

# *In Vitro* Activity of Polymyxin B in Combination with Various Antibiotics against Extensively Drug-Resistant *Enterobacter cloacae* with Decreased Susceptibility to Polymyxin B

Yiyi Cai,<sup>a,b</sup> Tze-Peng Lim,<sup>a,c</sup> Jocelyn Teo,<sup>a</sup> Suranthran Sasikala,<sup>a\*</sup> Winnie Lee,<sup>a</sup> Yanjun Hong,<sup>b\*</sup> Eric Chun Yong Chan,<sup>b</sup> Thean Yen Tan,<sup>d</sup> Thuan-Tong Tan,<sup>e</sup> Tse Hsien Koh,<sup>f</sup> Li Yang Hsu,<sup>g\*</sup> Andrea L. Kwa<sup>a,b,h</sup>

Singapore General Hospital, Department of Pharmacy, Singapore<sup>a</sup>; National University of Singapore, Department of Pharmacy, Singapore<sup>b</sup>; Duke-NUS Medical School, Office of Clinical Sciences, Singapore, Singapore<sup>c</sup>; Changi General Hospital, Department of Laboratory Medicine, Singapore<sup>d</sup>; Singapore General Hospital, Department of Infectious Diseases, Singapore<sup>e</sup>; Singapore General Hospital, Department of Pathology, Singapore<sup>f</sup>; National University of Singapore, Yong Loo Lin School of Medicine, Department of Medicine, Singapore<sup>g</sup>; Duke-NUS Medical School, Emerging Infectious Diseases, Singapore<sup>h</sup>

**Against extensively drug-resistant (XDR) *Enterobacter cloacae*, combination antibiotic therapy may be the only option. We investigated the activity of various antibiotics in combination with polymyxin B using time-kill studies (TKS). TKS were conducted with four nonclonal XDR *E. cloacae* isolates with 5 log<sub>10</sub> CFU/ml bacteria against maximum, clinically achievable concentrations of polymyxin B alone and in two-drug combinations with 10 different antibiotics. A hollow-fiber infection model (HFIM) simulating clinically relevant polymyxin B and tigecycline dosing regimens was conducted for two isolates over 240 h. Emergence of resistance was quantified using antibiotic-containing (3 × MIC) media. Biofitness and stability of resistant phenotypes were determined. All XDR *E. cloacae* isolates were resistant to all antibiotics except for polymyxin B (polymyxin B MIC, 1 to 4 mg/liter). All isolates harbored metallo-β-lactamases (two with NDM-1, two with IMP-1). In single TKS, all antibiotics alone demonstrated regrowth at 24 h, except amikacin against two strains and polymyxin B and meropenem against one strain each. In combination TKS, only polymyxin B plus tigecycline was bactericidal against all four XDR *E. cloacae* isolates at 24 h. In HFIM, tigecycline and polymyxin B alone did not exhibit any killing activity. Bactericidal kill was observed at 24 h for both isolates for polymyxin B plus tigecycline; killing was sustained for one isolate but regrowth was observed for the second. Phenotypically stable resistant mutants with reduced *in vitro* growth rates were observed. Polymyxin B plus tigecycline is a promising combination against XDR *E. cloacae*. However, prolonged and indiscriminate use can result in resistance emergence.**

*Enterobacter cloacae* isolates are nosocomial pathogens responsible for a diversity of infections. These organisms exhibit a remarkable adaptive capability and can acquire resistance to several antibiotics (1). All bacteria of the *Enterobacter* genus are intrinsically resistant to ampicillin and narrow-spectrum cephalosporins; in addition, resistance to carbapenems is often conferred through porin loss plus overexpression of beta-lactamases and/or by acquisition of plasmid-mediated carbapenemases (2). Over the past decade, there has been an exponentially increasing number of publications reporting the occurrence of *Klebsiella pneumoniae* carbapenemase (KPC) enzymes and New Delhi metallo-β-lactamase-1 (NDM-1) carbapenemases in *E. cloacae* (3). Strains carrying these enzymes often also harbor a diversity of resistance levels to other classes of antibiotics, resulting in the development of extensively drug-resistant (XDR) and pan-drug-resistant (PDR) *E. cloacae*.

The development of such extensive resistance, coupled with a lack of novel antibiotics in the drug development pipeline, has created a demand for a reevaluation of current antibiotic regimens (4). Polymyxin B is a polypeptide antibiotic commercially released in the 1950s (5). Despite being available for more than 50 years, it has only recently been revived as a last resort against extensively drug-resistant Gram-negative bacteria (XDR-GNB). The use of polymyxin B as a monotherapy against XDR-GNB is debatable. Increasing reports of polymyxin heteroresistance against several Gram-negative organisms have suggested that rapid resistance to polymyxins can develop as a result of polymyxin B monotherapy, and that polymyxin B is best administered as part of a combina-

tion therapy (6, 7). Unfortunately, to date the optimal antibiotic combinations against XDR *E. cloacae* remain unclear. While there is an increasing number of studies exploring effective combinations against XDR *E. cloacae*, most studies reported effective combinations *in vitro* against XDR *E. cloacae* using time-kill studies (TKS) or checkerboard assays, and none have validated the suggested combinations in a hollow-fiber infection model (HFIM), where the bacteria are subjected to clinically relevant fluctuating concentrations (8, 9).

In this study, we (i) investigated the *in vitro* activity of various antibiotics in combination with polymyxin B using TKS against drug-resistant *E. cloacae* using clinically relevant antibiotic con-

Received 2 February 2016 Returned for modification 22 April 2016

Accepted 11 June 2016

Accepted manuscript posted online 20 June 2016

Citation Cai Y, Lim T-P, Teo J, Sasikala S, Lee W, Hong Y, Chan ECY, Tan TY, Tan T-T, Koh TH, Hsu LY, Kwa AL. 2016. *In vitro* activity of polymyxin B in combination with various antibiotics against extensively drug-resistant *Enterobacter cloacae* with decreased susceptibility to polymyxin B. *Antimicrob Agents Chemother* 60:5238–5246. doi:10.1128/AAC.00270-16.

