



Evaluating Polymyxin B-Based Combinations against Carbapenem-Resistant *Escherichia coli* in Time-Kill Studies and in a Hollow-Fiber Infection Model

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ABSTRACT Polymyxin B-based combinations have emerged as a mainstay treatment against carbapenem-resistant *Escherichia coli* (CREC). We investigated the activity of polymyxin B-based two-antibiotic combinations against CREC using time-kill studies (TKS) and validated the findings in a hollow-fiber infection model (HFIM). TKS were conducted using 5 clinical CREC strains at 5 log₁₀ CFU/ml against 10 polymyxin B-based two-antibiotic combinations at maximum clinically achievable concentrations. HFIMs simulating dosing regimens with polymyxin B (30,000U/kg/day) and tigecycline (100 mg every 12 h) alone and in combination were conducted against two CREC strains at 5 log₁₀ CFU/ml over 120 h. Emergence of resistance was quantified using antibiotic-containing media. Phenotypic characterization (growth rate and stability of resistant phenotypes) of the resistant isolates was performed. All five CREC strains harbored carbapenemases. Polymyxin B and tigecycline MICs ranged from 0.5 mg/liter to 2 mg/liter and from 0.25 mg/liter to 8 mg/liter, respectively. All antibiotics alone did not have bactericidal activity at 24 h in the TKS, except for polymyxin B against two strains. In combination TKS, only polymyxin B plus tigecycline demonstrated both bactericidal activity and synergy in two out of five strains. In the HFIM, polymyxin B alone was bactericidal against both CREC strains before regrowth was observed at 8 h. Phenotypically stable polymyxin B-resistant mutants were observed for both strains, with a reduced growth rate observed in one strain. Tigecycline alone resulted in a slow reduction in bacterial counts. Polymyxin B plus tigecycline resulted in rapid and sustained bactericidal killing up to 120 h. Polymyxin B plus tigecycline is a promising combination against CREC. The clinical relevance of our results warrants further investigations.

KEYWORDS hollow-fiber infection model, antibiotic combination testing, polymyxin B, carbapenem-resistant *Enterobacteriaceae*

Over the past decade, the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) has challenged the utility of carbapenems worldwide (1, 2). Infections caused by carbapenem-resistant *Escherichia coli* (CREC) are particularly problematic for several reasons. First, *E. coli* is a major etiologic agent for a range of life-threatening infections, such as ventilator-associated pneumonia and complicated intra-abdominal infections

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TABLE 1 MICs for the five *Escherichia coli* strains employed in this study

Antibiotic	MIC (mg/liter) for strain:				
	CREC01	CREC02	CREC03	CREC04	CREC05
Amikacin	≥128	≥128	≥128	64	32
Levofloxacin	≥64	≥64	≥64	≥64	≥64
Rifampin	16	32	≥64	≥64	≥64
Polymyxin B	2	1	1	0.5	0.5
Tigecycline	0.5	0.5	0.25	1	8
Cefepime	≥64	≥64	≥64	≥64	≥64
Meropenem	≥64	32	≥64	16	≥64
Doripenem	≥16	8	≥16	4	≥16
Imipenem	≥32	≥32	≥32	≥32	≥32
Aztreonam	≥128	≥128	≥128	≥128	≥128
Piperacillin-tazobactam	≥256/4	≥256/4	≥256/4	≥256/4	≥256/4

(2, 3). Second, genes encoding carbapenem resistance in *E. coli* (e.g., those encoding *K. pneumoniae* carbapenemases [KPCs] and New Delhi metallo-beta-lactamases [NDM]) are located on mobile genetic elements, facilitating the propensity for rapid spread in both the hospital and community settings (1–3). Third, CREC often exhibits broad-spectrum resistance to other classes of antibiotics, including beta-lactams, aminoglycosides, and fluoroquinolones (1–3). This has resulted in a paucity of effective treatment options against CREC, leaving clinicians in a major dilemma when faced with such infections.

In light of the futility of carbapenems against CREC, clinicians are now turning to polymyxin B, an antibiotic once sidelined due to concerns about unacceptable nephrotoxicity risks, for the treatment of infections caused by CREC (4). Unfortunately, increasing reports of polymyxin heteroresistance have suggested that rapid resistance to polymyxins can develop upon treatment with polymyxin B monotherapy, especially upon exposure to subtherapeutic polymyxin concentrations (5, 6). To circumvent this phenomenon, experts have advocated that polymyxins should be used in combination with one or more antibiotics for the treatment of CREC (4, 7, 8). When selecting a polymyxin B-based combination against CREC, one must consider the pharmacokinetic/pharmacodynamic (PK/PD) properties of the antibiotics and optimize the PK/PD target attainment of both polymyxin B and the adjuvant antibiotic employed (4, 7, 8).

In this study, we evaluated the activities of multiple antibiotics in combination with polymyxin B *in vitro* against CREC, using 24-h time-kill studies (TKS) to identify the most promising polymyxin B-based two-antibiotic combination against CREC. We then validated the activity of the most active combination using a hollow-fiber infection model (HFIM), subjecting the CREC strains to clinically relevant fluctuating concentrations of both polymyxin B and the adjuvant antibiotic. Additionally, we described the phenotypic properties of any resistant isolates that emerged when CREC was subjected to the fluctuating antibiotic concentrations in the HFIM.

