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Evaluation of antibacterial activities of colistin, rifampicin and meropenem combinations against NDM-1-producing *Klebsiella pneumoniae* in 24 h *in vitro* time–kill experiments

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Objectives: To investigate the activity of colistin alone or in double and triple combination with rifampicin and meropenem against NDM-1-producing *Klebsiella pneumoniae*.

Methods: Eight isolates of NDM-1-producing *K. pneumoniae* were exposed to clinically relevant antibiotic concentrations in 24 h time–kill experiments. Three colistin concentrations were used for two of the strains. Resistance development was assessed with population analysis and sequencing of the *mgrB* and *pmrB* genes.

Results: Initial killing was achieved with colistin alone, but with considerable regrowth at 24 h. Combinations including colistin and rifampicin were bacteriostatic or bactericidal against all strains. Colistin plus meropenem was bactericidal against one strain, but, overall, meropenem showed little additive effects. Higher concentrations of colistin did not enhance antibacterial activity. Resistant populations and deletion or mutations in the *mgrB* and *pmrB* genes were frequently detected in endpoint samples after exposure to colistin alone.

Conclusions: Based on the results of this and previous studies, the combination of colistin and rifampicin seems promising and should be further explored *in vivo* and considered for clinical evaluation. Meropenem seems less useful in the treatment of infections caused by high-level carbapenem-resistant NDM-1-producing *K. pneumoniae*. Higher colistin concentrations did not result in significantly better activity, suggesting that combination therapy might be superior to monotherapy also when colistin is prescribed using high-dose regimens in accordance with current recommendations.

Introduction

The increasing prevalence of carbapenemase-producing Enterobacteriaceae (CPE) is an immediate threat because of limited treatment options and high mortality of infected patients.[1,2] To improve the efficacy of the remaining antibiotics, combination therapy is widely used for infections caused by these bacteria.[3–5] However, the clinical benefits of combination treatment may have been overestimated due to methodological problems and suboptimal dosing in existing retrospective studies.[6,7] In the paucity of clinical data *in vitro* studies can provide additional information. Previous studies have demonstrated synergistic effects of colistin, β-lactams, aminoglycosides, tigecycline, fosfomycin, rifampicin and other antibiotics against CPE.[6] Most studies have addressed KPC- or VIM-producing *Klebsiella pneumoniae* and data for the XDR NDM-1 producers are still scarce.

The aim of this study was to explore the effects of colistin alone and in combination with meropenem and rifampicin against NDM-1-producing *K. pneumoniae*. To assess the potential impact of higher dosages three colistin concentrations were used in experiments with two of the strains. Emergence of resistance was assessed with population analysis and sequencing of the *mgrB* and *pmrB* genes, known to confer colistin resistance in *K. pneumoniae*.

Materials and methods

Bacteria and media

Eight previously described clinical isolates of NDM-1-producing *K. pneumoniae* were used.[8] Mueller–Hinton II broth (MHBII) and Mueller–Hinton II (MHII) agar plates were used for all experiments (Becton Dickinson & Co., USA). MICs were determined with the gradient test method (Etest, bioMérieux, France) and interpreted according to EUCAST definitions.[9] The Check-MDR CT103 array (Check-Points, the Netherlands) was used to identify genes encoding carbapenemases, ESBLs and AmpCs.[10]

Time–kill experiments

A single bacterial colony was added to 2 mL of MHBII and grown for 15–18 h at 37°C in a rocking water bath. Twenty microlitres of the

Table 1. Summary of mean bacterial concentrations at 0, 1 and 24 h during the time–kill experiments performed against eight NDM-1-producing *K. pneumoniae*