Address correspondence to Andrea L. Kwa, andrea.kwa.lh@sgh.com.sg.

\* Present address: Sasikala Suranthran, A\*STAR, Biopolis, Singapore; Yanjun Hong, Hong Kong Baptist University, Hong Kong; Li-Yang Hsu, Mount Elizabeth Novena Specialist Centre, Singapore.

Y.C. and T.-P.L. contributed equally to this study.

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TABLE 1 Simulated antibiotic dosing regimens and corresponding drug concentrations

Drug(s)	Simulated dosing regimen	Concn (mg/liter)	Reference
Amikacin	15–20 mg/kg of body wt every 24 h	80	13
Cefepime	2 g every 8 h	200	14
Levofloxacin	750 mg every 24 h	8	15
Rifampin	600 mg every 12 h	2	16
Tigecycline	100 mg every 12 h	2	17
Meropenem	2 g every 8 h (infused over 3 h)	64	18
Polymyxin B	30,000 IU/kg/day or at least 1 MU every 12 h	2	19
Imipenem	1 g every 6 h (infused over 0.5 h)	32	20
Doripenem	1 g every 8 h (infused over 4 h)	13	21
Aztreonam	6 g every 24 h (infused over 24 h)	18	22
Piperacillin-tazobactam	4.5 g every 6 h (infused over 4 h)	75/15	23

centrations, (ii) validated the findings in an HFIM, where the organisms will be subjected to clinically relevant fluctuating drug concentrations, and (iii) detected further emergence of resistance of these XDR *E. cloacae* strains when subjected to the clinically relevant fluctuating drug concentrations and characterized the phenotypic changes and overall biofitness associated with this further emergence of resistance development.

(This study was presented in part at the 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 9 to 12 September 2012, and the 23rd European Congress of Clinical Microbiology and Infectious Diseases, Berlin, Germany, 27 to 30 April 2013.)

## MATERIALS AND METHODS

**Bacterial isolates.** Four nonclonal clinical carbapenem-resistant *E. cloacae* strains (ECL9800, ECL15118, ECL2197, and ECL6217) employed in this study were identified from a 1,700-bed tertiary public hospital in Singapore from 2009 to 2011, collected as part of a national surveillance study. Genus identity was previously determined using Vitek 2 ID-GN cards (bioMérieux, Inc., Hazelwood, MO). The isolates were stored at  $-70^{\circ}\text{C}$ , and fresh isolates were subcultured twice on 5% blood agar plates (Thermo Scientific, Malaysia) for 24 h at  $35^{\circ}\text{C}$  before each experiment.

**Susceptibility testing and molecular mechanisms of resistance.** MICs of all tested antibiotics were determined for all isolates using commercial custom-made broth microdilution panels (Trek Diagnostics, East Grinstead, United Kingdom), performed in accordance with the manufacturer's recommendations. The studies were conducted in duplicate and were repeated at least once on a separate day. Fresh isolates were subcultured twice on 5% blood agar plates for 24 h at  $35^{\circ}\text{C}$  before MIC testing. A multiplex PCR assay with five different primer pairs was employed to detect genes encoding commonly acquired metallo- $\beta$ -lactamases (MBLs) (*bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>SIM</sub>, *bla*<sub>GIM</sub>, and *bla*<sub>SPM</sub>) (10). The presence of genes encoding extended-spectrum beta-lactamases (ESBLs), plasmid-mediated AmpCs, NDM, and KPCs were determined using PCR (10). Changes in porin gene expression (OmpC and OmpF) were determined using reverse transcriptase PCR, and the presence of efflux pumps was determined using the efflux pump inhibitor phenyl-arginine- $\beta$ -naphthylamide (PA $\beta$ N) (50  $\mu\text{g}/\text{ml}$ ) (11, 12).

**Antimicrobial agents.** A total of 11 antibiotics were employed (Table 1). Stock solutions of all antimicrobial agents except tigecycline and rifampin were prepared in sterile water, aliquoted, and stored at  $-70^{\circ}\text{C}$ . Tigecycline in solution was freshly prepared before each experiment. Rifampin was dissolved in dimethyl sulfoxide (DMSO) and was then serially diluted in sterile water to the desired final drug concentration. The final DMSO concentration ( $<1\%$ , vol/vol) had no effect on *E. cloacae* growth. Prior to each experiment, the drug was thawed and diluted to the desired concentrations with cation-adjusted Mueller-Hinton II broth (Ca-MHB) (BBL, Sparks, MD).

**TKS.** TKS was performed for each antibiotic individually and in 2-drug combination with polymyxin B, using clinically achievable unbound concentrations (13–23). The simulated steady-state free peak concentrations of the following antibiotics and their corresponding representative doses are shown in Table 1. To perform TKS, an overnight bacterial culture was prepared using Ca-MHB and incubated at  $35^{\circ}\text{C}$  until log-phase growth. The bacteria were further diluted, and 15 ml was transferred to sterile flasks, each containing 1 ml of drug(s) at 16 times the target concentration. This gave a final inoculum concentration of approximately  $5 \log_{10}$  CFU/ml ( $1 \times 10^5$  CFU/ml to  $5 \times 10^5$  CFU/ml) in each flask. The flasks were incubated in a shaker water bath at  $35^{\circ}\text{C}$ , and serial samples were obtained in duplicate at 0, 2, 4, 8, and 24 h. Each sample was centrifuged at  $10,000 \times g$  for 15 min (Eppendorf 5417R centrifuge; Hamburg, Germany) and reconstituted with sterile normal saline to its original volume to minimize drug carryover effect. Total bacterial count was quantified by depositing serial 10-fold dilutions of the broth sample onto Mueller-Hinton agar (MHA) plates using a spiral plater (Interscience, St. Nom La Breteche, France). Inoculated plates were incubated at  $35^{\circ}\text{C}$  for 18 to 24 h and enumerated visually. The lower limit of detection for the colony counts was  $2.6 \log_{10}$  CFU/ml.