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RESULTS

Phenotypic and genotypic characterization. The MICs of the five CREC isolates to various antibiotics are shown in Table 1. All five isolates were resistant to amikacin, levofloxacin, rifampin, cefepime, meropenem, doripenem, imipenem, and aztreonam. No interpretative standards are provided by the CLSI for *Enterobacteriaceae* against polymyxin B or tigecycline (9). Polymyxin B MICs ranged from 0.5 mg/liter to 2 mg/liter, while tigecycline MICs ranged from 0.25 mg/liter to 8 mg/liter. The type of beta-lactamase gene, presence of changes in porin gene expression, and presence of efflux pumps in the five CREC isolates are shown in Table 2. All five CREC isolates harbored genes encoding carbapenemases; three isolates (CREC01, CREC03, and CREC05) harbored *bla*_{NDM}, one (CREC02) harbored *bla*_{OXA-48}, and one (CREC04) harbored *bla*_{KPC-2}. The presence of efflux pumps was exhibited in all five CREC isolates based on pheno-

TABLE 2 Molecular mechanisms of resistance of the five *Escherichia coli* strains employed in this study

Mechanism of resistance	Presence in strain:				
	CREC01	CREC02	CREC03	CREC04	CREC05
Genetic	<i>bla</i> _{CTXM-1} , <i>bla</i> _{CMY} , <i>bla</i> _{NDM}	<i>bla</i> _{TEM} , <i>bla</i> _{CTXM-1} , <i>bla</i> _{OXA-48}	<i>bla</i> _{CTXM-1} , <i>bla</i> _{CMY} , <i>bla</i> _{NDM}	<i>bla</i> _{TEM} , <i>bla</i> _{KPC-2}	<i>bla</i> _{CTXM-1} , <i>bla</i> _{CMY} , <i>bla</i> _{NDM}
Downregulation of porin gene expression	OmpF	OmpC, OmpF	OmpC, OmpF	None	None
Presence of efflux pumps	Yes	Yes	Yes	Yes	Yes

typic testing; in addition, three isolates (CREC01, CREC02, and CREC03) had downregulation of porin gene expression.

TKS. The time-kill profiles of the single and combination antibiotics over 24 h are shown in Fig. 1 and 2, respectively. As shown, all antibiotics alone did not have bactericidal activity at 24 h, except for polymyxin B against CREC04 and CREC05. In combination time-kill studies (TKS), a reduction in bacterial counts was observed for all polymyxin B-based combinations against all five CREC strains at 2 to 4 h, followed by various degrees of regrowth (an initial reduction from the starting inoculum followed by an increase in bacterial counts) for most combinations. Effective combinations appeared to be highly strain specific, and all tested combinations except polymyxin B plus tigecycline demonstrated antagonism against one or more CREC strains (Table 3). Polymyxin B plus amikacin, polymyxin B plus tigecycline, polymyxin B plus meropenem, polymyxin B plus imipenem, and polymyxin B plus aztreonam were bactericidal against 2/5 strains each; among these, only polymyxin B plus tigecycline also demonstrated synergy against both strains.

HFIM. We validated polymyxin B plus tigecycline in our hollow-fiber infection model (HFIM) against CREC01 and CREC02 based on our TKS results. The time courses of the

