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NOTE

Effect of the meropenem MIC on the killing activity of meropenem and polymyxin B in combination against KPC-producing *Klebsiella pneumoniae*

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INTRODUCTION

Infections caused by multidrug resistant *Klebsiella pneumoniae* that produce *K. pneumoniae* carbapenemase (KPC) typically require treatment with two or more antimicrobial agents.[1,2] Although carbapenems are hydrolyzed by KPCs, combined therapy with a carbapenem and a polymyxin (colistin or polymyxin B) significantly lowers mortality in critically ill patients infected with polymyxin-susceptible *K. pneumoniae* that produce KPC.[3,4] However, KPC-producing Enterobacteriaceae often exhibit a range of carbapenem MICs.[5] Mortality rates are higher in patients with isolates displaying higher levels of carbapenem resistance suggesting that carbapenem and polymyxin combinations may require modification based on the level of carbapenem resistance to improve outcomes.[4] To better understand the pharmacodynamics and clinical utility of carbapenem and polymyxin combinations, we evaluated the time-kill activity and the emergence of polymyxin resistance of meropenem and polymyxin B alone and in combination against KPC-producing *K. pneumoniae* with varying MICs of meropenem.

EXPERIMENTAL PROCEDURES

We evaluated four clinical isolates of *K. pneumoniae* producing KPC-3, previously identified by PCR. Isolates were obtained from the University of Kentucky HealthCare System and designated KP 22, KP 24, KP 34 and KP 44. Of those, KP 22, KP 24 and KP 44 underwent whole-genome sequencing by the University of Kentucky Genomics Core Laboratory. IRB approval was obtained and the requirement for informed consent was waived. MIC testing for meropenem, polymyxin B and colistin were performed using broth microdilution in accordance with CLSI guidelines, whereas MIC testing of other beta-lactam antimicrobials were determined by the BD Phoenix Automated Microbiology System as part of routine clinical testing.[6] Antimicrobial agents were obtained from Sigma-Aldrich (St Louis, MO, USA). Since Enterobacteriaceae are without CLSI-approved breakpoints for polymyxin B, isolates were considered 'susceptible' based upon CLSI-approved interpretive criteria for *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[6] Polymyxin B susceptible strains representing a wide range of meropenem MICs

Table 1 Polymyxin and beta-lactam MIC results

	KP 34	*KP 22*	*KP 24*	*KP 44*
Carbapenems				
Meropenem[a]	4	16	32	128
Ertapenem	>4	>4	>4	>4
Polymyxins				
Polymyxin B[a]	0.125	0.06	0.125	0.06
Colistin[a]	0.125	0.06	0.125	0.06
Penicillins				
Ampicillin	>16	>16	>16	>16
Cephalosporins				
Cefazolin	>16	>16	>16	>16
Cefepime	⩽1	>16	16	⩽1
Cefoxitin	⩽4	>16	>16	>16
Ceftazidime	2	>16	>16	>16
Ceftriaxone	>32	>32	>32	>32
Cefuroxime	>16	>16	>16	>16
β-lactam/β-lactamase inhibitors				
Ampicillin/sulbactam	>16	>16	>16	>16
Piperacillin/tazobactam	>64/4	>64/4	>64/4	>64/4
Monobactams				
Aztreonam	⩽2	>16	>16	>16

[a]Meropenem, polymyxin B and colistin MIC data reported from broth microdilution assays. All other MIC data are reported from BD Phoenix Automated Testing Systems.

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were selected for time-kill studies. Those isolates, in order of ascending meropenem MIC, were KP 34 (MIC 4 mg l^{-1}), KP 22 (MIC 16 mg l^{-1}), KP 24 (MIC 32 mg l^{-1}) and KP 44 (MIC 128 mg l^{-1}).

Time-kill studies of meropenem and polymyxin B alone and in combination were performed over 48 h using cation-adjusted Mueller–Hinton broth according to CLSI guidelines with a starting inocula of 10^6 CFU per ml for each isolate.[7] Meropenem and polymyxin B alone were evaluated at three (4, 16 and 64 mg l^{-1}) and seven (0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 mg l^{-1}) clinically relevant concentrations, respectively.[8] Polymyxin B concentrations of 0.25 and 1 mg l^{-1} were reflective of minimum concentrations of polymyxin exposure,[9] and were evaluated in combination with all three concentrations of meropenem against KP 22, KP 24 and KP 44. However, for the highly meropenem resistant isolate (KP 44), polymyxin B at 4 mg l^{-1} was also evaluated in combination with the three concentrations of meropenem. Against KP 34, only polymyxin B concentrations 0.25 and 1 mg l^{-1} in combination with meropenem 4 and 16 mg l^{-1} were evaluated because we observed maximal killing with meropenem 64 mg l^{-1} alone. All time-kill assays were performed at least in duplicate on different days with samples collected at 0, 1, 2, 4, 8, 24 and 48 h. Samples were diluted as necessary, and aliquots (50 μl) were logarithmically plated onto Mueller–Hinton agar using a spiral plater, which controlled for antibiotic carryover.[10] Colonies were counted using a laser colony counter with a lower limit of quantification of 10^2 CFU per ml. Repeat MICs for polymyxin B were determined for all regrowing colonies with emergence of resistance being defined as at least a four-fold increase in the MIC.