**HFIM.** To validate the activity of bactericidal combinations against the XDR *E. cloacae* strains in our TKS, an HFIM in which the bacteria were exposed to clinically relevant fluctuating drug concentrations over time (due to repeated dosing and constant elimination) was employed. A schematic diagram of the HFIM has been described previously (24). The antibiotic(s) was directly injected into the central reservoir to clinically achievable peak concentrations. Fresh, drug-free Ca-MHB was continuously infused from the diluent reservoir into the central reservoir to simulate drug elimination in humans, with equal volumes of drug-containing medium removed concurrently to maintain an isovolumetric system. Bacteria were inoculated into the extracapillary compartment of the hollow-fiber cartridge (Fibercell Systems, Inc., Frederick, MD). The bacteria were confined to the extracapillary compartment but exposed to the fluctuating drug concentration(s) in the central reservoir by means of an internal circulatory pump in the bioreactor loop. The experimental setup was slightly modified for the combination setup to account for the different half-lives ( $t_{1/2}$ ) of the two antibiotics (25).

**Experimental setup.** Two XDR *E. cloacae* strains (ECL15118 and ECL9800) were employed in the HFIM testing. HFIM testing was conducted for up to 240 h in a humidified incubator set at  $35^{\circ}\text{C}$ . To prepare the inoculum, overnight cultures of the isolates were prepared as described above, and 20 ml of each *E. cloacae* suspension was inoculated into the extracapillary compartment of the hollow-fiber cartridge. The final inoculum concentration in the extracapillary compartment was approximately  $5 \log_{10}$  CFU/ml ( $1 \times 10^5$  CFU/ml to  $5 \times 10^5$  CFU/ml). The most promising combination regimen based on the activity in TKS was selected for the HFIM experiment. For objective comparison, the respective single-drug regimens of the selected combination and a placebo control were tested. Each infection model was subjected to different drug exposures,

TABLE 2 Polymyxin B and tigecycline MICs (mg/liter) and mechanisms of resistance in the 4 XDR *E. cloacae* strains

<i>Enterobacter cloacae</i> strain	MIC (mg/liter)		Resistance mechanism(s)
	Polymyxin B MIC	Tigecycline MIC	
ECL15118	1	2	SHV, TEM, NDM; porin loss
ECL9800	4	4	SHV, TEM, IMP-1; presence of efflux; porin loss
ECL6217	1	0.5	NDM; presence of efflux; porin loss
ECL2197	1	1	SHV, TEM, IMP-1; presence of efflux; porin loss

simulating steady-state pharmacokinetic profiles of unbound drug corresponding to clinical doses simulated in the TKS, with maintenance doses to reattain the targeted concentrations. The effectiveness of the different regimens was compared based on the observed viable bacterial burden over time, as described below.

**Microbiological response.** To determine the effect of the drug exposures on the total and resistant bacterial burden over time in the HFIM, serial samples were obtained from each infection model at 0 (baseline), 4, 8, 12, 24 (predose), 28, 32, 36, 48, 60, 72, 84, 96, 120, 144, 168, 192, 216, and 240 h in duplicate and quantified as described above. For total bacterial population, the samples were cultured on drug-free MHA plates, while for resistant bacterial subpopulations, the samples were cultured on MHA supplemented with the exposed agent at 3 times the MIC of the organism.

**Pharmacokinetic validation and drug assay.** Serial drug samples were obtained from the central reservoir of the infection models and kept frozen at  $-70^{\circ}\text{C}$  until analysis (within 1 month from the completion of

the hollow-fiber infection model studies). Drug concentrations in these samples were assayed using a validated liquid chromatography-mass spectrophotometry (LC-MS) method. Assay of tigecycline was performed on an Agilent 1290 Infinity ultraperformance liquid chromatography (UPLC) system (Santa Clara, CA) interfaced with an Agilent 6430 triple-quadrupole mass spectrometer (QQQ MS) equipped with an electrospray ionization (ESI) source (Santa Clara, CA), as described in a previous study (26). For polymyxin B, the LC-MS consisted of an Agilent 1290 Infinity UPLC (Santa Clara, CA, USA) interfaced with the AB SCIEX QTRAP 5500 tandem mass spectrometer (MS/MS) (Framingham, MA, USA) equipped with an ESI source. The column and autosampler temperatures were maintained at  $60^{\circ}\text{C}$  and  $4^{\circ}\text{C}$ , respectively. The chromatographic column (Acquity UPLC BEH HILIC column;  $1.7\ \mu\text{m}$  volume, 2.1 mm by 100 mm; Waters, Milford, MA, USA) was operated with mobile phases consisting of 0.1% formic acid and 10 mM ammonium formate in acetonitrile (solvent A) and water (solvent B) delivered at 0.45 ml/min. The optimized elution conditions were a linear gradient of 5 to 35% B (0 to 2.5 min), isocratic conditions of 95% B (2.51 to 3.5 min), and reequilibration to 5% (3.51 to 4 min). Chromatographic peak integration was performed using the Analyst software. The concentration-time profiles of both antibiotics were modeled by fitting a one-compartment linear model to the observations using ADAPT II software (27).

