In Vitro Pharmacodynamics of Various Antibiotics in Combination against Extensively Drug-Resistant *Klebsiella pneumoniae*

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Extensively drug-resistant (XDR) *Klebsiella pneumoniae* is an emerging pathogen in Singapore. With limited therapeutic options available, combination antibiotics may be the only viable option. In this study, we aimed to elucidate effective antibiotic combinations against XDR *K. pneumoniae* isolates. Six NDM-1-producing and two OXA-181-producing *K. pneumoniae* strains were exposed to 12 antibiotics alone and in combination via time-kill studies. A hollow-fiber infection model (HFIM) with pharmacokinetic validation was used to simulate clinically relevant tigecycline-plus-meropenem dosing regimens against 2 XDR *K. pneumoniae* isolates over 240 h. The emergence of resistance against tigecycline was quantified using drug-free and selective (tigecycline at 3× the MIC) media. The *in vitro* growth rates were determined and serial passages on drug-free and selective media were carried out on resistant isolates obtained at 240 h. Both the polymyxin B and tigecycline MICs ranged from 1 to 4 mg/liter. In single time-kill studies, all antibiotics alone demonstrated regrowth at 24 h, except for polymyxin B against 2 isolates. Tigecycline plus meropenem was found to be bactericidal in 50% of the isolates. For the isolates that produced OXA-181-like carbapenemases, none of the 55 tested antibiotic combinations was bactericidal. Against 2 isolates in the HFIM, tigecycline plus meropenem achieved a >90% reduction in bacterial burden for 96 h before regrowth was observed until 10⁹ CFU/ml at 240 h. Phenotypically stable and resistant isolates, which were recovered from tigecycline-supplemented plates post-HFIM studies, had lower growth rates than those of their respective parent isolates, possibly implying a substantial biofitness deficit in this population. We found that tigecycline plus meropenem may be a potential antibiotic combination for XDR *K. pneumoniae* infections, but its efficacy was strain specific.

Broad-spectrum resistance to antimicrobials is a well-recognized problem among Gram-negative bacteria (GNB) worldwide and in Singapore (1, 2). In a recent study published in 2012, our group found a significant trend of increasing carbapenem resistance in *Enterobacteriaceae* in Singapore (3). In fact, this trend started to emerge since 2008 (4). In the era of rising antimicrobial resistance, coupled with a continued dwindling pipeline of drugs to treat these infections (5), it is evident that combination therapy will be the most likely approach for treatment against carbapenem-resistant *Enterobacteriaceae* (CRE) infections.

Carbapenems are broad-spectrum antimicrobial agents that have potent microbiological activities against a wide variety of bacteria. They have increasingly been used as first-line therapies in institutions where there are high levels of resistance to other classes of antimicrobial agents (aminoglycosides, fluoroquinolones, and cephalosporins) (6). However, resistance to carbapenems has also emerged, and there have been reports of outbreaks involving carbapenem-resistant GNB (7–10). The carbapenems, especially ertapenem, have been used in the treatment of multidrug-resistant (MDR) *Enterobacteriaceae*. However, the increasing prevalence of CRE has rendered them ineffective when used alone.

Carbapenemase-producing *Klebsiella pneumoniae* strains are of grave clinical concern, as this frequently occurring pathogen is associated with severe nosocomial infections (8, 11–13). The more common resistance mechanisms include *K. pneumoniae*-producing carbapenemases (KPC), OXA-48 type carbapenemases, metallo-β-lactamases (MBLs) consisting mainly of IMP-type and VIM-type MBLs, and New Delhi-type metallo-β-lactamases (NDM). Recent studies have demonstrated clinical efficacy in the treat-

ment of serious infections with the use of combination therapy (14–16). However, the recommendations have been mixed, and there is no consensus of which common antibiotic combinations should be employed against these infections. In addition, most of the studies examined *K. pneumoniae* strains that produce specific carbapenemases, but these organisms still retained susceptibility to some antibiotics (14–16). The primary aim of this study was to evaluate various antibiotic combinations and identify the effective combinations to be validated via the hollow-fiber infection model (HFIM) against extensively drug-resistant (XDR) *K. pneumoniae* that produces a variety of carbapenemases (17). The secondary aim of the study was to detect further the emergence of resistance of these XDR *K. pneumoniae* strains when exposed to clinically relevant fluctuating concentrations of the aforementioned effec-