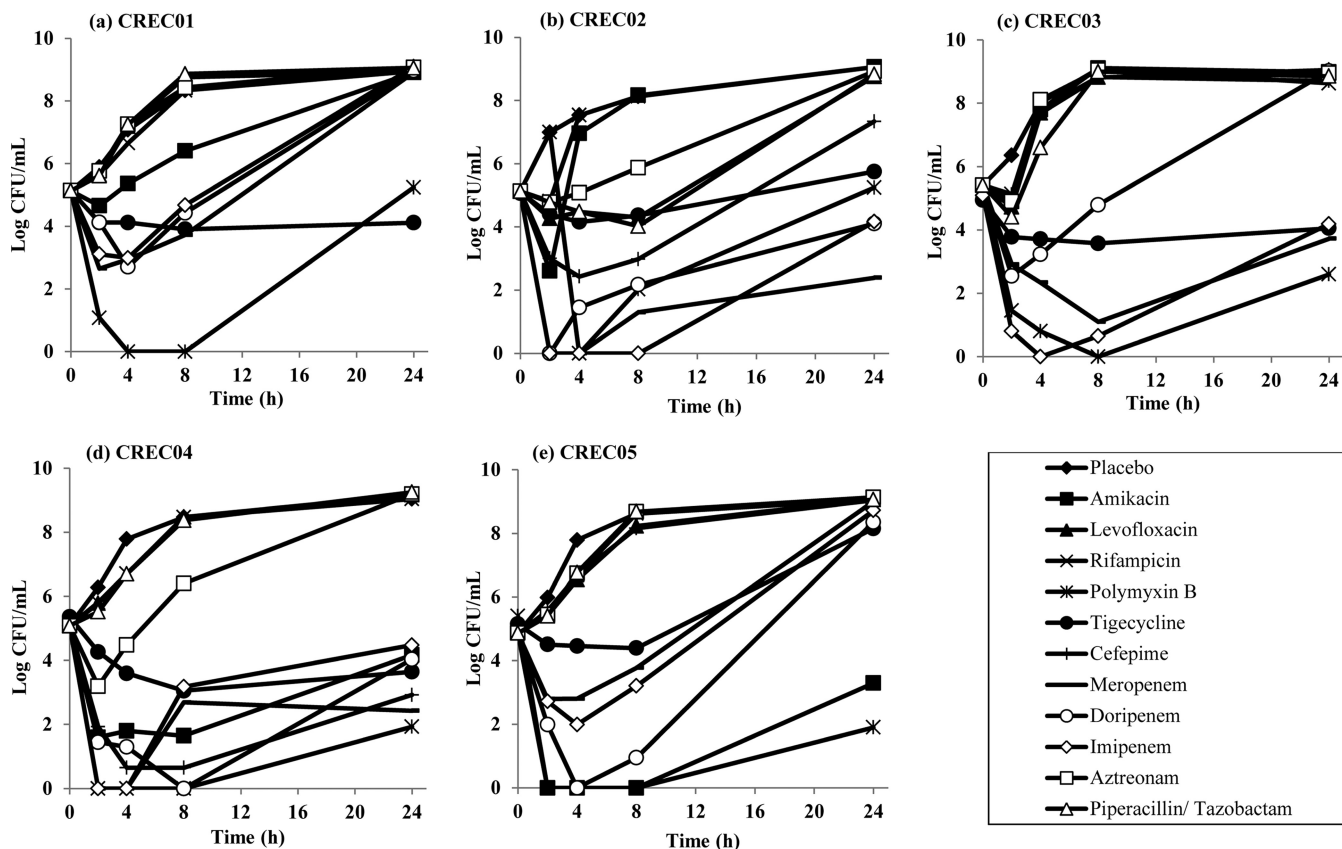


FIG 1 Microbiological response of CREC01 (a), CREC02 (b), CREC03 (c), CREC04 (d), and CREC05 (e) over 24 h upon exposure to various single-antibiotic regimens in TKS.

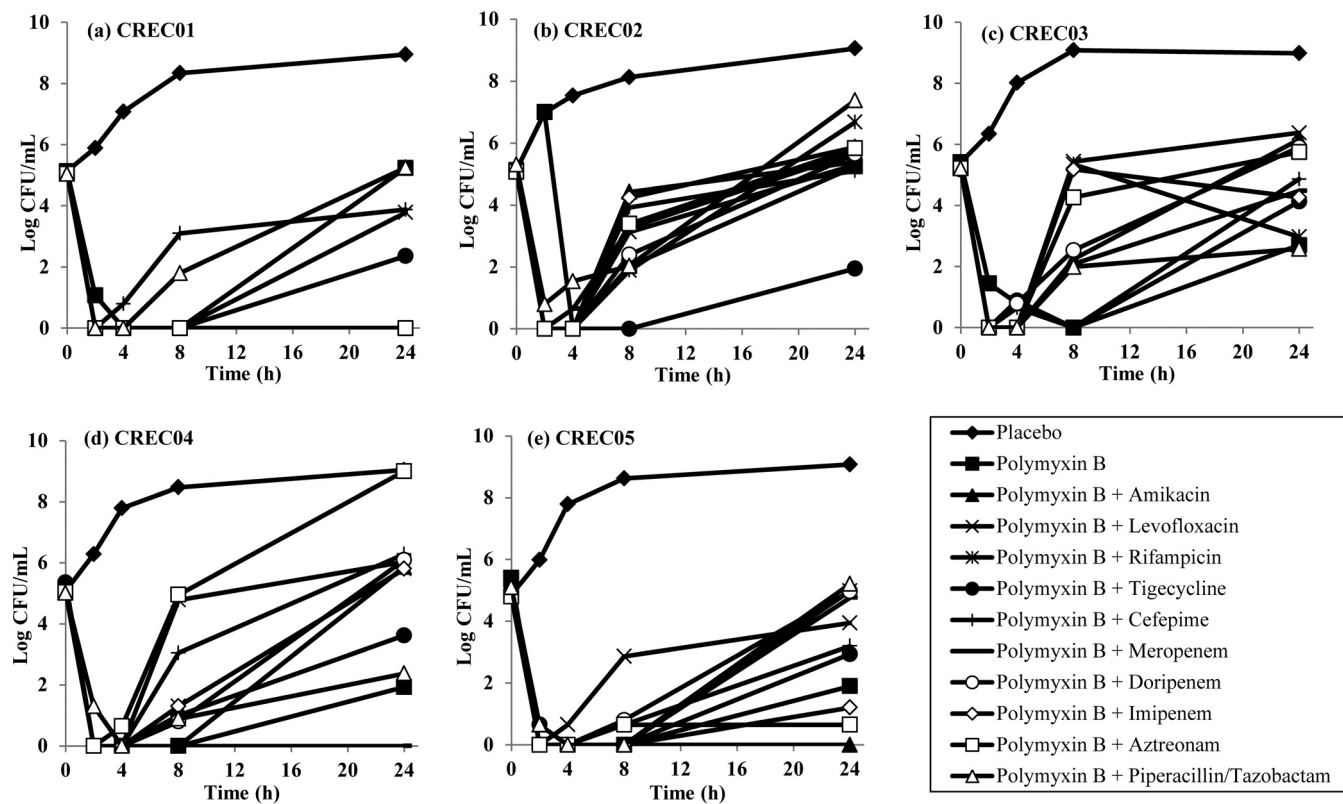


FIG 2 Microbiological response of CREC01 (a), CREC02 (b), CREC03 (c), CREC04 (d), and CREC05 (e) over 24 h upon exposure to polymyxin B alone and to various polymyxin B-based 2-antibiotic combinations in TKS.