Table 2 Bacterial strain genome assembly metrics

Strain	*# reads*	*Coverage*	*Contigs*	*Assembly size (Mb)*
KP 22	728 531	×46	176	5.74
KP 24	903 841	×52	186	5.71
KP 44	684 557	×49	148	5.29

For whole-genome sequencing, barcoded Nextera libraries were generated by using ~50 ng of each bacterial DNA sample in individual tagmentation reactions, according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The tagmented DNA was purified using Zymo-Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) and then used as a template in a PCR amplification using reagents from the Nextera kit. The amplified products were then purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). The concentration and sizes of the amplification products were determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA. USA) and library quality was assessed via quantitative PCR, using the KAPA library quantification kit (KAPA Biosystems, Wilmington, MA, USA). Amplification conditions were as described in the manufacturer's instructions. The libraries were then pooled in

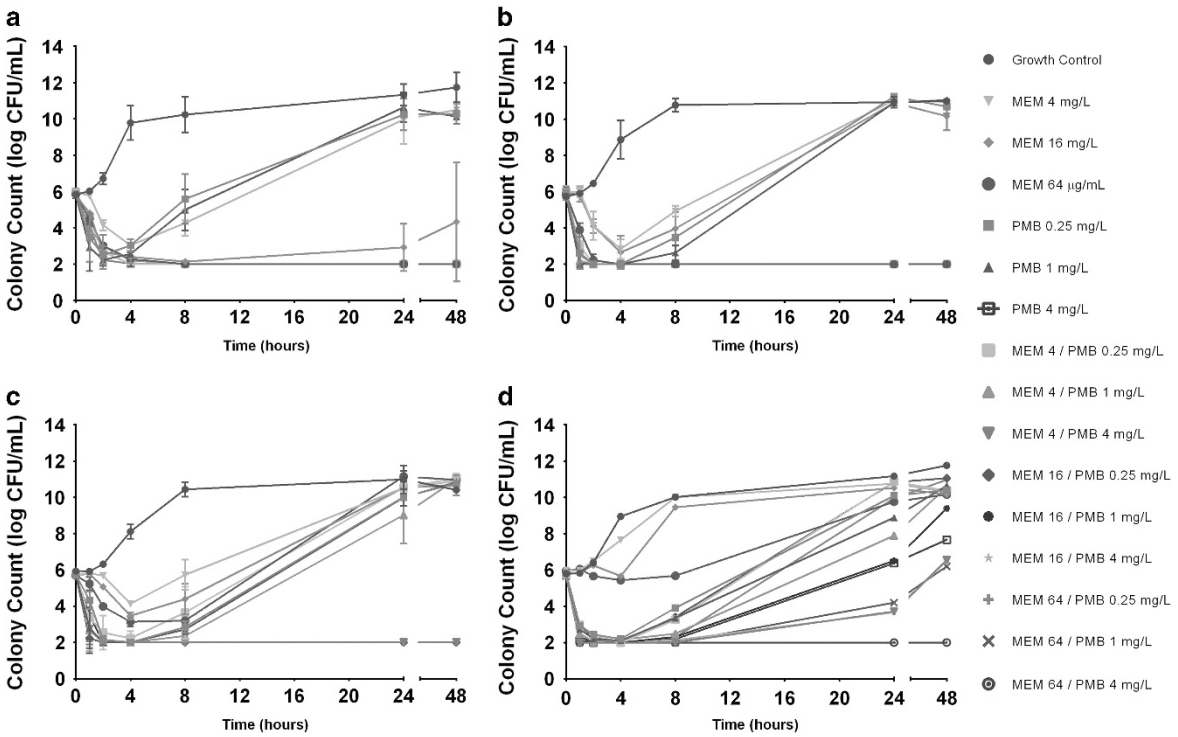

Figure 1 Time-kill curves of meropenem (MEM) and polymyxin B (PMB) alone and in combination against *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* strains: (**a**) KP 34 (MICs: MEM 4 mg l^{-1}, PMB 0.125 mg l^{-1}); (**b**) KP 22 (MICs: MEM 16 mg l^{-1}, PMB 0.06 mg l^{-1}); (**c**) KP 24 (MICs: MEM 32 mg l^{-1}, PMB 0.125 mg l^{-1}); and (**d**) KP 44 (MICs: MEM 128 mg l^{-1}, PMB 0.06 mg l^{-1}). Data points are geometric means of replicate experiments (n=2–4). The lower limit of quantification was 10^2 CFU per ml. For all panels, blue filled circles represent growth controls, red filled inverted triangles represent MEM 4 mg l^{-1}, red filled diamonds represent MEM 16 mg l^{-1}, red filled circles represent MEM 64 mg l^{-1}, green filled squares represent PMB 0.25 mg l^{-1}, green filled triangles represent PMB 1 mg l^{-1}, green open squares represent PMB 4 mg l^{-1}, purple filled squares represent MEM 4/PMB 0.25 mg l^{-1}, purple filled triangles represent MEM 4/PMB 1 mg l^{-1}, purple filled inverted triangles represent MEM 4/PMB 4 mg l^{-1}, purple filled diamonds represent MEM 16/PMB 0.25 mg l^{-1}, purple filled circles represent MEM 16/PMB 1 mg l^{-1}, purple filled stars represent MEM 16/PMB 4, purple plus signs represent MEM 64/PMB 0.25 mg l^{-1}, purple crosses represent MEM 64/PMB 1 mg l^{-1}, purple open circles represent MEM 64/PMB 4 mg l^{-1}. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