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TABLE 1 Simulated antibiotic concentrations used in time-kill studies

Antibiotic (reference)	Simulated concn (mg/liter)
Amikacin (23)	65
Levofloxacin (25)	8
Rifampin (20)	2
Polymyxin B (21)	2
Tigecycline (22)	2
Cefepime (26)	200
Meropenem (30)	64
Doripenem (31)	13
Imipenem (27)	32
Aztreonam (24)	18
Piperacillin-tazobactam (29)	35/7
Ertapenem (28)	15

tive antibiotic combination and to evaluate the overall biofitness associated with this further emergence of resistance.

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MATERIALS AND METHODS

Microorganisms. The clinical *K. pneumoniae* isolates used in the study were isolates that had been identified from 2 major public hospitals (1,800-bed and 800-bed hospitals) from November 2008 to December 2013. This pool of isolates was collected as part of a national surveillance study. Eight XDR *K. pneumoniae* isolates were selected for this study. They were previously described as harboring NDM-1 and OXA-181 carbapenemases (3). The bacteria were stored at -70°C in Microbank (Pro-Lab Diagnostics, Round Rock, TX, USA) storage vials. Fresh isolates were

subcultured twice on 5% blood agar plates (Thermo Scientific, Singapore) for 24 h at 35°C prior to each experiment.

Susceptibility studies. The MICs to various antibiotics were determined using commercial broth microdilution panels (Trek Diagnostics, East Grinstead, United Kingdom), performed according to the manufacturer's recommendations. Fresh isolates were subcultured twice on 5% blood agar plates for 24 h at 35°C before each experiment. Tigecycline susceptibility was performed using freshly prepared broth.

Antimicrobial concentrations. A total of 12 antibiotics were used in the study. They were tested singly and in 2-drug combinations at clinically relevant unbound concentrations (Table 1). Prior to each experiment, an aliquot of the drug was thawed and diluted to the desired concentrations with cation-adjusted Mueller-Hinton II broth (CA-MHB) (BBL, Sparks, MD).

Time-kill studies. Time-kill studies were conducted with various antibiotics alone and in combination at clinically achievable free drug concentrations (when maximum indicated clinical doses were used) in serum or tissue (20–31). The antibiotics were chosen based on the unique mechanism of action from the various antibiotic classes and the availability in our hospitals' pharmacies. The antibiotics chosen for the study included amikacin (15 to 20 mg/kg of body weight every 24 h), levofloxacin (750 mg every 24 h), rifampin (600 mg every 12 h), polymyxin B (25,000 to 30,000 IU [2.5 to 3.0 mg]/kg/day or ≥ 1 million units (MU) [100 mg] every 12 h), tigecycline 100 mg every 12 h, cefepime (2 g every 8 h), meropenem (2 g every 8 h as a 3-h infusion), doripenem (1 g every 8 h as a 4-h infusion), imipenem (1 g every 6 h as a 0.5-h infusion), aztreonam (6 g every 24 h as a 24-h infusion), and piperacillin-tazobactam (4.5 g every 6 h as a 4-h infusion). Time-kill studies involving ertapenem (1 g every 24 h as a 0.5-h infusion) were conducted with imipenem, meropenem, and doripenem alone and in combination to examine the "double-carbapenem strategy" proposed by Bulik and Nicolau (32). In total, we performed >500 time-kill experiments with 55 different antibiotic combinations for each isolate (Table 2).

An overnight culture of each isolate was diluted into prewarmed CA-MHB and incubated further at 35°C until reaching log-phase growth. The bacterial suspension was diluted with CA-MHB, according to its absor-

TABLE 2 Antimicrobial drug susceptibilities and resistance mechanism of 8 XDR *K. pneumoniae* isolates in Singapore