bacterial burden over 120 h against polymyxin B plus tigecycline are shown in Fig. 3. Against both strains, polymyxin B alone exhibited bactericidal activity up to 8 h. This was followed by rapid regrowth, reaching approximately 9 log₁₀ CFU/ml at 60 h to 72 h. This increase in bacterial burden was accompanied by an increase in the polymyxin B-resistant subpopulation in the polymyxin B-supplemented medium. Exposure to tigecycline alone in the HFIM resulted in a slow reduction in bacterial counts, with a decrease of 2 log₁₀ CFU/ml from the baseline inoculum observed only after 48 h. No tigecycline-resistant subpopulation was observed upon exposure to tigecycline alone. Rapid bactericidal killing was observed within 4 h for both CREC01 and CREC02 when exposed to clinically fluctuating concentrations of polymyxin B plus tigecycline; this suppression was sustained for both isolates up to 120 h.

TABLE 3 Summary of the pharmacodynamic endpoints achieved by the polymyxin B-based two-antibiotic combinations in the *in vitro* TKS at 24 h against the five CREC isolates

Combination	Result ^a for strain:				
	CREC01	CREC02	CREC03	CREC04	CREC05
Polymyxin B + amikacin	B/S	I	A	A	B
Polymyxin B + levofloxacin	I	I	A	A	A
Polymyxin B + rifampin	B/S	I	I	A	A
Polymyxin B + tigecycline	B/S	B/S	I	I	I
Polymyxin B + cefepime	I	I	A	A	I
Polymyxin B + meropenem	B/S	I	A	B	A
Polymyxin B + doripenem	B/S	I	A	A	A
Polymyxin B + imipenem	B/S	I	I	A	B
Polymyxin B + aztreonam	B/S	I	A	A	B
Polymyxin B + piperacillin-tazobactam	I	I	I	I	A

^aA, antagonism; B, bactericidal; I, indifference; S, synergism.

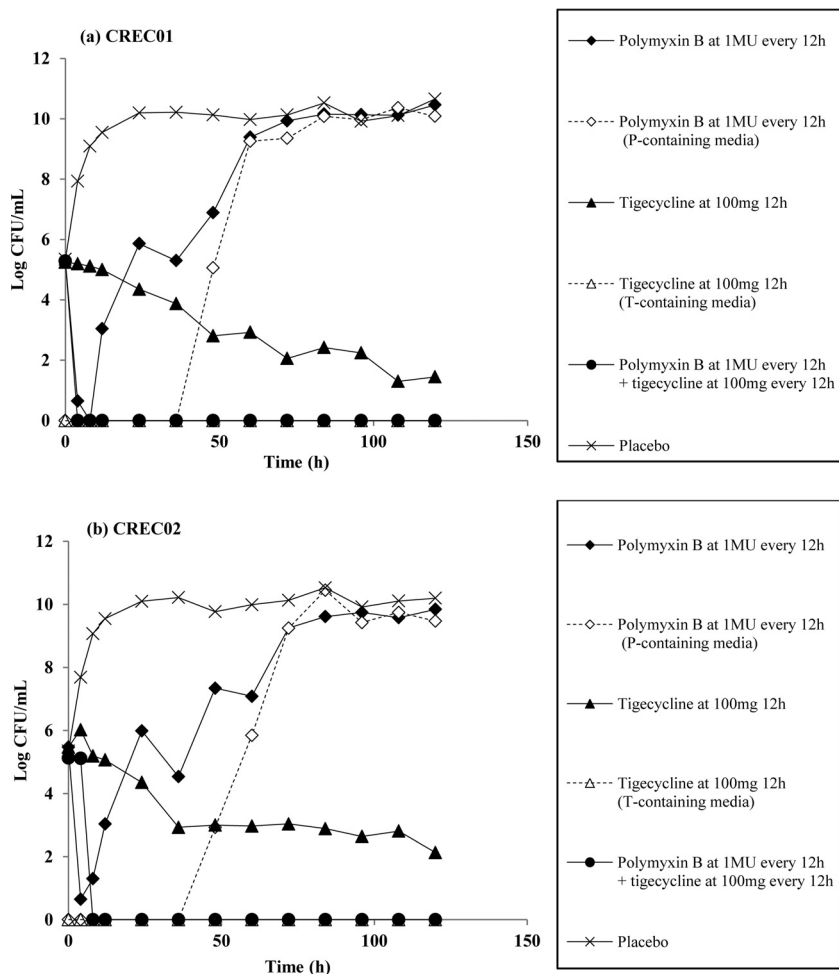


FIG 3 Microbiological response of CREC01 (a) and CREC02 (b) over 120 h upon exposure to polymyxin B and tigecycline singly and in combination in an HFIM.

PK validation. The simulated polymyxin B and tigecycline exposures in the HFIM are shown in Fig. 4. All simulated polymyxin B and tigecycline exposures were satisfactory; the simulated antibiotic exposures had an r^2 value of 0.98.