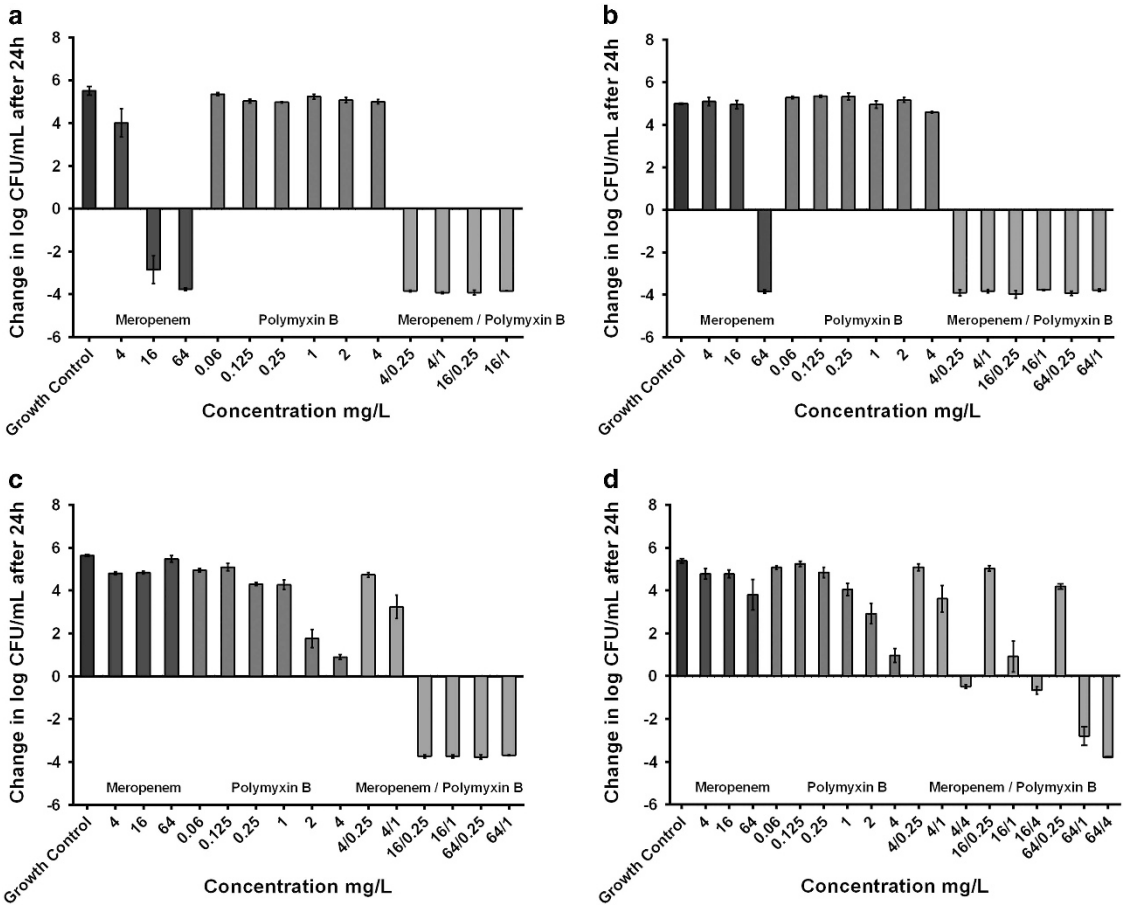

Figure 2 24 h change in colony count for meropenem (MEM) and polymyxin B (PMB) alone and in combination against *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae*: (**a**) KP 34 (MICs: MEM 4 mg l^{-1}, PMB 0.125 mg l^{-1}); (**b**) KP 22 (MICs: MEM 16 mg l^{-1}, PMB 0.06 mg l^{-1}); (**c**) KP 24 (MICs: MEM 32 mg l^{-1}, PMB 0.125 mg l^{-1}); (**d**) KP 44 (MICs: MEM 128 mg l^{-1}, PMB 0.06 mg l^{-1}). Data are differences of geometric means at time points 0 and 24 h with s.d. of replicate experiments ($n=2$–4). Blue bars represent growth control. Red bars represent meropenem concentrations evaluated alone. Green bars represent polymyxin B concentrations alone. Purple bars represent meropenem and polymyxin B evaluated in combination. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

equimolar fashion to a final concentration of 4 nM in a total volume of 10 µl. The libraries were denatured by adding an equal volume of 0.2 N NaOH and then neutralized by adding 980 µl of Illumina hybridization buffer. Six hundred microliters of the denatured libraries was used for sequencing. Sequence data (250 bp, paired-end reads) were acquired using the MiSeq platform (Illumina). Genome assemblies were generated with Newbler v2.9 (Roche, Indianapolis, IN, USA), in paired-end mode and using default parameters. The presence and absence of known resistance mechanisms for KP 22, 24 and 44 were identified using two databases, ResFinder and ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation).[11]

RESULTS

MIC testing and whole-genome sequencing

Meropenem MIC's ranged from 4 to 128 mg l^{-1} whereas polymyxin B/colistin MIC's ranged from 0.06 to 0.125 (Table 1). Isolates were resistant to most other beta-lactam antimicrobials (Table 1). Whole-genome sequencing identified KP 22, KP 24 and KP 44 as sequence type 258 and verified the presence of *blaKPC-3*. However, we found other beta-lactamase genes in each isolate as well. We identified *blaOXA-9*, *blaSHV-12* and *blaTEM-213* in KP 22 and KP 24; but *blaOXA-9*, *blaSHV-11* and *blaTEM-1A* in KP 44. Genome assembly metrics are provided (Table 2).