**Emergence-of-resistance studies.** To characterize the resistant strains isolated during the course of HFIM testing, resistant isolates recovered from antibiotic-supplemented plates (at baseline and throughout the experiment) were stored, and MIC testing with the agent used was repeated on at least two bacterial colonies to determine the presence of resistance. Susceptibility to the other antimicrobial agents was performed using commercial custom-made broth microdilution panels, as described above, to look for potential changes in resistance. To determine the stability of the resistant phenotypes, resistant isolates (if present) were subjected to 20 days of passaging on drug-free MHA and MHA supplemented with the

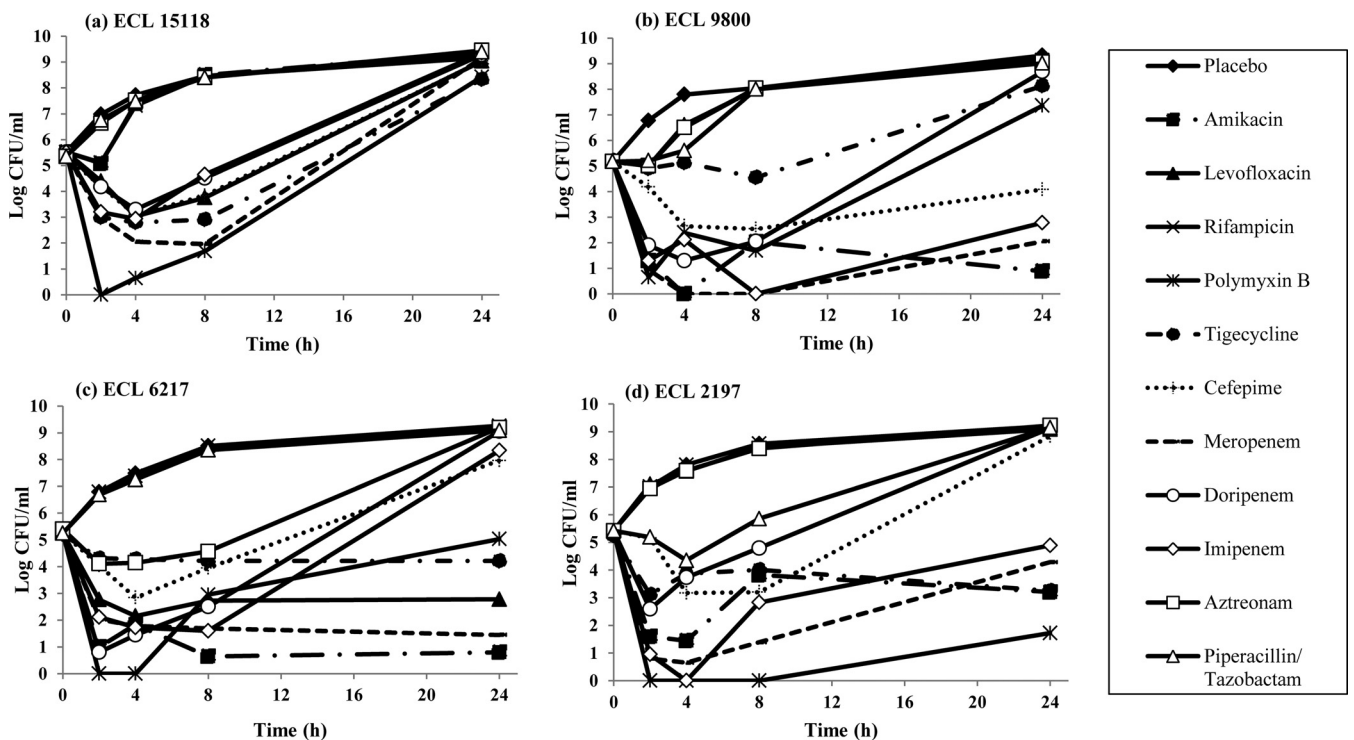


FIG 1 Microbiological response of ECL15118 (a), ECL9800 (b), ECL6217 (c), and ECL2197 (d) over 24 h when exposed to various single antibiotics in TKS. In single-drug TKS, amikacin (filled square) was bactericidal against ECL9800 and ECL6217 at 24 h, while meropenem (dash) and polymyxin B (asterisk) were bactericidal at 24 h against ECL6217 and ECL2197, respectively.