Phenotypic characterization of the resistant isolates. The MICs of the resistant isolates recovered from polymyxin-B supplemented plates at 120 h (the end of the HFIM experiments) are shown in Table 4. An increase in MICs to polymyxin B was observed for both the CREC01 (polymyxin B MIC = 8 mg/liter) and CREC02 (polymyxin B MIC = 16 mg/liter) colonies recovered from the polymyxin B-supplemented plates. No changes in the susceptibilities to other antibiotics were observed. After 10 and 20 days of serial passages on antibiotic-free medium, no reversal of polymyxin B MICs was observed, which suggested the stability of the resistance phenotype in the postexposure isolates. A slight reduction in growth rate was observed in the post-polymyxin B exposure isolates (mean best-fit growth rate constant [K_g] = 1.451/h) for CREC01 compared to the parent strain (K_g = 1.477/h). In contrast, a large reduction in growth rate was observed for the CREC02 post-polymyxin B exposure isolates (K_g of CREC02 parent strain = 1.706/h; K_g of CREC02 postexposure subpopulation = 1.120/h), suggesting a large reduction in biofitness upon polymyxin B exposure for CREC02.

DISCUSSION

Our study investigated the activity of multiple antibiotics in combination with polymyxin B *in vitro* against CREC in TKS and validated the most promising polymyxin

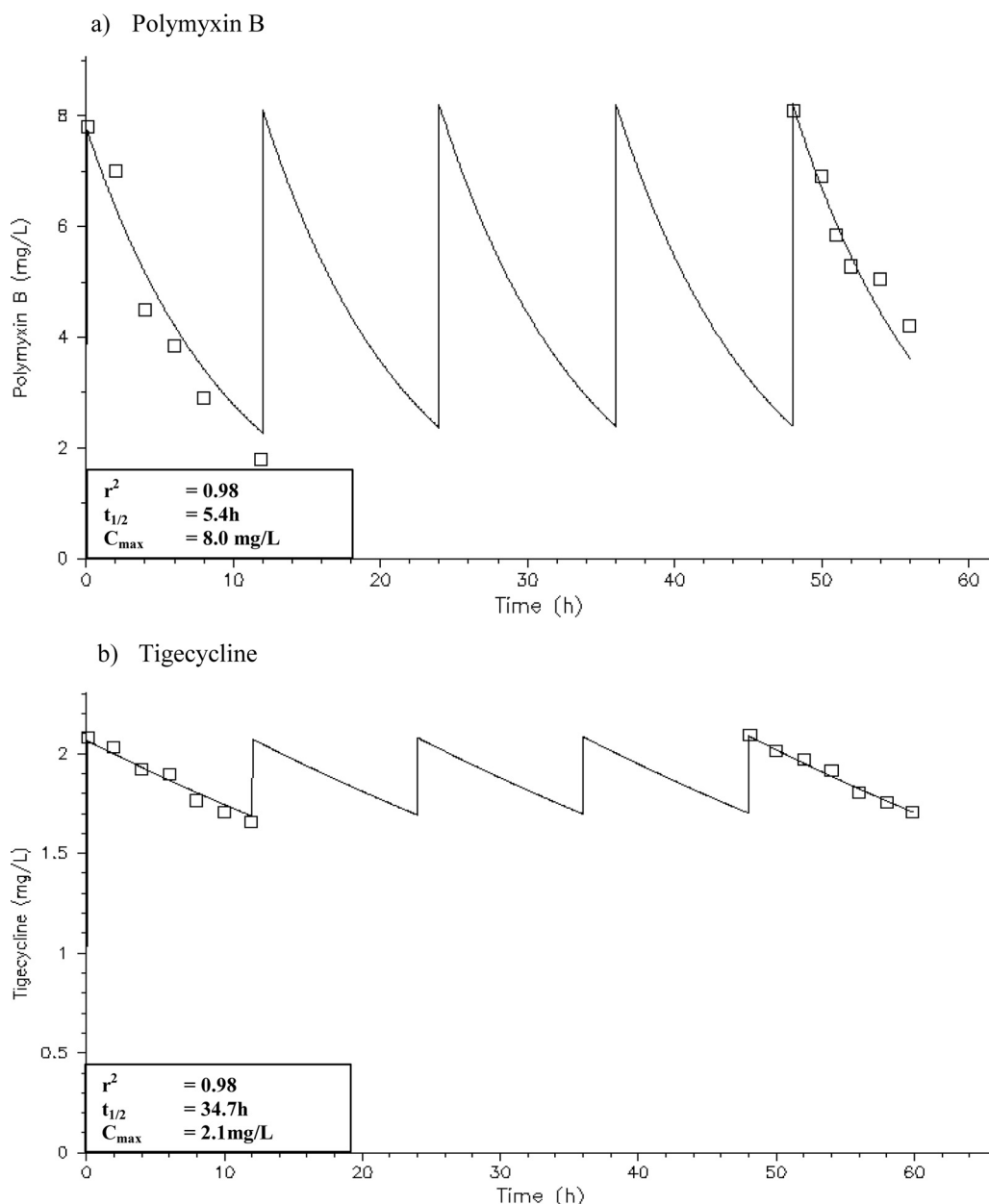


FIG 4 Pharmacokinetic profiles in the HFIM with polymyxin B at 30,000 U/kg/day or at least 1 MU every 12 h (a) and tigecycline at 100 mg every 12 h (b).

B-based combination in an HFIM. The results from our HFIM experiments showed that the combination of polymyxin B and tigecycline was rapidly bactericidal within 4 h of antibiotic exposure and limited the emergence of polymyxin B resistance up to 120 h.

