

In vitro pharmacodynamics of colistin against multidrug-resistant *Klebsiella pneumoniae*

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Background: Resistance to colistin is emerging in multidrug-resistant Gram-negative bacteria and no solid pharmacodynamic data are available for colistin against *Klebsiella pneumoniae*.

Methods: Twenty-one multidrug-resistant clinical *K. pneumoniae* isolates from 16 different clinical sites worldwide were employed. The genetic relatedness of these isolates was examined with PFGE. *In vitro* pharmacodynamic properties of colistin (sulphate) were investigated by studying the MICs, mutation prevention concentrations, time–kill kinetics, population analysis profiles and the post-antibiotic effect (PAE). Time–kill was studied with three clinical isolates plus ATCC 13883 at concentrations ranging from 0.5 to 64× MIC. The PAE was examined after 20 min of exposure of these isolates.

Results: The 22 isolates belonged to 18 different PFGE groups. For susceptible isolates, colistin MICs ranged from 0.125 to 1 mg/L. Six isolates were colistin-resistant with MICs of ≥32 mg/L. Colistin heteroresistance was observed in 15 of 16 isolates considered colistin-susceptible based on MICs. For susceptible isolates, colistin showed extremely rapid killing; however, regrowth was observed as early as 2 h after treatment and substantial regrowth at 24 h even at concentrations up to 64× MIC for some isolates. Colistin exhibited no or very modest PAE against the isolates tested.

Conclusions: The data suggest that monotherapy with colistin methanesulfonate, the parenteral form of colistin, and long dosage intervals may be problematic for the treatment of infections caused by multidrug-resistant *K. pneumoniae*, particularly for colistin-heteroresistant strains. Further investigation on combination therapy of colistin with other antibiotics is warranted.

Keywords: heteroresistance, killing kinetics, post-antibiotic effect, mutation prevention concentration, population analysis profile

Introduction

Klebsiella pneumoniae is emerging as an important nosocomial pathogen due to rapidly increasing resistance to all currently available antibiotics, in particular carbapenems.^{1,2} It is an opportunistic Gram-negative pathogen that may cause wound infection, urinary tract infection and other life-threatening hospital-acquired infections such as pneumonia, bacteraemia and post-operative meningitis.³ Data from the National Nosocomial

Infection Surveillance (NNIS) System (2003 versus 1998–2002)⁴ showed that, in the nine selected antimicrobial-resistant pathogens associated with nosocomial infections in intensive care unit patients, the 47% increase in the prevalence of resistance to third-generation cephalosporins (either ceftriaxone, cefotaxime or ceftazidime) in *K. pneumoniae* was by far the largest change encountered.³ The recent emergence of KPC-type carbapenemases in *K. pneumoniae* resistant to other drug classes serves to highlight the important therapeutic role of polymyxins

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such as colistin.⁵ No new antibiotic classes against multidrug-resistant Gram-negative bacteria are expected to be commercially available within the next several years.^{3,6} Even more worrying, the emergence of resistance to colistin, the only available active antibiotic against multidrug-resistant Gram-negative bacteria,^{7,8} is rapidly emerging in *K. pneumoniae*.^{2,9}

Colistin (also known as polymyxin E) is a multi-component polypeptide antibiotic discovered in the 1950s.¹⁰ Owing to its significant (currently high) activity against Gram-negative 'superbugs', including *K. pneumoniae*, colistin is now being administered as 'salvage' therapy in patients in whom none of the other available antibiotics are active against their isolate.^{7,11} Although aminoglycosides replaced colistin in the 1970s because the former were regarded as 'less' toxic, recent studies indicate that colistin may be less toxic than the aminoglycosides.¹² Because colistin was developed before contemporary drug development and approval procedures were introduced, there are substantial gaps in knowledge of its pharmacokinetics, pharmacodynamics and toxicodynamics.^{7,11,13} The aim of the present study was to examine the *in vitro* pharmacodynamic properties, namely MICs, mutation prevention concentrations (MPCs), population analysis profiles (PAPs), bacterial killing and the post-antibiotic effect (PAE), of colistin against clinical isolates of multidrug-resistant *K. pneumoniae*. Colistin sulphate was employed in the current study because sodium colistin methanesulfonate (CMS), the form of colistin for parenteral administration in humans, is a non-active pro-drug.¹⁴ The study was intended to provide important pharmacodynamic information for future evaluation of pre-clinical and clinical pharmacokinetics/pharmacodynamics of colistin against this problematic pathogen.