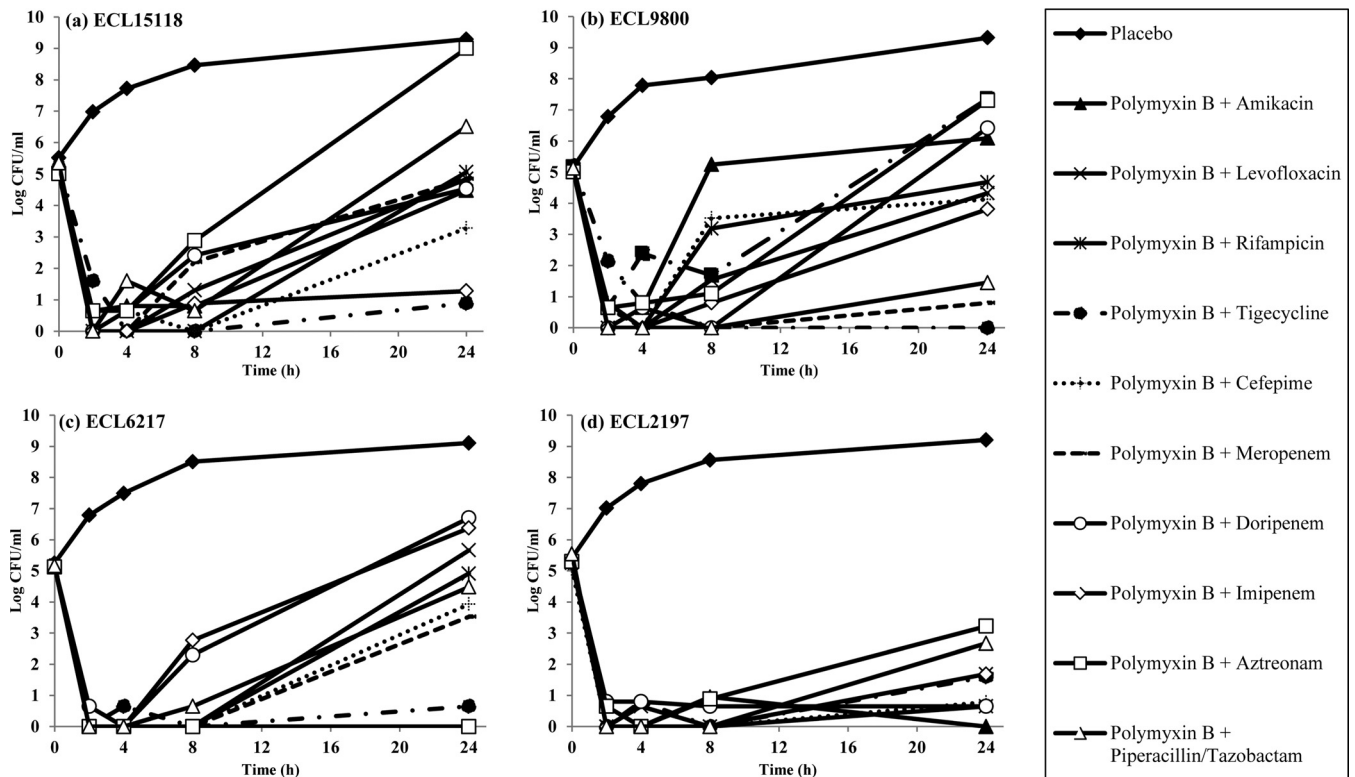


FIG 2 Microbiological response of ECL15118 (a), ECL9800 (b), ECL6217 (c), and ECL2197 (d) over 24 h when exposed to polymyxin B-containing combinations in TKS. Polymyxin B in combination with tigecycline (filled circle) was the only combination that was consistently bactericidal against all four *E. cloacae* isolates at 24 h.

exposed antibiotic at 3 times the MIC, and susceptibility testing for all of the antimicrobial agents was performed at the 10-day and 20-day time points. Finally, the growth kinetics of the resistant isolates was compared to that of the original isolate using *in vitro* time-growth studies. To carry out the time-growth studies, overnight culture of the isolates was prepared as described above, and 24 ml of the suspension was transferred to 50-ml sterile conical flasks until the final concentration of the bacterial suspension in each flask was approximately  $5 \log_{10}$  CFU/ml ( $1 \times 10^5$  CFU/ml to  $5 \times 10^5$  CFU/ml). The flasks were then incubated in a shaker water bath at 35°C, serial samples were obtained from each flask at 0 (baseline), 1, 2, 3, 4, 5, 6, and 24 h after incubation, and bacterial load was determined by quantitative cultures as described above. The exponential growth of the bacterial population over 24 h was analyzed using an adapted mathematical model (28).

**Definitions and pharmacodynamic endpoints.** XDR was defined as nonsusceptibility to at least one agent in all but two or fewer antimicrobial categories (29). Bactericidal activity (primary endpoint) was defined as a 3  $\log_{10}$  CFU/ml decrease (99.9% kill) in the colony count from the initial inoculum at 24 h (30). Synergy (secondary endpoint) was defined as a 2  $\log_{10}$  CFU/ml decrease in the colony count by the drug combination compared with its most active constituent and a 2  $\log_{10}$  CFU/ml decrease from the initial inoculum at 24 h, while 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 (30).

## RESULTS

### Susceptibility testing and molecular mechanisms of resistance.

All isolates were resistant to penicillins, cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides (data not shown). No interpretative standards are provided by the Clinical and Laboratory Standards Institute (CLSI) for *Enterobacteriaceae* against

polymyxin B or tigecycline. The MICs of the four isolates for polymyxin B and tigecycline, as well as molecular mechanisms of resistance, are shown in Table 2. Of note, all four XDR *E. cloacae* isolates harbored genes encoding metallo-beta-lactamases (two with *bla*<sub>IMP-1</sub>, two with *bla*<sub>NDM-1</sub>). Reductions in OmpC and OmpF porin expression were observed in all four isolates. Addition of PAβN resulted in a decrease in levofloxacin MICs in three strains, suggesting the presence of efflux pumps in these strains.

**TKS.** In single-drug TKS, all antibiotics alone demonstrated regrowth at 24 h, except amikacin against ECL9800 and ECL6217 and meropenem and polymyxin B against ECL6217 and ECL2197, respectively (Fig. 1). In combination time-kill studies, few two-drug combinations (2 to 3 combinations for each strain) were found to be bactericidal against ECL15118, ECL9800, and ECL6217. In contrast, a large number of two-drug combinations showed bactericidal activity against ECL2197. Polymyxin B plus tigecycline was the only combination that was consistently bactericidal against all four *E. cloacae* isolates, and it demonstrated synergism against 3/4 strains. Polymyxin plus amikacin, polymyxin plus meropenem, and polymyxin plus imipenem were bactericidal against 2/4 isolates and demonstrated synergism in 2/4, 1/4, and 1/4 isolates, respectively (Fig. 2).

**Hollow-fiber infection model.** Polymyxin B plus tigecycline was chosen for HFIM studies, as it was the only two-drug combination that was consistently bactericidal against all four *E. cloacae* isolates. The time courses of bacterial burden for ECL15118 and ECL9800 against polymyxin B and tigecycline are shown in Fig. 3. Overall, observations in HFIM were in agreement with findings in