The antibiotic concentrations employed in the TKS and the dosage regimens simulated in the HFIM experiments were based on careful consideration of the concentration-time profiles of the unbound antibiotics that can be achieved in patients when administered at maximum doses (10, 11). The polymyxin B concentrations achieved in our HFIM were similar to those in the most recent pharmacokinetic studies on polymyxin B; in a population pharmacokinetic study by Sandri et al., exposure to a polymyxin B dose of 30,000 U/kg/day (administered every 12 hours) resulted in a median steady-state peak unbound concentration of 8.51 mg/liter and a median steady-state unbound trough concentration of 2.25 mg/liter (12). The tigecycline concentrations employed in our HFIM experiments were based on achievable unbound

TABLE 4 Comparison of susceptibilities of resistant isolates CREC01 and CREC02 before and after exposure to polymyxin B in an HFIM

Antibiotic	MIC (mg/liter) for strain:			
	CREC01		CREC02	
	Before polymyxin B exposure	After polymyxin B exposure	Before polymyxin B exposure	After polymyxin B exposure
Amikacin	≥128	≥128	≥128	≥128
Levofloxacin	≥64	32	≥64	≥64
Rifampin	≥64	≥64	≥64	≥64
Polymyxin B	2	8	1	16
Tigecycline	0.5	≤0.25	0.5	0.5
Cefepime	≥64	≥64	≥64	≥64
Meropenem	≥64	≥64	32	32
Doripenem	≥16	≥16	8	≥16
Imipenem	≥32	≥32	≥32	≥32
Aztreonam	≥128	≥128	≥128	≥128
Piperacillin-tazobactam	≥256/4	≥256/4	≥256/4	≥256/4

tissue concentrations instead of serum concentrations (13). This is because tigecycline distributes widely into tissues and has no utility in the treatment of bacteremia in clinical practice but is instead commonly employed for the treatment of skin/soft tissue and intra-abdominal infections (14).

We employed five clinical CREC strains harboring an array of carbapenem resistance mechanisms, including carbapenemases, enhanced efflux pump expression, and/or altered membrane permeability due to porin loss, in our study to reflect the variability of carbapenem resistance mechanisms encountered in our local setting (15). An NDM-producing strain and an OXA-producing strain were eventually selected for the HFIM experiments, as these carbapenemases were reported to be the most common among *Enterobacteriaceae* in Singapore (15, 16). In the TKS experiments, all five strains were susceptible to polymyxin B (MIC range, 0.5 to 2 mg/liter); however, regrowth was demonstrated in three out of the five strains exposed to polymyxin B alone, suggesting potential heteroresistance among these strains. No single polymyxin B-based combination was effective against all five strains, which may be attributable to the differences in the mechanisms of molecular resistance. Interestingly, despite similar molecular resistance mechanisms mediating carbapenem resistance in CREC01 and CREC03, the effective polymyxin B-based combinations against each strain appeared to be highly strain specific. Of note, all polymyxin B-carbapenem combinations were bactericidal against CREC01 at 24 h, while none were bactericidal against CREC03. We postulate that the observed difference may be attributed to the differences in the type and/or degree of porin loss; a more detailed work-up on the resistance mechanisms in these strains will be required (17). Antagonism was observed in all polymyxin B-based combinations except polymyxin B plus tigecycline against one or more CREC strains. This finding suggested that the empirical selection of polymyxin B-based combinations can be counterproductive and emphasized the importance of *in vitro* testing in guiding the selection of effective combinations (18, 19).

Our findings in the HFIM experiments corroborated our TKS results. Exposure to polymyxin B alone resulted in an initial bactericidal killing followed by regrowth, which is possibly due to the selective amplification of the preexisting polymyxin B-resistant subpopulations when exposed to polymyxin B alone. Upon exposure to fluctuating concentrations of tigecycline alone, a slow reduction in bacterial burden was observed for both CREC isolates, with less than 1 log₁₀ CFU/ml reduction after 24 h. Our findings were similar to those in previous reports, which showed that tigecycline demonstrated a slow but sustained bacteriostatic effect against *Enterobacteriaceae* (20). There was no emergence of tigecycline-resistant subpopulations despite prolonged treatment with tigecycline monotherapy, which is contrast to a previous HFIM study by Lim et al., who demonstrated that tigecycline-resistant *Klebsiella pneumoniae* mutants were isolated

from an initially tigecycline-susceptible strain after 240 h of tigecycline exposure at clinically relevant concentrations (11).

To estimate the biological costs associated with resistance development, we conducted a pairwise time-growth study between the resistant isolates recovered from the antibiotic-supplemented plates in the HFIM and their corresponding ancestral strains (21). Interestingly, a significant reduction in the growth rate of the polymyxin B-resistant isolates recovered from the antibiotic-supplemented plates was seen in CREC02, while only a slight reduction in growth rate was observed in the CREC01 post-polymyxin B exposure isolates. This suggested that the magnitude of biological costs that is imposed by the development of resistance mutations can vary among strains and may be dependent on a multitude of factors, including the initial biofitness of the ancestral strain and the type and degree of resistance acquired upon antibiotic exposure. It should be noted that the lack of measureable cost based on growth experiments under *in vitro* conditions does not necessarily mean that the costs of resistance are nonexistent, as an *in vitro* biofitness deficit in bacteria does not equate to *in vivo* biofitness (21). Further *in vivo* work-up will be required to fully investigate the cost of resistance development on *in vivo* virulence of these postexposure isolates.