Materials and methods

Bacterial strains and antibiotics

Twenty-one *K. pneumoniae* clinical isolates were obtained from patients at the Austin Hospital (Melbourne, Australia), the SENTRY surveillance programme (Asia-Pacific), Teaching Hospital Nitra in the Slovak Republic and Hospital Saint-Joseph in Paris (France). These isolates were from 16 medical centres. *K. pneumoniae* ATCC 13883 (VA, USA) was included as a reference strain. A pair of isolates (16 and 17) was isolated from the same patient before (imipenem-susceptible) and after (imipenem-resistant) imipenem therapy, respectively. All isolates were stored at -80°C and sub-cultured onto horse-blood agar plates (Medium Preparation Unit, University of Melbourne, Australia) before the experiments.

Colistin stock solutions were prepared by dissolving colistin sulphate powder (Lot095K1048, Sigma-Aldrich, Castle Hill, NSW, Australia) in Milli-Q water (Millipore, Australia) and sterilizing by passage through 0.20 μm filters (Sartorius, Australia).

Genotyping

Clonotypes of the 21 clinical isolates and ATCC 13883 were investigated by PFGE at the Microbiology Department, Austin Health (VIC, Australia), using a previously described method (http://www.cdc.gov/pulsenet/protocols/ecoli_salmonella_shigella_protocols.pdf). A PFGE dendrogram was created in GelCompar II (Applied Maths, Belgium) to calculate similarity coefficients and to perform unweighted pair group analysis using arithmetic mean clustering.

Dice coefficient with 0.5% optimization and 1.0% position tolerance was used. The band patterns were interpreted according to the criteria suggested by Tenover *et al.*,¹⁵ and PFGE patterns were designated by letters A to R.

Susceptibility and colistin MIC and MPC measurements

Susceptibility to various antibiotics was examined in the isolates including ATCC 13883 by broth microdilution in Mueller–Hinton broth (Oxoid Australia, West Heidelberg, VIC, Australia), according to the CLSI standards.¹⁶ Multidrug resistance was defined as isolates intermediate or resistant to at least three drugs in the following classes: third-generation cephalosporins, carbapenems, aminoglycosides or fluoroquinolones using CLSI breakpoints.¹⁶ MICs of colistin (sulphate, Sigma-Aldrich) were determined for all the isolates by broth microdilution in cation-adjusted Mueller–Hinton broth (CAMHB).¹⁶ The MIC breakpoint of colistin for *K. pneumoniae* has not been developed by the CLSI or BSAC. In the present study, isolates with MIC ≥ 4 mg/L were regarded as colistin-resistant. This value was chosen according to the CLSI breakpoints for colistin susceptibility in *Acinetobacter baumannii* (susceptible, ≤ 2 mg/L; resistant, ≥ 4 mg/L) and *Pseudomonas aeruginosa* (susceptible, ≤ 2 mg/L; intermediate, 4 mg/L; resistant, ≥ 8 mg/L).¹⁶ MPCs were measured for all the isolates including ATCC 13883 using a previously reported method; tested colistin concentrations were 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 16, 32, 64 and 128 mg/L.¹⁷

PAPs

Analysis of colistin-heteroresistant subpopulations of bacteria by PAPs was conducted for all the isolates plus ATCC 13883 with a previously reported method.¹⁸ Full 24 h cultures ($\sim 10^8$ cfu/mL) were employed. Samples of bacterial cell suspension (50 μL) were spirally plated on nutrient agar plates (Medium Preparation Unit), using a Whitley automatic spiral plater (WASP, Don Whitley Scientific, West Yorkshire, UK). Colonies were counted by a ProtoCOL automated colony counter (Synbiosis, Cambridge, UK). The limit of detection was one colony for 50 μL sample (i.e. $1.3 \log_{10}$ cfu/mL), and the lower limit of quantification (LOQ) was 400 cfu/mL (i.e. $2.6 \log_{10}$ cfu/mL). Colistin heteroresistance was defined as a colistin-susceptible isolate (i.e. MIC ≤ 2 mg/L), with subpopulations growing in the presence of >2 mg/L colistin.