Drug	MIC (mg/liter) for <i>K. pneumoniae</i> strain ^a :							
	1883 (SHV-11, TEM-1, CTXM-1, NDM-1)	3229 (SHV-1, TEM-1, CTXM-1, NDM-1)	7433 (SHV-11, TEM-1, CTXM-1, DHA, NDM-1)	7522 (SHV-11, TEM-1, CTXM-1, NDM-1)	43320 (CTXM-1, NDM-1)	44591 (SHV-1, TEM-1, CTXM-1, CTXM-9, NDM-1)	21479 (OXA-181)	53879 (OXA-181, CTXM-15, CMY-4)
Amikacin	≥ 128	≥ 128	≥ 128	≥ 128	16	≥ 128	≥ 128	≥ 128
Gentamicin	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256
Levofloxacin	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64
Rifampin	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64
Polymyxin B	2	2	2	4	2	1	4	1
Tigecycline	4	2	4	2	1	4	2	4
Cefepime	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128
Meropenem	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64
Doripenem	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64
Imipenem	≥ 64	32	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64	≥ 64
Aztreonam	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128
Piperacillin-tazobactam	$\geq 256/4$	$\geq 256/4$	$\geq 256/4$	$\geq 256/4$	$\geq 256/4$	$\geq 256/4$	$\geq 256/4$	$\geq 256/4$
Ertapenem	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32
Ampicillin-sulbactam	$\geq 128/64$	$\geq 128/64$	$\geq 128/64$	$\geq 128/64$	$\geq 128/64$	$\geq 128/64$	$\geq 128/64$	$\geq 128/64$
Ciprofloxacin	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16
Ceftazidime	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128
Sulfamethoxazole-trimethoprim	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32

^a MICs were obtained using the broth microdilution method, according to CLSI guidelines. The resistance mechanisms are shown in parentheses after the strain designations.

bance (at 630 nm); 15 ml of the suspension was transferred to 50-ml sterile conical flasks, each containing 1 ml of a drug dilution at 16 times the target concentration. The final concentration of the bacterial suspension in each flask was approximately $5 \log_{10}$ CFU/ml (ranging from 1×10^5 CFU/ml to 5×10^5 CFU/ml).

The flasks were incubated in a shaker water bath at 35°C. Serial samples of broth were obtained from each flask at 0 (baseline), 2, 4, 8, and 24 h after incubation. The samples were obtained in duplicate at each time point. The extracted broth samples (0.5 ml) were first centrifuged at $10,000 \times g$ for 15 min and then reconstituted with sterile normal saline to their original volumes in order to minimize drug carryover. The total bacterial count for each sample was quantified by depositing serial 10-fold dilutions of broth samples onto Mueller-Hinton agar (MHA) plates (Thermo Scientific, Singapore) using a spiral plater (Spiral Biotech, Norwood, MA).

The inoculated plates were incubated in a humidified incubator (35°C) for 18 to 24 h, the bacterial colonies were visually counted, and the original bacterial density from the original sample was calculated based on the dilution factor. The lower limit of detection for the colony counts was $2.6 \log_{10}$ CFU/ml.

Pharmacodynamic endpoints. Bactericidal activity (primary endpoint) was defined as a $\geq 3\text{-log}_{10}$ CFU/ml decrease in the colony count at 24 h from that in the initial inoculum. Synergy (secondary endpoint) was defined as a $\geq 2\text{-log}_{10}$ CFU/ml decrease in the colony count by the drug combination compared with that of its most active constituent, as well as a $\geq 2\text{-log}_{10}$ CFU/ml decrease at 24 h from that of the initial inoculum, while indifference was defined as a $< 2\text{-log}_{10}$ CFU/ml change at 24 h by the combination compared with that by the most active single agent (33).

Hollow-fiber infection model. To selectively validate the quantitative assessment of combined killing with various antimicrobial agents in combination, a hollow-fiber infection model (HFIM) in which the bacteria were exposed to clinically relevant (fluctuating concentrations over time due to repeated dosing and constant elimination) drug exposures was used (34). Two bacterial isolates were used for the experiment. Drug(s) was directly injected into the central reservoir to reach clinically achievable peak concentrations. Fresh (drug-free) growth medium (CA-MHB) was continuously infused from the diluent reservoir into the central reservoir to dilute the drug in order to simulate drug elimination in humans. An equal volume of drug-containing medium was concurrently removed from the central reservoir to maintain an isovolumetric system. Bacteria were inoculated into the extracapillary compartment of the hollow-fiber cartridge (FiberCell Systems, Inc., Frederick, MD, USA); they were confined in the extracapillary compartment but were exposed to the fluctuating drug concentration in the central reserve by means of an internal circulatory pump in the bioreactor loop. The experimental setup was slightly modified if the elimination half-lives of the agents in a combination were considerably different (35).