	CST concentration (mg/L)	Antibiotics	0 h	SD (0 h)	1 h	SD (1 h)	24 h	SD (24 h)	Δ (1 h)	Δ (24 h)
IR8	1.2	CST	6.70	0.02	3.57	0.85	9.20	0.04	−3.13	2.50
		CST+RIF	6.90	0.09	1.42	0.49	4.38	1.90	−5.48	−2.52
		CST+MEM	6.86	0.10	3.07	0.43	8.92	0.52	−3.79	2.06
		CST+RIF+MEM	6.87	0.10	2.17	1.08	5.33	0.73	−4.70	−1.54
	2.5	CST	6.81	0.04	1.93	0.04	9.16	0.05	−4.88	2.35
		CST+RIF	7.17	0.67	2.33	0.88	4.25	2.86	−4.84	−2.92
		CST+MEM	6.87	0.13	2.11	0.29	9.20	0.15	−4.76	2.33
		CST+RIF+MEM	6.78	0.06	2.13	0.91	4.50	2.26	−4.65	−2.28
	4.2	CST	6.81	0.08	1.55	0.72	9.13	0.13	−5.26	2.32
		CST+RIF	6.95	0.06	1.39	0.55	3.03	2.87	−5.56	−3.92
		CST+MEM	6.87	0.08	2.92	0.06	9.22	0.06	−3.95	2.35
		CST+RIF+MEM	6.86	0.13	2.66	2.34	4.36	1.94	−4.20	−2.50
IR62E	1.2	CST	6.74	0.17	3.10	0.22	9.25	0.15	−3.64	2.51
		CST+RIF	6.80	0.03	3.61	0.86	5.67	0.81	−3.19	−1.13
		CST+MEM	6.80	0.11	2.60	0.98	5.09	0.01	−4.20	−1.71
		CST+RIF+MEM	6.72	0.05	3.28	0.11	3.54	2.21	−3.44	−3.18
	2.5	CST	6.70	0.15	2.69	1.50	9.31	0.14	−4.01	2.61
		CST+RIF	6.81	0.18	3.20	1.66	4.28	1.39	−3.61	−2.53
		CST+MEM	6.83	0.11	2.50	1.73	3.79	2.24	−4.33	−3.04
		CST+RIF+MEM	6.78	0.02	1.54	1.15	3.48	0.09	−5.24	−3.30
	4.2	CST	6.74	0.21	1.88	1.01	9.19	0.43	−4.86	2.45
		CST+RIF	6.97	0.68	1.64	1.49	3.22	1.93	−5.33	−3.75
		CST+MEM	6.70	0.19	2.12	0.15	7.31	3.33	−4.58	0.61
		CST+RIF+MEM	6.67	0.06	1.57	0.78	3.97	1.27	−5.10	−2.70
K1	1.2	CST	7.00	0.13	2.46	0.32	9.13	0.11	−4.54	2.13
		CST+RIF	6.92	0.08	2.40	0.66	4.35	0.44	−4.52	−2.57
		CST+MEM	7.00	0.08	2.72	0.41	9.26	0.11	−4.28	2.26
		CST+RIF+MEM	6.95	0.13	2.26	0.49	1.10	0.17	−4.69	−5.85
K6	1.2	CST	6.93	0.13	3.51	0.67	9.23	0.01	−3.42	2.30
		CST+RIF	6.91	0.03	3.51	0.60	1.00	0.00	−3.40	−5.91
		CST+MEM	6.89	0.12	3.24	0.36	9.37	0.20	−3.65	2.48
		CST+RIF+MEM	6.90	0.13	3.08	0.76	5.43	0.46	−3.82	−1.47
K9	1.2	CST	6.92	0.00	3.37	0.41	9.26	0.01	−3.55	2.34
		CST+RIF	7.04	0.10	2.87	0.68	5.31	0.71	−4.17	−1.73
		CST+MEM	6.97	0.17	3.25	0.31	9.39	0.19	−3.72	2.42
		CST+RIF+MEM	6.98	0.14	2.88	0.47	6.06	0.29	−4.10	−0.92
IR15	1.2	CST	6.89	0.10	2.37	0.40	9.47	0.12	−4.52	2.58
		CST+RIF	6.87	0.03	2.22	0.66	3.77	1.09	−4.65	−3.10
		CST+MEM	6.91	0.05	2.50	1.44	9.34	0.01	−4.41	2.43
		CST+RIF+MEM	6.82	0.22	2.35	0.59	5.52	0.92	−4.47	−1.30
IR18K	1.2	CST	6.80	0.15	3.02	0.14	8.55	0.16	−3.78	1.75
		CST+RIF	6.77	0.09	3.00	0.00	3.83	0.14	−3.77	−2.94
		CST+MEM	6.81	0.15	3.12	0.28	9.13	0.21	−3.69	2.32
		CST+RIF+MEM	6.62	0.21	2.81	0.28	6.22	0.55	−3.81	−0.40