Time–kill kinetics

Static time-killing kinetics of three clinical isolates [isolate 4 (colistin-heteroresistant with a low MIC of 0.125 mg/L), isolate 16 (colistin-heteroresistant with an MIC of 1 mg/L and imipenem-susceptible) and isolate 17 (colistin-susceptible with an MIC of 1 mg/L and imipenem-resistant, a paired isolate of isolate 16) and ATCC 13883 (reference strain) by colistin (sulphate)] was examined.¹⁸ The antibiotic was added to a logarithmic-phase broth culture (20 mL) of $\sim 10^6$ cfu/mL to yield concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32 and $64 \times$ MIC of the respective isolate. Viable counting was performed on samples (200 μL) collected at 0, 1, 2, 4, 6 and 24 h after antibiotic addition. The limit of detection and lower LOQ were the same as those stated earlier.

PAE

In vitro PAE was determined¹⁹ for the four isolates employed in the time–kill study. For each PAE experiment, *K. pneumoniae* ($\sim 10^6$ cfu/mL) in logarithmic-phase growth was exposed for 20 min

Table 1. Susceptibility of colistin against *K. pneumoniae*

Isolate	PFGE group	MIC (mg/L)	MPC (mg/L)	Susceptibility to colistin	Susceptibility to other antibiotics									
					PIP	CIP	AMK	GEN	MEM	AMP	SAM	AMC	CAZ	TOB
ATCC 13883	A	1	>128	susceptible and heteroresistance	S	S	S	S	S	R	—	S	S	S
1	B	0.5	32	susceptible and heteroresistance	R	R	S	R	S	R	R	R	R	R
2	C	0.125	128	susceptible and heteroresistance	R	S	S	R	S	R	S	S	S	S
3	D	0.25	128	susceptible and heteroresistance	R	S	S	R	S	R	R	S	R	R
4	E	0.125	>128	susceptible and heteroresistance	R	R	R	R	S	R	R	R	R	R
5	F	0.5	>128	susceptible and heteroresistance	R	R	R	R	S	R	—	R	R	R
6	G	>128	>128	resistant	R	R	S	R	S	R	—	R	R	R
7	G	>128	>128	resistant	R	R	S	R	S	R	—	R	R	R
8	G	>128	>128	resistant	R	R	S	R	S	R	—	R	R	R
9	H	32	>128	resistant	R	R	S	S	S	R	R	R	R	R
10	I	1	128	susceptible and heteroresistance	R	R	R	R	S	R	—	R	R	R
11	J	0.125	>128	susceptible and heteroresistance	R	S	S	S	S	R	S	S	S	S
12	K	0.25	>128	susceptible and heteroresistance	R	S	S	S	S	R	S	S	S	S
13	L	0.5	32	susceptible and heteroresistance	R	R	S	R	S	R	—	R	R	R
14	M	0.25	>128	susceptible and heteroresistance	S	S	S	S	S	R	S	S	S	S
15	N	0.125	>128	susceptible and heteroresistance	R	S	S	S	S	R	R	R	S	S
16	O1	1	32	susceptible and heteroresistance	R	R	R	R	S	R	—	R	R	R
17	O1	1	64	susceptible	R	R	R	R	R	R	—	R	R	R
18	O2	0.5	64	susceptible and heteroresistance	R	R	R	R	S	R	—	R	R	R
19	P	32	>128	resistant	S	S	S	S	S	R	—	S	S	S
20	Q	128	>128	resistant	R	R	S	S	S	R	R	R	R	R
21	R	0.25	32	susceptible and heteroresistance	R	S	S	R	S	R	S	S	S	S

PIP, piperacillin; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; MEM, meropenem; AMP, ampicillin; SAM, ampicillin/sulbactam; AMC, amoxicillin/clavulanic acid; CAZ, ceftazidime; TOB, tobramycin.