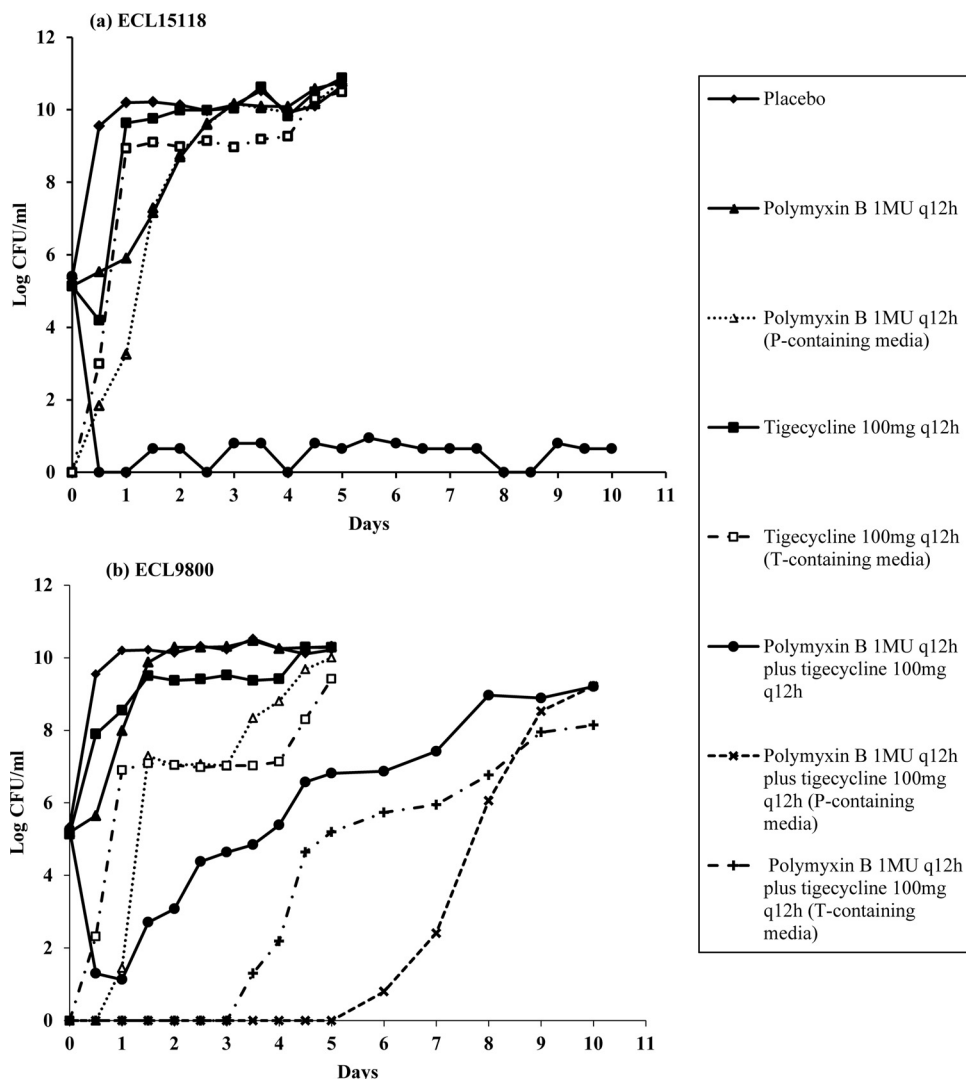


FIG 3 Microbiological responses of ECL15118 (a) and ECL9800 (b) against polymyxin B monotherapy, tigecycline monotherapy, and polymyxin B in combination with tigecycline in a hollow-fiber infection model.

the TKS: against both isolates, both tigecycline and polymyxin B alone did not exhibit any killing activity despite repeated dosing. In comparison, when polymyxin B was used in combination with tigecycline, bactericidal killing was seen at 24 h for both isolates; this suppression was sustained for ECL15118 for up to 240 h. For ECL9800, a slow but apparent regrowth was evident despite repeated dosing, reaching  $9 \log_{10}$  CFU/ml at 240 h. This increase in total bacterial burden was accompanied by an increase in polymyxin B- and tigecycline-resistant subpopulations in the respective drug-supplemented media.

**Pharmacokinetic validation.** Simulated drug exposures in the infection models were satisfactory (Fig. 4). Simulated drug exposures had an  $r^2$  value of 0.90 to 0.91; the simulated polymyxin B and tigecycline half-lives were 6.5 h and 34 h, respectively. Intraday and interday accuracy for the lower-quality control and higher-quality control samples had a coefficient of variation (CV) of <5% (data not shown).

**Emergence-of-resistance studies.** The MICs of isolates recovered from antibiotic-supplemented plates are shown in Table 3.

As expected, an increase in MICs to the respective antibiotics was observed in isolates postexposure to polymyxin B alone [ECL15118<sub>(post-P)</sub>,  $\geq 32$  mg/liter; ECL9800<sub>(post-P)</sub>, 8 mg/liter] and tigecycline alone [ECL15118<sub>(post-T)</sub>, 8 mg/liter, ECL9800<sub>(post-T)</sub>, 8 mg/liter]. Cross-resistance to other antimicrobial agents in the screening panel was not observed. For ECL9800 isolates recovered postexposure to polymyxin B in combination with tigecycline [ECL9800<sub>(post-P+T)</sub>], an increase in MIC to both polymyxin B (16 mg/liter) and tigecycline was observed (16 mg/liter). Susceptibilities to all other antibiotics remained the same. MICs of the postexposure isolates remained similar at day 10 and day 20 of serial passage on drug-free media, which confirmed the stability of the resistant phenotypes in the postexposure mutant strains.

The mean best-fit growth rate constant ( $K_g$ ) for ECL15118 and ECL9800 was  $1.57 \text{ h}^{-1}$  and  $1.66 \text{ h}^{-1}$ , respectively (Table 3). A similar growth rate was observed in the ECL15118<sub>(post-T)</sub> strains; in the ECL15118<sub>(post-P)</sub> strains, however, a lower  $K_g$  of  $1.44 \text{ h}^{-1}$  was observed, suggesting the development of a substantial biofitness deficit as resistance to polymyxin B developed. For ECL9800,