Meropenem and polymyxin B alone

Meropenem alone at 4, 16 and 64 mg l^{-1} achieved bactericidal activity (defined as a $\geqslant 3$ $\log_{10}$ CFU per ml decrease in colony count) within 4 h for KP 34 and KP 22. These two strains had the lowest levels of meropenem resistance. Regrowth in these strains was observed for the two lowest (4 and 16 mg l^{-1}) but not the highest (64 mg l^{-1}) meropenem exposures (Figures 1a and b). In contrast, the same concentrations of meropenem alone produced only bacteriostatic activity (defined as a decrease in colony count of <3 $\log_{10}$ CFU per ml) in KP 24 and KP 44 (Figures 1c and d). These were the two isolates with the highest levels of meropenem resistance. Regrowth for these two isolates began by 8 h. Polymyxin B alone, at all concentrations tested, produced bactericidal activity against all strains within 2 h, but regrowth occurred within 8 h in all instances (Figures 1a–d).

Meropenem and polymyxin B in combination

All combinations of meropenem (4, 16 or 64 mg l^{-1}) and polymyxin B (0.25 or 1 mg l^{-1}) concentrations achieved synergistic activity (defined

as a $\geqslant 2$ $\log_{10}$ CFU per ml lower colony count at 24 h compared to the most active agent alone) against KP 34 and 22 (Figures 2a and b) with no regrowth over 48 h (Figures 1a and b). Meropenem 4 mg l^{-1} in combination with polymyxin B 0.25 or 1 mg l^{-1} produced indifferent activity (defined as a colony count within 2 $\log_{10}$ CFU per ml at 24 h compared to the colony count of the most active agent alone) against KP 24 (Figure 2c) with regrowth occurring by 8 h (Figure 1c). All remaining combinations of meropenem 16 or 64 mg l^{-1} with polymyxin B 0.25 or 1 mg l^{-1} achieved synergistic activity with no regrowth over 48 h (Figures 1 and 2c) for KP 24. Combinations of meropenem 4, 16 or 64 mg l^{-1} with polymyxin B 0.25 or 1 mg l^{-1} displayed indifferent activity against KP 44 (Figure 2d) with regrowth (Figure 1d). Only the highest tested concentration of meropenem (64 mg l^{-1}) and polymyxin B (4 mg l^{-1}) produced synergistic activity against KP 44 and prevented regrowth (Figures 1 and 2d).