Continued

Table 1. *Continued*

	CST concentration (mg/L)	Antibiotics	0 h	SD (0 h)	1 h	SD (1 h)	24 h	SD (24 h)	Δ (1 h)	Δ (24 h)
IR19K	1.2	CST	6.91	0.14	2.74	0.49	9.37	0.15	−4.17	2.46
		CST+RIF	6.79	0.07	2.34	0.61	3.47	2.14	−4.45	−3.32
		CST+MEM	6.82	0.05	2.55	0.37	9.27	0.09	−4.27	2.45
		CST+RIF+MEM	6.80	0.04	1.56	0.45	3.35	1.87	−5.24	−3.45

CST, colistin; RIF, rifampicin; MEM, meropenem.
Experiments using colistin at 1.2 mg/L, which represents the median non-protein-bound drug concentration in patient plasma at steady state, were performed with all strains. Two higher concentrations that are still achievable in treated patients (2.5 and 4.2 mg/L) were also used against IR8 and IR62E. Meropenem and rifampicin were added to obtain concentrations of 6.8 mg/L and 1.7 mg/L, which correspond to the detected mean plasma concentrations of non-protein-bound substances. All experiments were performed at least in duplicate. The standard deviation (SD) at each time point and change (Δ) in bacterial concentrations in $\log_{10}$ cfu/mL at 1 and 24 h compared with the starting inoculum (0 h) are shown. Bacteriostatic and bactericidal effects (<3 $\log_{10}$ and ≥3 $\log_{10}$ reduction in cfu/mL after 24 h, respectively) are highlighted in light and dark grey, respectively.

overnight culture was added to 2 mL of MHBII, and further pre-cultured for 1.5 h to achieve starting inocula of ~1×10^7 cfu/mL. Antibiotics were added to obtain the following concentrations: 1.2, 2.5 and 4.2 mg/L for colistin;[11] 6.8 mg/L for meropenem;[12] and 1.7 mg/L for rifampicin.[13] Samples were taken at 0, 1, 2, 4, 6 and 24 h, serially diluted, spread on to plates and incubated at 37°C. Colonies were counted after 24 h. Bacterial concentrations <10 cfu/mL were counted as 1.0 $\log_{10}$ cfu/mL, which was the lower limit of detection. A bacteriostatic effect was defined as <3 $\log_{10}$ decrease in cfu/mL after 24 h compared with the starting inoculum and a bactericidal effect as ≥3 $\log_{10}$ decrease. In the comparison of bacterial reduction differences >1 $\log_{10}$ cfu/mL were considered significant.

Resistant subpopulations

Population analysis was performed for IR8 and IR62E before and after exposure to colistin, using plates containing 0, 0.125, 0.25, 0.625, 1.25, 2.5, 5 and 10 mg/L colistin. Resistant endpoint populations growing on 10 mg/L colistin were selected from each experiment, re-streaked on plates containing 10 mg/L colistin and explored with PCR and sequencing. Colonies grown on non-antibiotic-containing plates before the experiments were sequenced for comparison.

PCR was performed as follows: 95°C for 15 min; 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 2 min and 72°C for 7 min. PCR products were purified using exonuclease I and FastAP thermosensitive alkaline phosphatase (Thermo Fisher) and sequenced by Macrogen Inc. (South Korea). The *mgrB* and *pmrB* genes were amplified using primers mgrB_ext_F 5′-AAGGCGTTCATTCTACCACC-3′ and mgrB_ext_R 5′-TTAAGAAGGCCGTGCTATCC-3′[14] and pmrB_ext_F 5′-ACCTACGCGAAAAGATTGGC-3′ and pmrB_ext_R 5′-GATGAGGATAGCGCCCATGC-3.[15] The EEmgrB_ext_F (5′-GGCTATGGCGAGGATAATGAG-3′) and EEmgrB_ext_R (5′-GCTGTGATGTAAGCGTCTGGTG-3′) primers, spanning a larger region of the *mgrB* locus, were used to confirm the deletion of this gene.[16] Sequences were analysed using BioNumerics 7.1 (Applied Maths, Belgium) and compared with sequences submitted to NCBI GenBank using BioEdit version 7.2.5. Amino acid sequences were deduced using BioEdit.