Experimental setup. For each study, the inoculum was prepared as described above. Twenty milliliters of *K. pneumoniae* suspension at approximately 10^5 CFU/ml (ranging from 1×10^5 CFU/ml to 5×10^5 CFU/ml) was used. The experiment was conducted for 240 h in a humidified incubator set at 35°C. The infection models were subjected to different drug exposures simulating various steady-state pharmacokinetic profiles of unbound drugs. The maintenance doses were given according to the clinical dosing frequencies of the individual drugs to reattain the targeted clinically unbound concentration (Table 1).

Microbiological response. Serial samples were also obtained from each infection model at 0 (baseline), 4, 8, 12, 24, 28, 32, 36, 48, 60, 72, 84, 96, 120, 144, 168, 192, 216, and 240 h in duplicate. The total and resistant bacterial populations were determined in duplicate by quantitative culture, as described above, to examine the effects of various drug exposures on the bacterial population over time. For the total bacterial population, the samples were cultured on Mueller-Hinton agar (MHA) plates, while for the resistant bacterial subpopulations, the samples were cultured on MHA supplemented with the exposed agent at 3 times its MIC. Since

susceptibility testing was performed in serial 2-fold dilutions, and one 2-fold-dilution (two times the concentration) difference was commonly accepted as a reasonable interday variation, quantitative cultures on drug-supplemented (at 3 times the MIC) medium plates would allow for a reliable detection of bacterial subpopulations with reduced susceptibilities.

Pharmacokinetic validation. Serial samples were obtained from the infection models and kept frozen at -80°C until analysis. The drug concentrations in these samples were assayed by validated methods, as described below. The concentration-time profiles were modeled by fitting a one-compartment linear model to the observations using ADAPT II (36).

Drug assays. The liquid chromatography-mass spectrometry (LC-MS) system consisted of 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. The column and autosampler temperatures were maintained at 45°C and 4°C, respectively. The UPLC-tandem mass spectrometry (MS/MS) was controlled by the Agilent MassHunter Workstation software. Chromatographic separation was achieved using an Acquity UPLC ethylene bridged hybrid (BEH) high-strength silica (HSS) T3 1.8- μm , 100 by 2.1-mm inside diameter (i.d.) column (Waters). The mobile phases were 1% formic acid in water (solvent A) and acetonitrile (solvent B) delivered at 0.45 ml/min. The optimized elution conditions for the samples were linear gradient, 10 to 30% B (0 to 4.8 min); isocratic, 30% B (4.8 to 5.94 min), and reequilibration to 10% (5.95 to 6.5 min). The multiple-reaction monitoring (MRM) experimental parameters of all compounds were optimized using the Agilent MassHunter Optimizer software. Further UPLC-MS/MS analysis was performed to optimize the MRM parameters and chromatographic retention of the analytes and internal standard (ceftazidime). The MS source conditions for all compounds were as follows: gas temperature, 350°C; gas flow, 12 liters/min; nebulizer gas pressure, 40 lb/in.²; and capillary voltage 3,500 V. Chromatographic peak area integration was performed using the Agilent MassHunter quantitative analysis software.

Emergence of resistance studies. The resistant isolates recovered from the antibiotic-supplemented plates (at baseline and throughout the experiment) were stored, and testing of susceptibility (MIC) to the antibiotic was repeated to confirm the presence of resistance. In addition, testing of susceptibility to the other antimicrobial agents in the screening panel was performed to screen for potential changes in resistance. Next, resistant isolates in duplicate (if present) were subjected to 20 days of passaging on drug-free MHA and MHA supplemented with the antibiotic at 3 times the MIC. Testing of susceptibility to all the antimicrobial agents was performed at the 10-day and 20-day time points to screen for the stability of the resistance phenotypes.