To date, a limited number of previous studies have investigated the activity of polymyxin (polymyxin B or colistin)-based combinations against CREC; however, most of these studies only conducted TKS or checkerboard assays over 24 h (20, 22, 23). Betts et al. further explored the efficacy of tigecycline plus colistin against three CREC strains in a *Galleria mellonella* model, but the relevance of an invertebrate model in predicting human therapeutics remains questionable (23). In contrast, our present study is the first to investigate polymyxin B-based combinations against CREC using an HFIM, subjecting the CREC strains to clinically relevant concentrations of antibiotics for a prolonged duration. The main limitation of our study was that we were able to validate only a single polymyxin B-based combination in the HFIM experiments due to their labor-intensive nature. Given the strain-specific activity of various polymyxin B-based combinations in our TKS, future studies will be required to further validate the activity of other polymyxin B-based combinations in an HFIM system.

Conclusion. We have shown in our study that the combination of polymyxin B and tigecycline at clinically relevant fluctuating concentrations was rapidly bactericidal against CREC and suppressed the emergence of polymyxin B resistance. Our future studies will include validating other combinations found effective against CREC in an HFIM system, as well as investigating the *in vivo* and clinical relevance of these combinations.

MATERIALS AND METHODS

Bacterial isolates. Five nonclonal, clinical strains of CREC (CREC01 to CREC05), previously collected as part of a nationwide surveillance study from 2011 to 2012, were obtained from the largest tertiary hospital in Singapore (1,700 beds). Genus identity was ascertained using Vitek 2 ID-GN cards (bioMérieux, Inc., Hazelwood, MO). The CREC strains were stored at -80°C in Cryobank (Thermo Scientific, Singapore) storage vials, and fresh isolates were subcultured twice on 5% sheep blood agar plates (Thermo Scientific, Singapore) for 24 h at 35°C before each experiment.

Phenotypic and genotypic characterization. MICs to multiple antibiotics were determined in cation-adjusted Mueller-Hinton II broth (Ca-MHB) (BBL, Sparks, MD) using commercial broth microdilution panels (Trek Diagnostics, East Grinstead, UK) and interpreted in accordance to the CLSI guidelines (9, 10). All experiments involving tigecycline were performed using freshly prepared Ca-MHB. PCR was employed to determine the presence of genes encoding extended-spectrum β -lactamases (ESBLs) (*bla*_{TEM} and *bla*_{SHV}), plasmid-mediated AmpCs, metallo- β -lactamases (MBLs) (*bla*_{VIM}, *bla*_{IMP}, *bla*_{SIM}, *bla*_{GIM}, *bla*_{SPM}, and *bla*_{NDM}), and KPCs (24). Expression of the *ompC* and *ompF* porin genes was examined by quantitative reverse transcription-PCR (RT-PCR), with expression levels normalized to the housekeeping *rpoB* gene (25). Relative quantities of mRNA of each gene of interest were determined using the comparative threshold cycle method, calibrating against standard *E. coli* ATCC 25922. A reduction in porin gene expression of ≥ 10 -fold was considered indicative of reduced porin gene expression (26). To elucidate the presence of efflux pumps, levofloxacin MICs were determined in the presence and absence of the efflux pump inhibitor phenylalanine-arginine β -naphthylamide (50 $\mu\text{g}/\text{ml}$), in accordance to a previously published protocol (27). Levofloxacin MICs that were ≥ 4 -fold lower in the presence of an efflux pump inhibitor were considered to be indicative of an elevated efflux mechanism (27).

Antimicrobial agents. Eleven antibiotics were used in the experiments. Aztreonam and rifampin were purchased from Toronto Research Chemicals. Cefepime was purchased from Kemimac(s) Pte Ltd.

TABLE 5 Simulated antibiotic dosing regimens and corresponding drug concentrations

Drug	Simulated dosing regimen	Concn (mg/liter)	Reference
Amikacin	15–20 mg/kg every 24 h	80	28
Aztreonam	6 g every 24 h (infused over 24 h)	18	29
Cefepime	2 g every 8 h	200	30
Doripenem	1 g every 8 h (infused over 4 h)	13	31
Imipenem	1 g every 6 h (infused over 0.5 h)	32	32
Levofloxacin	750 mg every 24 h	8	33
Meropenem	2 g every 8 h (infused over 3 h)	64	34
Piperacillin-tazobactam	4.5 g every 6 h (infused over 4 h)	75/15	35
Polymyxin B	30,000 IU/kg/day or at least 1 MU every 12 h	2	36
Rifampin	600 mg every 12 h	2	37
Tigecycline	100 mg every 12 h	2	13

Amikacin, levofloxacin, and polymyxin B were purchased from Sigma-Aldrich. Doripenem was obtained from Shionogi & Co. Imipenem was provided by Merck & Co. Meropenem was provided by Astra Zeneca Inc. Tigecycline and piperacillin-tazobactam were provided by Pfizer Inc. Aliquots of stock solutions of all antibiotics except rifampin were prepared in sterile water and stored at -80°C . Prior to each experiment, the aliquot was thawed and diluted to the desired concentrations with Ca-MHB. Rifampin was dissolved in dimethyl sulfoxide (DMSO) before being serially diluted in sterile water to the desired concentration. The final DMSO concentration ($<1\%$, vol/vol) did not affect bacterial growth (9).