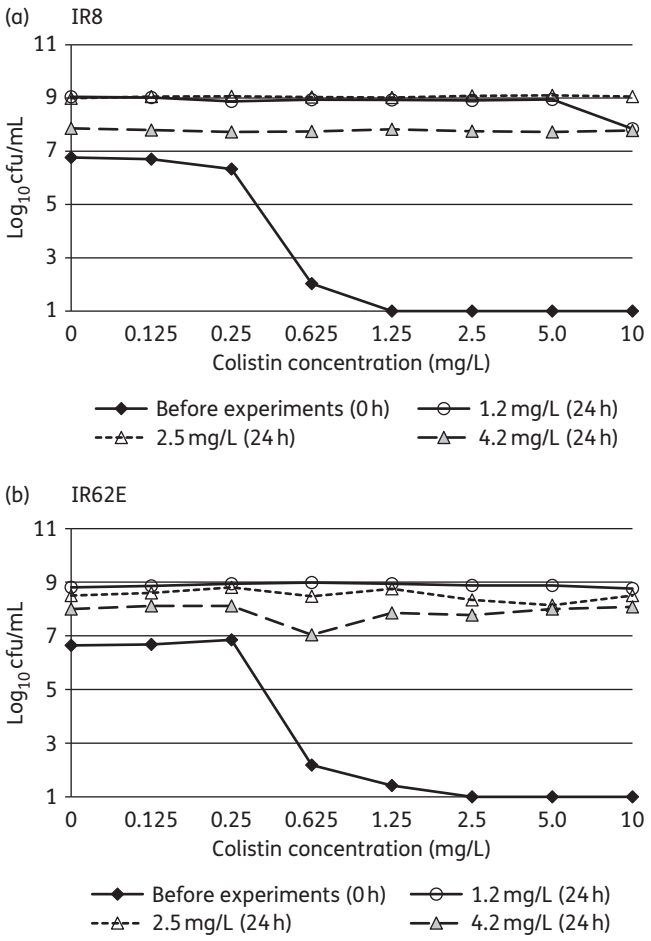

Figure 1. Population analysis of the time–kill experiments with NDM-1-producing *K. pneumoniae* strains IR8 (a) and IR62E (b) exposed to colistin at 1.2, 2.5 or 4.2 mg/L. Samples were taken before (0 h) and after (24 h) the experiments and spread on to plates containing colistin at concentrations 0–10 mg/L for determination of bacterial counts ($\log_{10}$ cfu/mL).

Results

All strains were susceptible to colistin (MICs ≤0.25 mg/L) as shown in Table S1 (available as Supplementary data at *JAC* Online). IR8 was intermediate to meropenem (MIC 4 mg/L), whereas all other strains were resistant (MICs ≥16 mg/L). Rifampicin MICs were 16 mg/L for K6 and IR15 and ≥32 mg/L for the other strains. The following genes encoding ESBLs and pAmpC were detected: $bla_{\text{CTX-M-1 group}}$ (K1, IR8, IR15, IR18, IR19 and IR62E) and $bla_{\text{CMY-II}}$ (IR18, IR19 and IR62E).

Initial >3 $\log_{10}$ reductions in cfu/mL occurred during the first hour of all time–kill experiments (Table 1 and Figures S1 and S2). Marked regrowth was observed with colistin alone, resulting in ~2–2.5 $\log_{10}$ cfu/mL higher bacterial concentrations after 24 h as compared with the starting inocula. Colistin plus rifampicin and the triple combination of colistin, rifampicin and meropenem showed bacteriostatic or bactericidal effects against all strains. Colistin plus meropenem was bacteriostatic (1.2 mg/L colistin) and bactericidal (2.5 mg/L colistin) against IR62E, but showed negligible effects against the other strains.

Higher concentrations of colistin alone (2.5 and 4 mg/L) resulted in enhanced initial killing of IR8 and IR62E, but regrowth was still observed (Table 1 and Figure S1). With higher colistin concentrations, the bacterial reduction at 24 h was improved for some combinations (e.g. colistin plus rifampicin). However, the results were variable and for some combinations, the effects were better when using lower colistin concentrations.