In vitro time-growth studies and modeling of bacterial growth kinetics for overall biofitness assessment. An overnight culture of the original/postantibiotic exposure isolate was diluted into prewarmed CA-MHB and incubated further at 35°C until reaching log-phase growth. The bacterial suspension was diluted with CA-MHB according to absorbance (at 630 nm); 24 ml of the suspension was transferred to 50-ml sterile conical flasks. The final concentration of the bacterial suspension in each flask was approximately $5 \log_{10}$ CFU/ml (ranging from 1×10^5 CFU/ml to 5×10^5 CFU/ml).

The flasks were incubated in a shaker water bath at 35°C. Serial samples of broth were obtained from each flask at 0 (baseline), 1, 2, 3, 4, 5, 6, 8, and 24 h after incubation. The samples were obtained in triplicate at each time point. The quantification of the bacterial samples was performed as described above. The exponential growth of the bacterial population over 24 h was analyzed using an adapted mathematical model (37).

RESULTS

Susceptibility studies. All isolates were found to be extensively drug resistant. As there were no CLSI breakpoints for polymyxin B

TABLE 3 List of 55 antimicrobial combinations studied against the 8 XDR *K. pneumoniae* isolates

Antibiotic combination
Amikacin + Levofloxacin Rifampin Polymyxin B Tigecycline Cefepime Meropenem Doripenem Imipenem Aztreonam Piperacillin-tazobactam
Levofloxacin + Rifampin Polymyxin B Tigecycline Cefepime Meropenem Doripenem Imipenem Aztreonam Piperacillin-tazobactam
Rifampin + Polymyxin B Tigecycline Cefepime Meropenem Doripenem Imipenem Aztreonam Piperacillin-tazobactam
Polymyxin B + Tigecycline Cefepime Meropenem Doripenem Imipenem Aztreonam Piperacillin-tazobactam
Tigecycline + Cefepime Meropenem Doripenem Imipenem Aztreonam Piperacillin-tazobactam
Cefepime + Meropenem Doripenem Imipenem Aztreonam Piperacillin-tazobactam
Meropenem + Aztreonam Piperacillin-tazobactam

TABLE 3 (Continued)

Antibiotic combination
Doripenem + Aztreonam Piperacillin-tazobactam
Imipenem + Aztreonam Piperacillin-tazobactam
Aztreonam + Piperacillin-tazobactam
Ertapenem + Meropenem Doripenem Imipenem

and tigecycline, both the polymyxin B and tigecycline MICs ranged from 1 to 4 mg/liter (Table 3). The types of β -lactamases produced by these isolates are described in Table 2 (3, 38).

Time-kill studies. In single-drug time-kill studies, all antibiotics alone (amikacin, levofloxacin, rifampin, polymyxin B, tigecycline, cefepime, meropenem, doripenem, imipenem, aztreonam, piperacillin-tazobactam, and ertapenem) demonstrated regrowth at 24 h, except for polymyxin B against *K. pneumoniae* strain 3229 (Fig. 1). In the combination time-kill studies, meropenem plus tigecycline was bactericidal against 4/8 isolates, and polymyxin B plus meropenem, polymyxin plus imipenem, tigecycline plus amikacin, and cefepime plus doripenem were bactericidal in 3/8 isolates. The number of antibiotic combinations that were bactericidal ranged from 2 to 20 out of 55 combinations tested for 6 isolates (Fig. 2). Cefepime plus doripenem, cefepime plus imipenem, polymyxin B plus amikacin, and amikacin plus meropenem were the most potent combinations for *K. pneumoniae* isolates 1883, 3229, 7522, and 43320, respectively. Polymyxin B plus imipenem was the most potent combination for *K. pneumoniae* isolates 7433 and 44591. For the remaining 2 isolates (*K. pneumoniae* 21479 and 53879) that produced OXA-181-like carbapenemases, none of the 55 tested antibiotic combinations was bactericidal. Interestingly, none of the dual-carbapenem combinations (ertapenem plus meropenem, ertapenem plus doripenem, and ertapenem plus imipenem) was bactericidal against all the *K. pneumoniae* isolates.

