± 0.27	1.00 ± 0.00	7.40 ± 0.31	-5.61	0.79
RIF-MEM-CST	6.57 ± 0.08	2.87 ± 1.79	3.16 ± 1.92	-3.70	-3.41
CIP	6.60 ± 0.07	7.39 ± 0.18	9.48 ± 0.11	0.79	2.88
CIP-CST	6.46 ± 0.06	1.20 ± 0.17	6.62 ± 1.18	-5.26	0.16
TGC	6.57 ± 0.04	7.11 ± 0.63	9.14 ± 0.35	0.54	2.57
TGC-CST	6.71 ± 0.10	1.00 ± 0.00	6.73 ± 0.31	-5.71	0.02
DAP	6.76 ± 0.11	7.36 ± 0.24	9.37 ± 0.11	0.61	2.61
DAP-CST	6.53 ± 0.18	1.80 ± 0.77	8.47 ± 1.49	-4.73	1.94
TLV	6.69 ± 0.13	7.36 ± 0.34	9.49 ± 0.16	0.67	2.80
TLV-CST	6.71 ± 0.18	1.89 ± 0.52	7.00 ± 2.08	-4.82	0.29
VAN	6.78 ± 0.20	7.50 ± 0.04	9.53 ± 0.25	0.72	2.75
VAN-CST	6.82 ± 0.08	1.66 ± 0.64	7.93 ± 1.34	-5.16	1.11

<sup>a</sup> CST, colistin; MEM, meropenem; ATM, aztreonam; FOF, fosfomycin; RIF, rifampin; CIP, ciprofloxacin; TGC, tigecycline; DAP, daptomycin; TLV, telavancin; VAN, vancomycin. The standard deviation (SD) at each time point and change in bacterial concentrations in log<sub>10</sub> CFU/ml (Δ) at 1 and 24 h compared with the starting inoculum (0 h) is shown. Bactericidal effect (≥3 log<sub>10</sub> reduction in CFU/ml after 24 h) is highlighted in gray.

synergistic against both NDM-KP strains in our study and has been effective against the majority of KPC-producing *K. pneumoniae* isolates in previous time-kill and checkerboard studies (14, 30). Fosfomycin-colistin, which showed synergistic and bactericidal effects against 3 of 4 strains in our study, has previously demonstrated synergy against 2 of 4 NDM-producing *Klebsiella* spp. in a study with the checkerboard method (15).

Some antibiotic combinations that have been effective against carbapenemase-producing *K. pneumoniae* in previous studies and recommended for clinical use showed no synergistic or bactericidal effects in our experiments. For example, meropenem-colistin was not bactericidal against any of the strains despite susceptibility to colistin in all strains and susceptibility or intermediate susceptibility to meropenem in two of the strains. The combina-

**TABLE 5** Summary of mean bacterial concentrations at 0, 1, and 24 h during time-kill experiments performed against NDM-producing *K. pneumoniae* strain NDM-KP IR8<sup>a</sup>

Antibiotic(s)	Concn (log <sub>10</sub> CFU/ml) ± SD at:			Δ Concn (log <sub>10</sub> CFU/ml) after:	
	0 h	1 h	24 h	1 h	24 h
CST	6.69 ± 0.09	1.87 ± 0.28	8.64 ± 0.42	-4.82	1.95
MEM	6.74 ± 0.12	1.63 ± 0.36	9.26 ± 0.33	-0.78	2.52
MEM-CST	6.83 ± 0.04	1.82 ± 1.16	6.83 ± 0.08	-5.67	0.00
ATM	6.77 ± 0.09	7.40 ± 0.06	9.46 ± 0.01	0.63	2.70
ATM-CST	6.56 ± 0.07	1.63 ± 0.21	8.65 ± 0.06	-4.93	2.09
ATM-MEM	6.56 ± 0.03	5.50 ± 0.05	9.33 ± 0.12	-1.07	2.77
ATM-MEM-CST	6.74 ± 0.19	1.00 ± 0.67	5.69 ± 0.69	-6.06	-1.05
FOF-CST	6.68 ± 0.01	2.06 ± 0.40	3.46 ± 0.37	-4.62	-3.22
FOF-MEM	6.68 ± 0.13	5.90 ± 0.18	9.18 ± 0.25	-0.77	2.51
FOF-MEM-CST	6.75 ± 0.03	2.92 ± 1.36	4.71 ± 0.21	-3.84	-2.05
RIF	6.82 ± 0.08	7.16 ± 0.00	9.44 ± 0.04	0.34	2.62
RIF-CST	6.66 ± 0.08	2.06 ± 1.49	3.87 ± 0.33	-4.61	-2.80
FOF	6.74 ± 0.01	6.79 ± 0.57	9.22 ± 0.27	0.04	2.48
RIF-MEM-CST	6.69 ± 0.04	1.00 ± 0.34	3.41 ± 0.21	-5.69	-3.28

<sup>a</sup> CST, colistin; MEM, meropenem; ATM, aztreonam; FOF, fosfomycin; RIF, rifampin. The standard deviation (SD) at each time point and change in bacterial concentrations in log<sub>10</sub> CFU/ml (Δ) at 1 and 24 h compared with the starting inoculum (0 h) is shown. Bactericidal effect (≥3 log<sub>10</sub> reduction in CFU/ml after 24 h) is highlighted in gray.