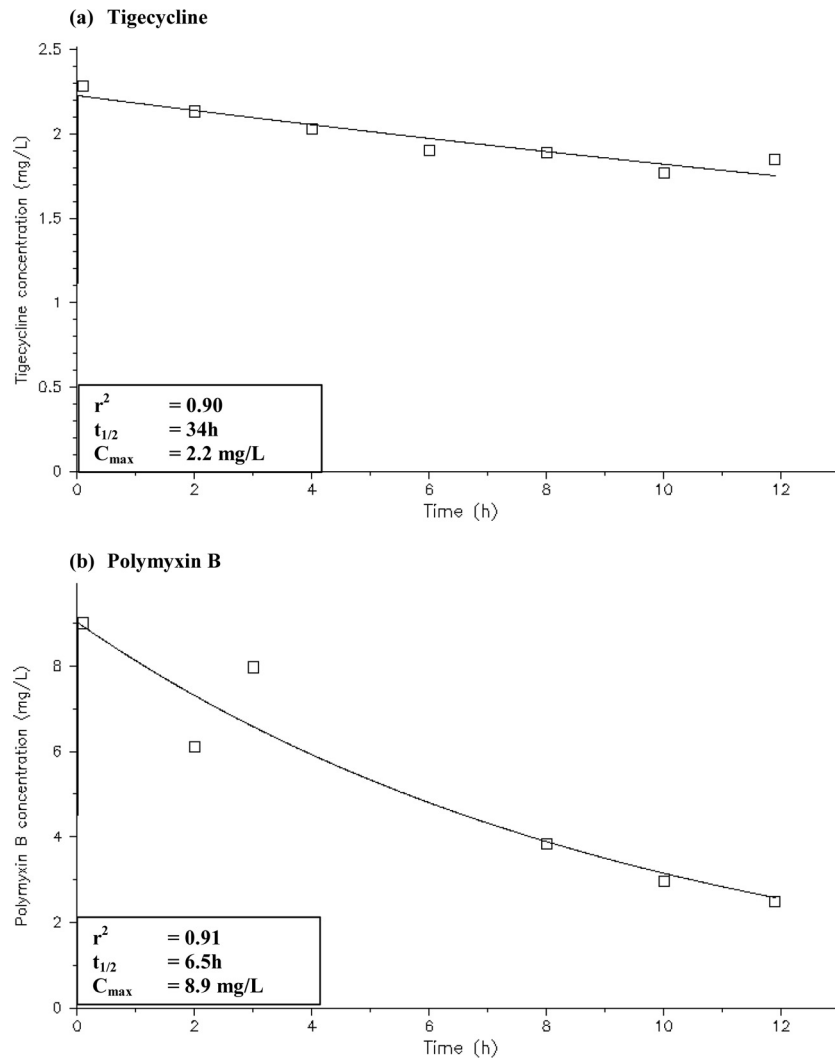


FIG 4 Pharmacokinetic profiles of the infection models tigecycline at 100 mg every 12 h (a) and polymyxin B at 30,000 IU/kg/day or at least 1 MU every 12 h (b).

lower  $K_g$  values were observed for all postexposure mutant strains [ECL9800<sub>(post-P)</sub>, 1.50 h<sup>-1</sup>; ECL9800<sub>(post-T)</sub>, 1.56 h<sup>-1</sup>; ECL9800<sub>(post-P+T)</sub>, 1.40 h<sup>-1</sup>], suggesting loss of biofitness in all strains after drug exposure as resistance to either polymyxin B and/or tigecycline developed.

## DISCUSSION

The emergence of novel beta-lactamases with direct carbapenem-hydrolyzing capabilities in *Enterobacteriaceae* constitutes an important public health threat (1). Genes encoding these beta-lactamases are usually associated with various mobile genetic structures, permitting their ease of dissemination into different *Enterobacteriaceae* species (2). As infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE) are associated with limited treatment options, combination antimicrobial therapy has been increasingly employed as the first-line therapy against CRE (2). However, the selection of the appropriate combination is a challenging process; in addition to achieving rapid bacterial kill, the selected combination should optimally suppress the development of resistance in order to preserve the utility of the antibiotic combination in the treatment of subsequent infection episodes (31).

To the best of our knowledge, ours is the first study to evaluate the activity of combinations *in vitro* against XDR *E. cloacae* using clinically relevant fluctuating drug concentrations. Unlike previous *in vitro* studies which had been conducted in KPC-producing carbapenem-resistant *E. cloacae* strains, our study focused on metallo-beta-lactamase (IMP and NDM)-harboring XDR *E. cloacae* strains, providing a fresh perspective compared to previous studies (8, 32). In addition, our study investigated the activity of polymyxin B in combination with various antibiotics, which is in contrast to most previous studies, which employed colistin in combination with other antibiotics against XDR *E. cloacae* (8, 9). Polymyxin B was employed in preference to colistin, as it has been shown to have superior clinical pharmacokinetics characteristics for infections (33). Unlike colistin, which may take several hours to reach desired peak concentrations even with loading doses, polymyxin B can rapidly achieve a desired plasma concentration that may then be maintained with a suitable daily dosage regimen (33). In addition, recent studies have suggested that polymyxin B is minimally eliminated renally; hence, adjustment of daily doses may not be required in patients with renal function (33, 34). As

TABLE 3 Emergence of resistance studies<sup>a</sup>

Strain	$K_g$ (h <sup>-1</sup> )	MIC										
		Amikacin	Levofloxacin	Rifampin	Polymyxin B	Tigecycline	Cefepime	Meropenem	Doripenem	Imipenem	Aztreonam	Ampicillin-sulbactam
ECL15118	1.57	≥128	≥64	≥64	1	2	≥32	≥16	≥32	≥128	≥128/4	≥256/4
ECL15118 (post-polymyxin B exposure)	1.44	≥128	≥64	≥64	≥32	1	≥32	≥16	≥32	≥128	≥128/4	≥256/4
ECL15118 (post-tigecycline exposure)	1.57	≥128	≥64	≥64	1	8	≥64	≥16	≥32	≥128	≥128/4	≥256/4
ECL9800	1.66	16	≥64	≥64	4	4	256	≥16	16	≥128	≥128/4	≥256/4
ECL9800 (post-polymyxin B exposure)	1.5	16	≥64	≥64	8	4	256	≥16	16	≥128	≥128/4	≥256/4
ECL9800 (post-tigecycline exposure)	1.56	16	≥64	≥64	4	8	256	≥16	16	≥128	≥128/4	≥256/4
ECL9800 (post-polymyxin B plus tigecycline exposure)	1.4	16	≥64	≥64	16	16	256	≥16	16	≥128	≥128/4	≥256/4

<sup>a</sup> Shown are the susceptibility and growth rate of ECL15118 and ECL9800, pre- and post-exposure to polymyxin B, tigecycline, and polymyxin B in combination with tigecycline.

adequate dosing of antibiotics is pertinent in the treatment of CRE infections, we employed maximally clinically achievable free or unbound concentrations simulating maximally possible antibiotic doses for all tested antibiotics (Table 1). These concentrations were simulated based on parameters and results reported in previously published population pharmacokinetics studies and ensured that the killing effect observed in our TKS mimicked as closely as possible the killing that takes place *in vivo* (13–23).