TKS. Time-kill studies (TKS) were performed on all five CREC strains with amikacin, aztreonam, cefepime, doripenem, imipenem, levofloxacin, meropenem, piperacillin-tazobactam, rifampin, and tigecycline singly and in two-antibiotic combinations with polymyxin B. The antibiotic concentrations used were based on maximal clinically relevant unbound concentrations when maximum antibiotic doses were administered and are shown in Table 5 (13, 28–37). The procedures for the TKS have been described in detail in our previous study (10). Briefly, 15 ml of log-phase bacterial suspensions in Ca-MHB was transferred to sterile flasks containing 1 ml of antibiotics and placed into a shaker water bath at 35°C (final bacterial concentration, approximately $5 \log_{10}$ CFU/ml [1×10^5 CFU/ml to 5×10^5 CFU/ml]). At 0, 2, 4, 8, and 24 h, samples were obtained in duplicate from each flask and washed with sterile normal saline to minimize drug carryover. Viable counts were obtained by dropping serial 10-fold dilutions of the reconstituted samples onto Mueller-Hinton agar (MHA) plates (Thermo Scientific, Singapore) and enumerating at 24 h. The lower limit of detection for the colony counts was $2.6 \log_{10}$ CFU/ml.

Experimental setup for the HFIM. A hollow-fiber infection model (HFIM) (FiberCell Systems, Frederick, MD), first described by Blaser, was used to determine the killing activity of the antibiotic combinations when bacteria were exposed to clinically relevant fluctuating antibiotic concentrations (38). A schematic diagram of the HFIM has been previously published (11, 38, 39). Two bacterial isolates (CREC01 and CREC02) were tested against polymyxin B plus tigecycline (the most active antibiotic combination in the TKS). The single-antibiotic regimens and a placebo control were also tested. Twenty milliliters of log-phase bacteria ($\sim 5 \log_{10}$ CFU/ml), prepared as described above, was injected into the extracapillary compartment of the hollow-fiber cartridge and subjected to the different antibiotic exposures for up to 120 h in a humidified incubator set at 35°C . The antibiotic exposures simulated the steady-state pharmacokinetic profiles when polymyxin B at 30,000 IU/kg/day, or least 1 MU every 12 h, or tigecycline at 100 mg every 12 h was administered to patients with multidrug resistant Gram-negative bacterial infections (10, 12). Maintenance doses were given in accordance with clinical dosing frequencies for the individual drugs to reattain the target concentrations in the HFIM.

Microbiological response. Serial samples were collected aseptically from the HFIM at 0 h (baseline), 4 h, 8 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, 96 h, 108 h, and 120 h in duplicates to determine the total and resistant bacterial subpopulation viable cell count. For the total bacterial population, the samples were cultured on MHA plates as described above; for resistant bacterial subpopulations, the samples were cultured on MHA supplemented with the exposed antibiotic at $3 \times$ the MIC of the exposed agent. The lower limit of detection for the colony counts was $2.6 \log_{10}$ CFU/ml.

Pharmacokinetic validation. Serial 1-ml samples were obtained every two hours from the central reservoir of the HFIM from 0 h to 12 h and 48 h to 60 h in triplicates and kept at -80°C until analysis. The antibiotic concentrations in the samples were assayed using a validated liquid chromatography-mass spectrometry (LC-MS) method as described previously (10). The pharmacokinetic models of the antibiotics were obtained by fitting a one-compartment linear model to the observations using ADAPT II (40). All samples were analyzed within 1 month from the completion of the HFIM studies.

Phenotypic characterization of the resistant isolates. The following phenotypic tests were conducted to characterize the resistant isolates (if any) that emerged in the HFIM experiments: (i) susceptibility testing using commercial broth microdilution panels to screen for potential phenotypic changes in resistance patterns, (ii) serial passages on drug-containing and drug-free media to evaluate stability of the resistant phenotypes, and (iii) time-growth studies to evaluate the biofitness of the resistant subpopulation. MIC testing for the exposed antibiotic(s) was performed on at least two colonies of the resistant isolates recovered from antibiotic-supplemented plates throughout the HFIM experiment to confirm the presence of resistance. MICs to other antibiotics were also determined with at least two colonies of the resistant isolates obtained at 120 h. For the serial passages, the resistant isolates were

subjected to 20 days of passages on drug-free MHA and MHA supplemented with the exposed antibiotic at $3\times$ the MIC, and MIC testing for all the antimicrobial agents was performed at the 10-day and 20-day time points. To carry out the time-growth studies, 24 ml of log-phase bacteria ($\sim 5 \log_{10}$ CFU/ml), prepared as described above, was transferred to 50-ml sterile conical flasks and incubated in a shaker water bath at 35°C. At 0 (baseline), 1, 2, 3, 4, 5, 6, and 24 h, serial 1-ml samples were obtained (in triplicate) and quantified as described above. The exponential growth of the bacterial population over 24 h was analyzed using an adapted mathematical model (41).

Pharmacodynamic endpoints. Bactericidal activity was defined as a $\geq 3 \log_{10}$ CFU/ml decrease in the colony count from the initial inoculum at 24 h (11). Synergy was defined as a $\geq 2 \log_{10}$ CFU/ml decrease in the colony count by the drug combination compared with its most active constituent and a $\geq 2 \log_{10}$ CFU/ml decrease from the initial inoculum at 24 h (11). Indifference was defined as a $< 2 \log_{10}$ CFU/ml change at 24 h by the combination compared with that by the most active single agent (11). Antagonism was defined as defined as an increase of $\geq 2 \log_{10}$ CFU/ml with the combination in comparison with the viable bacterial count obtained with the most active single agent (11).

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