in CAMHB to colistin (sulphate) at concentrations of 0.5, 1, 2, 4, 8, 16, 32 and 64 × MIC. A 20 min exposure was used due to the very rapid bactericidal effect of colistin. The antibiotic was removed by centrifuging three times at 3000 g for 10 min. Viable counts were performed at 0, 1, 2, 3, 4, 5 and 6 h onto nutrient agar. A growth control was performed in the same fashion, but without exposure to colistin. The colonies were counted after 24 h of incubation at 35°C. PAE was calculated as described previously.¹⁹

Results

PFGE patterns of ATCC 13883 and the 21 clinical isolates showed that they belonged to 18 different clonotypes. Isolates 6–8 (Group G, from the Teaching Hospital Nitra in the Slovak Republic) and 16–18 (Group O, from the Austin Hospital in Melbourne) were from the same PFGE clonotypes, respectively. All the other isolates had different PFGE patterns. Susceptibility to various antibiotics of all the isolates is summarized in Table 1. According to our definition, 13 (including isolates 4, 16 and 17) of the 21 clinical isolates were multidrug-resistant. Meropenem showed good activity against all isolates except isolate 17. ATCC 13883 and 15 clinical isolates were susceptible to colistin, with MICs ranging from 0.125 to 1 mg/L (Table 1). Six clinical isolates (isolates 6–9 from PFGE Groups G and H and isolates 19 and 20 from PFGE Groups P and Q) were resistant to colistin, with MICs of 32 or ≥128 mg/L (Table 1). MPCs for the colistin-susceptible isolates were from 32 to ≥128 mg/L, with ratios of MPC/MIC ≥32 (Table 1). All the colistin-resistant isolates had MPCs >128 mg/L.

Figure 1 shows the PAPs of ATCC 13883 and the clinical isolates. Resistant subpopulations (able to grow in the presence of >2 mg/L colistin sulphate) were observed in the reference strain and in 14 of the 15 colistin-susceptible clinical isolates, even though the isolates had MICs between 0.125 and 1 mg/L. The proportion of resistant subpopulations was of the order of 6.03×10^{-9} – 1.29×10^{-5} in these isolates (Figure 1). Surprisingly, isolate 17 (imipenem-resistant) had no subpopulations

able to grow in the presence of colistin (sulphate) at concentrations >2 mg/L, while its parent isolate 16 (imipenem-susceptible) was colistin-heteroresistant with 1.22×10^{-7} of the total population growing at 4 mg/L colistin. Heteroresistance to colistin in the four isolates employed in the time–kill studies below was stable after three daily passages in colistin-free CAMHB.

In time–kill studies, colistin showed rapid killing even at the lowest colistin concentration (Figure 2). At 64 × MIC, the bacterial killing for all the strains was very rapid; even at 2 × MIC, no bacteria could be detected (lower limit of detection 20 cfu/mL) for 1 h. Regrowth was observed at 4 h with some tested colistin concentrations in all isolates except isolate 17 (Figure 2), an isolate that was only susceptible to colistin and resistant to all other antibiotics including imipenem. At 24 h, substantial regrowth had occurred at high multiples of the respective MICs [16 × MIC, 64 × MIC and 32 × MIC for ATCC 13883 (MIC 1 mg/L), isolate 4 (MIC 0.125 mg/L) and isolate 16 (MIC 1 mg/L), respectively], except for the imipenem-resistant isolate 17 (MIC 1 mg/L), which did not regrow even at 1 × MIC. The emergence of resistance to colistin was clearly demonstrated by the changes of PAPs of ATCC 13883 and of isolates 4 and 16 after exposure to colistin during the 24 h time–kill experiments (Figure 3). This could not be examined with isolate 17 due to lack of regrowth. Negligible PAEs of colistin on the tested isolates were observed. Very modest PAE (1.6 h) was observed for ATCC 13883 only at concentrations of 64 × MIC. No PAE (≤0.5 h) was observed for all the clinical isolates, even at 64 × MIC.