Hollow-fiber infection model. Two bacterial isolates (*K. pneumoniae* 7522 and 44591) were selected for the HFIM studies. Both produce NDM-1 metallo- β -lactamases. We chose to validate the combination of tigecycline plus meropenem in this study, as (i) this was the most effective antibiotic combination in our study, (ii) XDR *K. pneumoniae* isolates were often found to be the pathogen in intra-abdominal infections at our institution, and these two antibiotics will likely be the choice of treatment due to their intrinsic pharmacokinetic characteristics. The time courses of the bacterial burden associated with tigecycline plus meropenem are shown in Fig. 3. Overall, these observations were generally in agreement with our previous assessment of single-antibiotic killing activity in time-kill studies. As depicted in Fig. 3A and B against *K. pneumoniae* 7522 and 44591, respectively, the tigecycline and meropenem single regimens did not exhibit any killing

FIG 1 Representative time-kill studies against *K. pneumoniae* 3229, depicting single antibiotics with bactericidal activity.

activity despite repeated dosing within 24 h. In comparison, when tigecycline was used concurrently with meropenem, a sustained suppression of the bacterial population (>90%) was seen with repeated dosing up to 84 to 96 h for both isolates. However, regrowth was slow and apparent in both isolates, despite repeated dosing of tigecycline plus meropenem, and reached 9 log CFU/ml at 240 h. The tracking of bacterial subpopulations with reduced susceptibilities to tigecycline showed that the total bacterial populations were slowly replaced by mutants with reduced susceptibility to tigecycline over time.

Pharmacokinetic validation. The simulated drug exposures in the infection models were satisfactory (Fig. 4). All the simulated drug exposures had an r^2 value of 0.90, and the tigecycline and meropenem half-lives were within the target ranges of 37 to 42 h and 1 to 2 h, respectively. The validated calibration for tigecycline and meropenem was linear ($r^2 = 0.992$ to 0.995). The intraday and interday quality control samples for the lower quality controls and higher quality controls have a coefficient of variation (CV) of <5% (data not shown).

Emergence-of-resistance studies. The resistant isolates were recovered from tigecycline-supplemented plates at the end of the HFIM experiments (bacteria exposed to tigecycline alone and tigecycline plus meropenem). We did not repeat this experiment on the meropenem-supplemented plates, as the meropenem MICs were already very high (≥ 64 mg/liter) for both isolates, and investigating the nature of the meropenem-induced mutants with potentially much higher MICs has no added clinical relevance. Tigecycline resistance was confirmed with repeated susceptibility testing of 2 bacterial colonies from *K. pneumoniae* 7522 (MIC, 8 mg/liter) and 44591 (MIC, 16 mg/liter). The susceptibilities to other antibiotics remained the same. The MICs at day 10 and day 20 of passaging on drug-free agar and tigecycline-supplemented agar remained largely similar, confirming the stability of the “new” phenotype of the tigecycline-resistant mutants.

In vitro time-growth studies. The mean best-fit growth rate constants ($K_{g \max}$) for *K. pneumoniae* 7522 and 44591 were 1.289 h^{-1} and 1.242 h^{-1} , respectively. For the tigecycline-exposed mutants, the growth rate constants were 1.056 to 1.098 h^{-1} and 1.017 to 1.034 h^{-1} for post-HFIM *K. pneumoniae* 7522 and post-HFIM *K. pneumoniae* 44591, respectively (Table 4). Clearly, this demonstrated that the growth rate of the resistant population was much lower than that of the respective parent isolate, possibly implying a substantial biofitness deficit in this population.

DISCUSSION

The advent of carbapenem-resistant *Enterobacteriaceae* (CRE) is a critical wake-up call. Frequent nosocomial outbreaks caused by CRE can cause high mortality for hospitalized patients who come to the hospital for the treatment of their ailments. Against a pressing need for viable treatment options for a wide-ranging repertoire of clinical scenarios caused by XDR *K. pneumoniae* isolates, optimal antibiotic combination therapy may well be the first-line therapy until new antimicrobial agents become available.