Population analysis showed growth of bacteria on plates containing up to 1.25 mg/L colistin before the experiments with IR8 and IR62E (Figure 1). Highly resistant populations were selected with colistin alone with all concentrations tested and at 24 h there was no significant bacterial reduction on plates containing up to 10 mg/L colistin.

The original DNA sequences of the *mgrB* and *pmrB* genes in IR8 and IR62E were identical between the two strains and WT *K. pneumoniae* isolates.[14,15] Mutated resistant isolates were detected at 24 h in all experiments evaluated with population analysis. For IR8 exposed to 1.2 mg/L colistin, seven nucleotide substitutions were found in the *pmrB* gene. Two of these result in amino acid substitutions: Ala246Thr (G736A) and Gly256Arg (G766C), whereas five are silent mutations. After experiments with 2.5 and 4.2 mg/L colistin, a single point mutation (G116A) resulting in the amino acid substitution Cys39Tyr was detected in the *mgrB* gene. For IR62E, identical nucleotide substitutions as in IR8 were found in the *pmrB* gene of endpoint resistant isolates with all colistin concentrations. Despite repeated testing with EEmgrB_ext_F and EEmgrB_ext_R no amplification products were yielded in experiments using colistin at 2.5 and 4.2 mg/L, indicating that deletion of the *mgrB* gene had occurred in these isolates.[16]

Discussion

In this study, we have evaluated the antibacterial effects of combinations including colistin, rifampicin and meropenem against NDM-1-producing *K. pneumoniae*. Combinations including colistin and rifampicin were bacteriostatic or bactericidal against all strains. Synergistic and bactericidal effects have previously been found with these antibiotics against KPC-producing *K. pneumoniae*.[17] The presumed mechanism of synergy is that colistin facilitates the permeability of rifampicin through the outer bacterial membrane. To our knowledge, clinical evidence to support the use of rifampicin combinations against CPE is still lacking.

Colistin plus meropenem showed bacteriostatic and bactericidal effects against only one strain. This combination is sometimes advocated for the treatment of infections caused by carbapenemase-producing *K. pneumoniae*.[5] Of note, this recommendation is based on results from clinical studies that comprise mostly KPC- or VIM-producing strains and may not be valid for NDM-1 producers. Further, the additive value of meropenem is probably limited against strains exhibiting MICs >8 mg/L,[5] which was the case in seven of the eight NDM-1-producing *K. pneumoniae* tested in this study.

Prompt initial killing followed by regrowth was observed with colistin alone in all experiments, also when using concentrations higher than those usually achieved in patient plasma. Regrowth is well known to occur in susceptible Gram-negative bacteria exposed to colistin presumably caused by selection of pre-existing resistant subpopulations, *de novo* mutations, adaptive resistance or formation of persister cells.[18,19] Overall, bacterial killing was not enhanced with higher colistin concentrations in the combinations.

The population analysis revealed growth of bacteria on colistin at concentrations up to 10×MIC before the experiments and the variability in results between experiments can in part depend on differences in the prevalence of pre-existing resistant subpopulations. There was a substantial shift to more resistant populations during the experiments, up to 80×MIC, at all three colistin concentrations and deletion or mutations in the *mgrB* and *pmrB* genes were frequently detected. Inactivation of the *mgrB* gene causes upregulation of the PhoQ/PhoP signalling system,[20] whereas mutations in *pmrB* can affect the function of the PmrA/PmrB signalling system. In both cases this will eventually result in resistance to colistin by modifications of the lipopolysaccharide target.[14,16]

In conclusion, colistin and rifampicin combinations were active against all NDM-1-producing *K. pneumoniae* used in this study and should be further explored *in vivo* and considered for clinical evaluation. Meropenem had little additive effect and might be less useful in the treatment of infections caused by NDM-1 producers. Higher colistin concentrations did not result in significantly better activity, suggesting that combinations may be more effective than monotherapy, also when using the currently recommended high-dose regimens.

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Transparency declarations

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Supplementary data

Table S1 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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