Discussion

Clinicians have already been confronted with the reality of infections caused by Gram-negative bacteria, including *K. pneumoniae*, resistant to all currently available antibiotics including colistin.^{2,9,11} Heteroresistance to colistin was discovered recently in multidrug-resistant but ‘colistin-susceptible’ (based on MICs) *K. pneumoniae*.²⁰ As the pharmacodynamics of

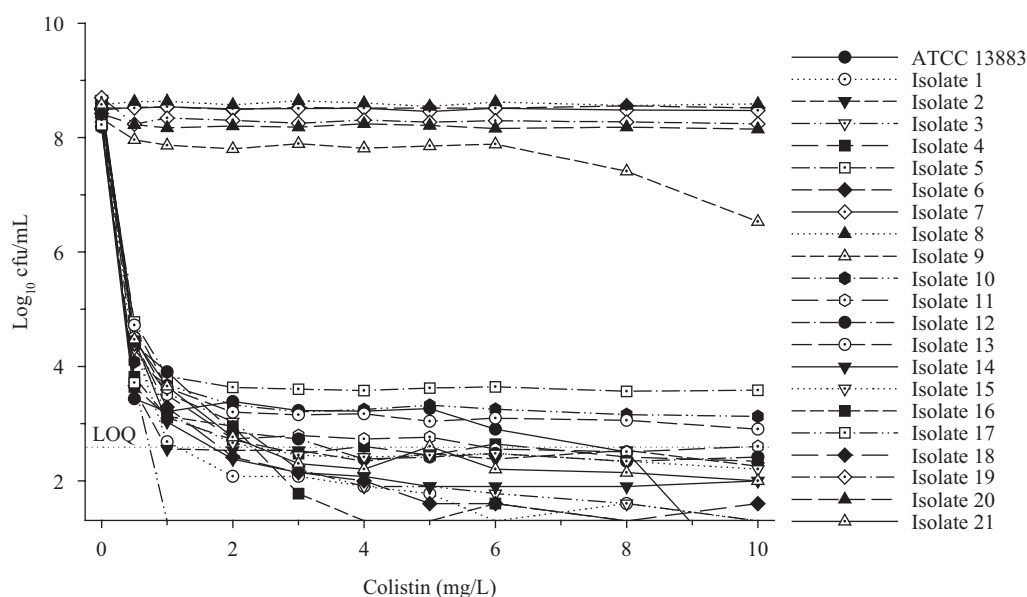


Figure 1. PAPs of 21 clinical isolates and ATCC 13883. The y-axis starts from the limit of detection and LOQ is indicated in the figure.