Emergence of polymyxin B resistance

The polymyxin B MICs of isolates exposed to polymyxin B alone or in combination with meropenem increased from 0.06 or 0.125 mg l^{-1} at baseline to 16–64 mg l^{-1} in those experiments where colonies grew by 48 h. In contrast, the polymyxin B MICs for regrowing bacteria exposed only to meropenem remained at 0.06 or 0.125 mg l^{-1}.

DISCUSSION

Synergy depends on the MIC of meropenem

Combinations of meropenem and polymyxin B, at clinically relevant concentrations, exhibited mainly bactericidal and synergistic activity against polymyxin B susceptible KPC-producing *K. pneumoniae* isolates with meropenem MICs $\leqslant 32$ mg l^{-1}. Almost all combinations suppressed both the regrowth observed with each drug alone and the emergence of resistance to polymyxin B. In contrast, for the *K. pneumoniae* strain with a meropenem MIC of 128 mg l^{-1}, synergistic activity and suppression of polymyxin B resistance was observed only with the highest concentrations safely attainable with conventional dosing in humans. If drug elimination and other pharmacokinetic factors associated with human dosing are considered, the above observations suggest that the activity of the meropenem and polymyxin B combination may be impacted by an increase in MIC of meropenem in *K. pneumoniae*, with the combination becoming progressively less effective in strains with higher levels of resistance to meropenem. These findings are in agreement with a recent clinical study that has reported incremental decreases in survival rates with meropenem and polymyxin B combinations in patients infected with *K. pneumoniae* strains having higher meropenem MICs.[4]

The enhanced activity, primarily synergy, that we observed with meropenem and polymyxin B in combination against KPC-producing *K. pneumoniae* has been reported by others. In fact, it is proposed that membrane permeabilization by polymyxins allow greater concentrations of carbapenems to reach penicillin-binding proteins in the cytoplasmic membrane, thereby overcoming hydrolysis by KPC enzymes.[12,13] The regrowth we observed in some of the combination experiments can be explained by the selection of subpopulations resistant to polymyxin B or through adaptive resistance where environmental stimuli (for example, polymyxin exposure) potentiate changes in the lipid membrane, conferring resistance to polymyxins.[14,15] This could be better characterized by future studies.

Polymyxin B rather than colistin in combination with a carbapenem

Studies that have evaluated polymyxins in combination with a carbapenem have typically used colistin or evaluated non-Enterobacteriaceae such as *P. aeruginosa* or *A. baumannii*. Furthermore, a recent meta-analysis included only three studies that evaluated polymyxin B in combination with a carbapenem against *K. pneumoniae*.[16] Because of this limited data, we evaluated polymyxin B rather than colistin, tested multiple concentrations for longer durations (48 h) and assessed the emergence of polymyxin B resistance following exposure to combination therapy.[12,13]

Others have suggested that polymyxin B may demonstrate more consistent synergistic activity against *K. pneumoniae* than colistin when combined with a carbapenem, but data are limited.[16] Additionally, polymyxin B, compared to colistin, does not require *in vivo* conversion for activity, does not require dosage adjustment for renal insufficiency and has a lower incidence of nephrotoxicity.[8,17] Therefore, data on the use of polymyxin B instead of colistin are needed to better understand where polymyxin B fits into antimicrobial therapy against carbapenem resistant *K. pneumoniae* infections.

Our data suggest that the probability of treatment success with meropenem in combination with polymyxin B may be dependent on the meropenem MIC in polymyxin B susceptible *K. pneumoniae* strains that produce KPC. Strains with meropenem MICs $\leqslant 32$ mg l^{-1} may be amenable to treatment using conventional dosing regimens of meropenem and polymyxin B in combination, whereas strains with meropenem MICs > 32 mg l^{-1} may require alternative dosing strategies and/or additional antimicrobial agents for optimal treatment. Additional *in vitro*, animal and ideally human studies are warranted to further elucidate the impact that the meropenem MIC has on the activity of meropenem and polymyxin B combinations against KPC-producing *K. pneumoniae*, including identification of resistance mechanisms because data are very limited.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)