In our TKS, we found that polymyxin B in combination with tigecycline is the most promising combination, demonstrating bactericidal activity against all four XDR *E. cloacae* strains at 24 h when exposed to static drug concentrations. As TKS poorly reflected antibiotic concentrations over time *in vivo*, we further validated the clinical applicability of the combinations in an HFIM. As expected, both tigecycline and polymyxin B monotherapies result in rapid regrowth for both strains, which was accompanied by the selective amplification of the resistant subpopulation over time, suggesting a strong selection pressure when nonoptimal antimicrobial regimens were used. The findings in our HFIM generally concurred with that of our TKS results, demonstrating bactericidal killing in both XDR *E. cloacae* strains in the HFIM at 24 h when polymyxin B was administered in combination with tigecycline. Interestingly, beyond 24 h, a slow and persistent regrowth was observed for ECL9800 but not ECL15118. This suggested the overall activity of antibiotic combinations was strain specific, and that prolonged and indiscriminate use of antibiotic combinations with initial bactericidal activity can result in the emergence of resistance in some XDR *E. cloacae* strains. The regrowth in ECL9800 was associated with the total bacterial population being replaced by mutants with reduced susceptibility to both polymyxin B and tigecycline over time, which ensued in the subsequent development of a polymyxin B- and tigecycline-resistant *E. cloacae* strain.

Upon further characterization of the resistant mutants, we found that the resistance phenotype was stable and did not revert to the susceptible phenotype despite repeated passages in drug-free media. This finding is similar to results published in a recent study by Lim et al. and is in contrast to previous findings in *Pseudomonas aeruginosa*, where susceptibility reversal was observed upon serial passage on drug-free medium (26, 35). Our findings suggest that the polymyxin B resistance in the XDR *E. cloacae* isolates is mutational and not adaptive in nature; however, this remains to be further studied. We observed a reduction in growth rate in most of the resistant mutants compared to the parent phenotype. This suggested that the development of several resistance mutations was associated with a corresponding biological fitness cost (36). Moving forward, the potential loss of virulence that may be associated with such loss of biofitness will be further explored.

To date, there have been a limited number of studies exploring antimicrobial combinations against XDR *E. cloacae*, and they were mainly conducted in KPC-harboring *E. cloacae* isolates. In a recent TKS study, polymyxin B plus carbapenems was found to be bactericidal against 6 KPC-producing CRE isolates, including two *E. cloacae* strains with high-level resistance to carbapenems (imipenem MIC, 64 mg/liter) (32). Polymyxin B plus tigecycline also showed bactericidal effect against both *E. cloacae* isolates; nonetheless, the reduction in colony count was lower than that of polymyxin B plus carbapenems (32). In another study, Betts et al. explored the activity of colistin plus tigecycline against CRE, including a tigecycline-resistant *E. cloacae* strain, by using standard

checkerboard and time-kill assays and a simple invertebrate model (8). They found that colistin plus tigecycline was synergistic against the tigecycline-resistant *E. cloacae* strain *in vitro*, with significantly improved survival compared to either antibiotic when administered as a monotherapy. Interestingly, these were similar to our study findings, despite our XDR *E. cloacae* isolates having different underlying mechanisms of resistance (metallo- $\beta$ -lactamases). The main advantages of our study were that, first, in addition to determining the activity of the potential combinations using static drug concentrations, we validated the combination in an *in vitro* HFIM system, subjecting the bacteria to humanized drug exposures. Second, we further characterized the resistant strains isolated during the course of testing, adding to the scarce literature on the properties of the resistant subpopulations in XDR *E. cloacae* that may develop during the course of treatment.

As with all *in vitro* studies, the main limitation of our study is that the clinical relevance of our findings, including the virulence of the resistant subpopulation, will need to be further confirmed. In addition, the usefulness of the TKS and HFIM studies is limited by their labor-intensive nature, resulting in an inability to provide combination results in a clinically relevant time frame. Moving forward, a reliable *in vitro* methodology with a rapid turnaround time will be valuable in identifying effective combinations for the treatment of patients with XDR *E. cloacae* infections.

**Conclusion.** In our study, we found that polymyxin B in combination with tigecycline is a promising treatment against XDR *E. cloacae*. However, it must be highlighted that prolonged and indiscriminate use, especially if employed at suboptimal doses, can result in the emergence of resistance strains. Future studies investigating polymyxin B combinations in animal models and in patients, with additional studies to characterize the virulence of the resistance strains, will be required.

## ACKNOWLEDGMENTS

We thank the staff of Singapore General Hospital and Changi General Hospital for assisting in collection of the bacterial isolates.

A.L.K., T.-P.L., and W.L. have received funding for research from Pfizer Inc. This work was supported in part by a Singapore General Hospital Research Grant (SRG/C3/02/2014), a National Medical Research Council Centre Grant (NMRC/CG/016/2013), and unrestricted grant funding from Pfizer Inc. (WS 832447 and WS 776979).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We have no conflicts of interest to declare.

## FUNDING INFORMATION

This work, including the efforts of Tze-Peng Lim, Jocelyn Teo, Winnie Lee, and Andrea L. Kwa, was funded by MOH | National Medical Research Council (NMRC) (NMRC/CG/016/2013). This work, including the efforts of Andrea L. Kwa, was funded by Pfizer (Pfizer Inc.) (WS 832447 and WS 776979). This work, including the efforts of Yiyi Cai, Tze-Peng Lim, Jocelyn Teo, and Winnie Lee, was funded by Singapore General Hospital (SGH) (SRG/C3/02/2014).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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