Pharmacodynamics of colistin against *Klebsiella pneumoniae*

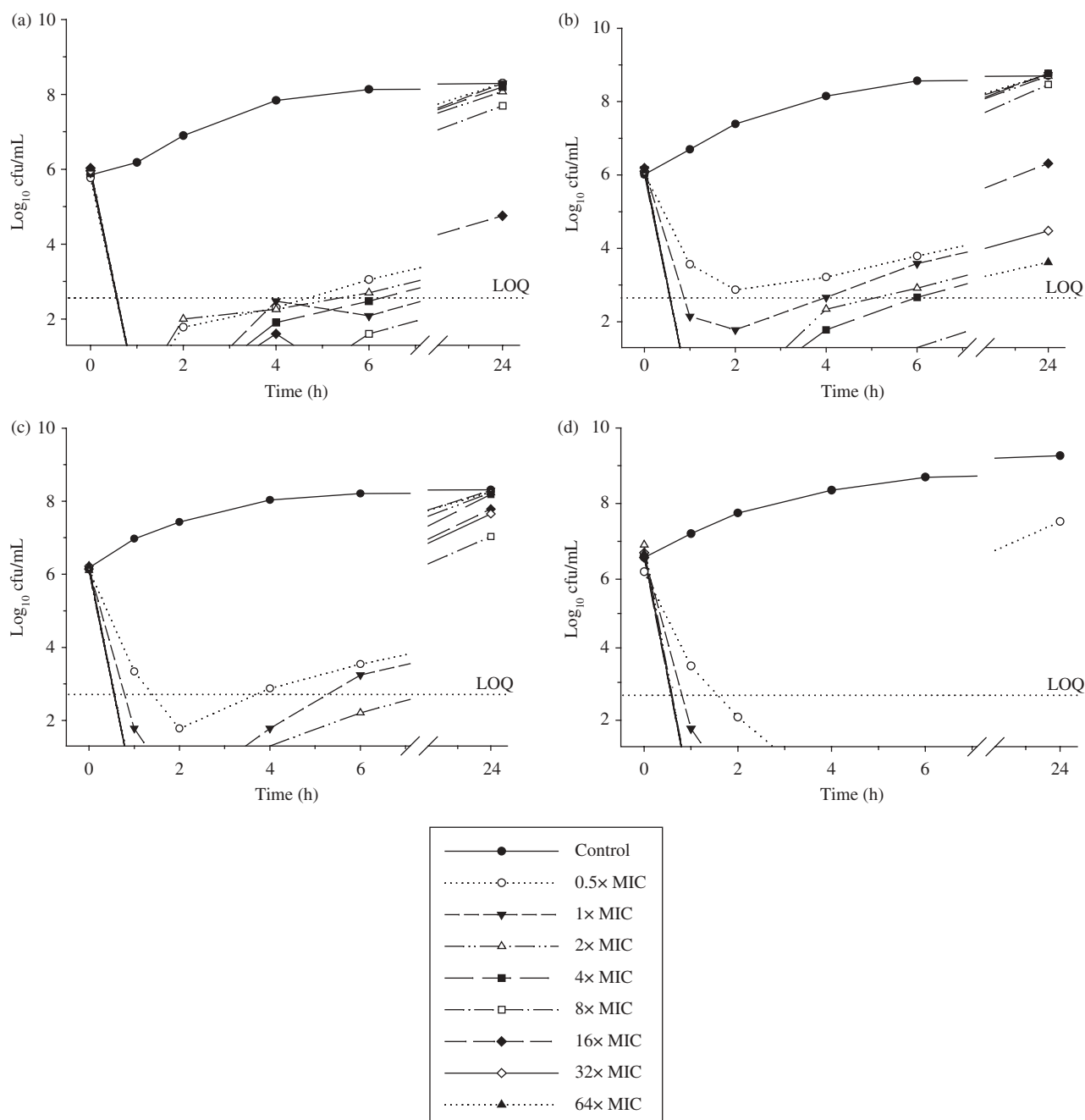


Figure 2. Killing curves of colistin against (a) ATCC 13883, (b) isolate 4, (c) isolate 16 and (d) isolate 17. The y-axis starts from the limit of detection and the LOQ is indicated in the figure. Some symbols are not shown at time points after 0 h because the density of viable cells was below the limit of 20 cfu/mL.

colistin have been reported to differ between *P. aeruginosa*¹⁹ and *A. baumannii*,²¹ it was important to examine the *in vitro* pharmacodynamics of colistin against *K. pneumoniae*.

Colistin was very active against colistin-susceptible *K. pneumoniae* based on the MICs (as low as 0.125 mg/L, Table 1). However, the MPCs were substantially higher than MICs (Table 1). Since PAPS (with an inoculum of $\sim 10^8$ cfu/mL using arithmetic drug concentrations which are clinically relevant) provide a level of detail not given by MPCs (using conventional 2-fold dilutions with an inoculum of 10^{10} cfu/mL), both were examined in this study. Similar to *A. baumannii*,¹⁸ colistin heteroresistance was observed in 14 of the 15 colistin-

susceptible clinical isolates examined plus ATCC 13883 (Figure 1). This is consistent with a recent ICAAC report.²⁰ Colistin heteroresistance (Figure 1) may partially explain the high MPC/MIC ratios (64 to >1024) for colistin-susceptible isolates.