**TABLE 6** Summary of mean bacterial concentrations at 0, 1, and 24 h during time-kill experiments performed against NDM-producing *K. pneumoniae* strain NDM-KP IR62E<sup>a</sup>

Antibiotic(s)	Concn (log <sub>10</sub> CFU/ml) ± SD at:			Δ Concn (log <sub>10</sub> CFU/ml) after:	
	0 h	1 h	24 h	1 h	24 h
CST	6.60 ± 0.16	2.67 ± 1.05	9.41 ± 0.02	-3.94	2.81
MEM	6.58 ± 0.08	6.55 ± 0.31	9.47 ± 0.01	-0.03	2.89
MEM-CST	6.63 ± 0.21	1.72 ± 0.33	6.94 ± 3.09	-4.91	0.31
ATM	6.65 ± 0.26	7.15 ± 0.25	9.46 ± 0.16	0.51	2.81
ATM-CST	6.56 ± 0.27	2.07 ± 1.08	9.44 ± 0.02	-4.50	2.88
ATM-MEM	6.49 ± 0.06	6.39 ± 0.24	9.41 ± 0.01	-0.10	2.92
ATM-MEM-CST	6.64 ± 0.22	1.82 ± 0.31	6.76 ± 3.55	-4.82	0.13
FOF	6.96 ± 0.01	5.23 ± 0.09	8.58 ± 0.26	-1.73	1.62
FOF-CST	6.86 ± 0.04	2.26 ± 0.21	3.68 ± 0.83	-4.60	-3.18
FOF-MEM	6.79 ± 0.00	4.58 ± 0.21	9.47 ± 0.06	-2.22	2.68
FOF-MEM-CST	6.85 ± 0.06	1.00 ± 0.49	1.00 ± 0.00	-5.85	-5.85
RIF	6.81 ± 0.30	7.37 ± 0.13	9.68 ± 0.05	0.56	2.87
RIF-CST	6.82 ± 0.17	3.02 ± 0.65	4.16 ± 0.82	-3.80	-2.66
RIF-MEM-CST	6.82 ± 0.06	1.83 ± 0.18	1.00 ± 0.00	-4.99	-5.82

<sup>a</sup> CST, colistin; MEM, meropenem; ATM, aztreonam; FOF, fosfomycin; RIF, rifampin. The standard deviation (SD) at each time point and change in bacterial concentrations in log<sub>10</sub> CFU/ml (Δ) at 1 and 24 h compared to the starting inoculum (0 h) is shown. Bactericidal effect (≥3 log<sub>10</sub> reduction in CFU/ml after 24 h) is highlighted in gray.

tion of tigecycline-colistin has demonstrated a synergistic and bactericidal effect against KPC-producing *K. pneumoniae* in previous studies with checkerboard and time-kill methods but was not effective against the VIM-KP isolates used in this study (14, 18). Further, meropenem-fosfomycin, which demonstrated synergistic and bactericidal effects against the majority of KPC-producing *K. pneumoniae* isolates in a previous time-kill study, was not effective in our experiments (16).

The combination of aztreonam-meropenem-colistin showed a synergistic or bactericidal effect against all strains, despite that the NDM-KP isolates were highly resistant to aztreonam and nonsusceptible to meropenem. One possible explanation for this finding is that aztreonam acts as a competitive MBL inhibitor and meropenem as a competitive inhibitor for other coproduced beta-lactamases (such as ESBLs or AmpCs) that hydrolyze aztreonam but not meropenem. Such competitive inhibitory activities have previously been demonstrated for aztreonam against MBLs and for meropenem against class A, C, and D beta-lactamases (31). The combined inhibitory activities of aztreonam and meropenem were not sufficient to achieve synergy against the MBL-producing *K. pneumoniae* strains used in this study. However, the addition of colistin resulted in synergistic activity, probably because of increased permeability leading to higher concentrations of aztreonam and meropenem in the periplasmic space with this combination (32).

In summary, we have found several double- and triple-antibiotic combinations of aztreonam, fosfomycin, meropenem, rifampin, and colistin with synergistic and bactericidal effects against MBL-producing *K. pneumoniae*. Bacterial regrowth at 24 h occurred for most combinations, but the clinical relevance is uncertain, because the experiments were performed at static antibiotic concentrations *in vitro*, where the effects of the immune system are not taken into account. Our results suggest that rifampin-meropenem-colistin is effective for infections with these strains and that aztreonam-colistin with or without meropenem might be a therapeutic option even when the causative bacteria are not susceptible to aztreonam. More studies are required to confirm the efficacy and evaluate the clinical use of these combinations. Yet

our results are encouraging and indicate that there is an antibacterial potential of antibiotic combinations against MBL-producing *K. pneumoniae*. Effective combinations might include antibiotics to which the bacteria are resistant as well as drugs not usually considered therapeutic options for Gram-negative bacteria. Our results are somewhat inconsistent with those previously reported for carbapenemase-producing *K. pneumoniae*, which might be explained in part by the fact that most studies to date have addressed KPC-producing rather than MBL-producing strains.

Because clinical studies are not easily performed with patients infected with these bacteria, therapeutic decisions will sometimes depend on the results from *in vitro* studies. Therefore, we believe that there is an urgent need for more time-kill studies aimed at finding effective antibiotic combinations for the treatment of MBL-producing *K. pneumoniae*.

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