Due to the rapid killing that occurred within 1 h, it is difficult to discern the extent of its concentration-dependent effect. Similar to *A. baumannii*,^{21,22} arguably, one of the most important findings in this study was that substantial regrowth can occur in *K. pneumoniae*; as an example, regrowth was observed with isolate 4 at 24 h even at 64x MIC (MIC 0.125 mg/L) (Figure 2). The regrowth is consistent with the heteroresistance

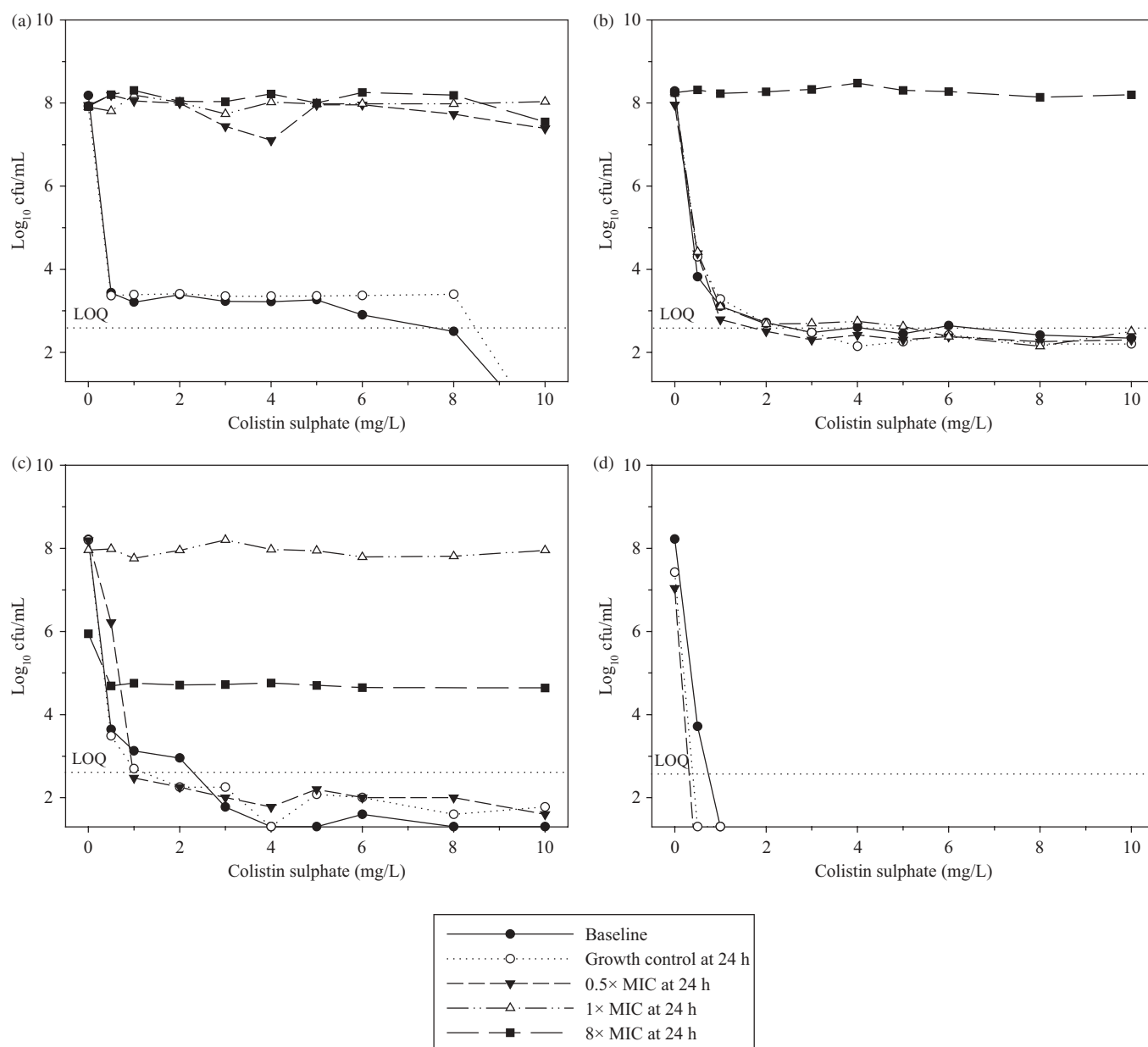


Figure 3. Changes in PAPs of (a) ATCC 13883, (b) isolate 4, (c) isolate 16 and (d) isolate 17 after exposure to selected colistin concentrations (0, 0.5×, 1× and 8× MIC) for 24 h. The y-axis starts from the limit of detection and the LOQ is indicated in the figure.

to colistin in these isolates (Figure 1). It is very likely that the more resistant subpopulations led to substantial regrowth after the killing of susceptible subpopulations; this is consistent with the changes of PAPs of ATCC 13883 and of isolates 4 and 16 after exposure to colistin for 24 h (Figure 3). Interestingly, the emergence of resistance observed at different concentrations is very different among these strains. For ATCC 13883 (Figure 3a), exposure to as low as 0.5×MIC led to substantial amplification of resistant subpopulations, while amplification occurred at 1× MIC for isolate 16 (Figure 3c) and at 8× MIC for isolate 4 (Figure 3b). Even more worrying, according to recent data on plasma concentrations of colistin (1–4 mg/L) after intravenous administration of CMS in humans,^{23,24} the concentrations required in the present study to inhibit any regrowth

at 24 h (up to 32 mg/L) are unachievable *in vivo*, even without considering plasma protein binding. This study strongly suggests that great care is required when CMS is administered parenterally as monotherapy for infections caused by *K. pneumoniae*, even where isolates appear susceptible based on MIC, because such isolates may harbour colistin-resistant populations. Thus, similar to our recent finding of colistin heteroresistance in *A. baumannii*,¹⁸ the MIC alone may not provide information to guide treatment in clinical settings for these problematic pathogens.

One pair of clinical isolates (16 and 17) obtained from a patient before and after imipenem therapy was employed in the present study. Although both isolates had the same colistin MIC (1 mg/L), the imipenem-resistant isolate did not appear to

Pharmacodynamics of colistin against *Klebsiella pneumoniae*

harbour a colistin-resistant subpopulation, while the imipenem-susceptible isolate did (Figure 1). This was demonstrated by the lack of regrowth of isolate 17 at 24 h for isolate 17 (Figure 2). Further investigation with a large number of paired clinical isolates is required to confirm this phenomenon.

Slightly different from *P. aeruginosa* against which colistin shows very modest PAE only at high concentrations ($>8 \times \text{MIC}$),¹⁹ colistin showed no PAE at up to $64 \times \text{MIC}$ against all the tested clinical isolates and ATCC 13883; the only exception was for ATCC 13883 at $64 \times \text{MIC}$, which is not achievable clinically.^{23,24} In contrast to *A. baumannii*,¹⁸ no significant negative PAE was observed with *K. pneumoniae*. Therefore, in view of the relatively short half-life (~ 4 h) of colistin after intravenous CMS^{23,24} and the lack of significant PAE observed at clinically achievable concentrations of colistin^{23,24} in the current study, infrequent dosing regimens (e.g. 24 h) of CMS²⁵ are unlikely to be a good option for the treatment of infections caused by *K. pneumoniae*. Further pharmacokinetic/pharmacodynamic investigation is warranted for the optimization of dosage regimens. Furthermore, the high frequency of colistin heteroresistance in multidrug-resistant *K. pneumoniae* suggests that colistin monotherapy may be inadequate for treatment.

A limitation of this study is the employment of static colistin concentrations in the *in vitro* experiments. However, important findings from the present study (i.e. heteroresistance, high MPCs, rapid time-kill, no significant PAE and emergence of resistance upon exposure to colistin) provide essential information for further pharmacodynamic investigations on dosage optimization of CMS against multidrug-resistant, and especially colistin-heteroresistant, *K. pneumoniae*.

In conclusion, the present study demonstrated initial rapid bacterial killing by colistin against susceptible *K. pneumoniae*. However, the concerning findings were a high frequency of colistin heteroresistance, the substantial regrowth within 24 h that occurred even at colistin concentrations up to $64 \times \text{MIC}$ and no significant colistin PAE. These findings suggest the potential risk that monotherapy with CMS and extended-interval dosage regimens may promote colistin resistance in multidrug-resistant *K. pneumoniae*. In addition, the different colistin effects, in terms of PAPs and killing kinetics of the paired imipenem-susceptible and -resistant isolates, may provide useful information on a rational strategy for combination therapy and require further investigation.

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Transparency declarations

None to declare.

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