Our results offered a new perspective compared with those of previous studies. We conducted a comprehensive time-kill screen using 12 different antibiotics, mainly against NDM-1-producing and OXA-181-producing XDR *K. pneumoniae* isolates. Most of the *in vitro* studies had centered on KPC-producing *K. pneumoniae* isolates, as they were found around most of the world. At the start of this study, no KPC-producing bacteria were found in Singapore and thus none were included in the analysis. However, recent surveillance in Singapore reported the presence of KPC-producing isolates (3). Of interest, these KPC-producing isolates were found to harbor other carbapenemases as well (NDM-1). These findings contrasted the reported results of outbreaks of KPC-producing *Enterobacteriaceae*, in which the organisms of interest were found to harbor only one type of carbapenemase (7, 11–13, 15). Rifampin plus colistin has been found to be bacteri-

FIG 2 Representative time-kill studies against *K. pneumoniae* strains 1883 (A), 3229 (B), 7433 (C), 7522 (D), 43320 (E), and 44591 (F) depicting bactericidal combinations.

(A) KP 7522

(B) KP 44591

FIG 3 Microbiologic response observed in the following infection models: meropenem, tigecycline (T), and meropenem plus tigecycline against *K. pneumoniae* strains 7522 (A) and 44591 (B). q8h, every 8 h.

cidal in KPC-producing *K. pneumoniae* isolates (39–41). Although we tested polymyxin B instead of colistin, the combination of rifampin and polymyxin B was bactericidal in only 1/8 isolates tested. None of the 55 two-antibiotic combinations was bactericidal against 2 of the 8 *K. pneumoniae* isolates; both were OXA-181 producers. This may support the need to perform time-kill studies using three-antibiotic combinations against OXA-181-producing *K. pneumoniae* isolates. The justification for using three-antibiotic combinations was evidenced by the recent study by Tängdén et al.

(41). They found that the combination of rifampin, meropenem, and colistin was bactericidal against MBL-producing *K. pneumoniae*. In another study by Urban, Mariano, and Rahal, the combination of rifampin, doripenem, and colistin was found to be bactericidal against KPC-producing *K. pneumoniae* isolates (42).

The selection of a tigecycline-resistant subpopulation of XDR *K. pneumoniae* was evident by the multiple-fold increase in the tigecycline MIC harvested after 240 h of “humanized” tigecycline exposure in the HFIM, resulting in the development of a pandrug-

FIG 4 Typical observed pharmacokinetic profiles in the following infection models: tigecycline at 100 mg every 12 h (A) and meropenem at 2 g every 8 h as a 3-h infusion (B).

TABLE 4 *In vitro* growth rates obtained from post-HFIM in the tigecycline only and tigecycline-plus-meropenem systems

<i>K. pneumoniae</i> isolate ^a	$K_{g\ max}\ (h^{-1})^b$
ATCC 13883	1.323 (1.133, 1.513)
7522 (parent strain)	1.289 (1.079, 1.499)
7522 colonies from 3× the MIC medium (T only)	1.098 (0.909, 1.284)
7522 colonies from 3× the MIC medium (T+M)	1.056 (0.7216, 1.390)
44591 (parent strain)	1.242 (0.797, 1.687)
44591 colonies from 3× the MIC medium (T only)	1.017 (0.534, 1.500)
44591 colonies from 3× the MIC medium (T+M)	1.034 (0.654, 1.414)

^a T, tigecycline; T+M, tigecycline plus meropenem.
^b Shown as mean (95% confidence interval [CI]).

resistant (PDR) *K. pneumoniae* mutant. It is believed to be a result of a selective amplification of preexisting resistant populations, leading to the emergence of resistance. Furthermore, the growth rate of the surviving population was much lower than that of the parent isolate, possibly implying a substantial biofitness deficit in this population. It has been proposed that the magnitude of this fitness cost is largely influenced by the rate of emergence of resistance and the stability of the resistance (43). Consistent with previously published studies, multiple resistance mutations were associated with various biological costs, often manifested as a reduction in growth rates (44, 45). Furthermore, we showed that the PDR phenotype is stable and does not revert to its parent phenotype after days of passaging in tigecycline-supplemented or

drug-free medium. Since an *in vitro* biofitness deficit in bacteria does not necessarily correlate with *in vivo* biofitness and virulence, investigations are under way to examine the clinical relevance of the *in vivo* virulence of the PDR-KP mutant isolate.

The literature has exploded with numerous publications regarding drug-resistant bacteria infections (46). However, the diversity in the recommended combinations for treatment has also reinforced the fact that effective combinations are often strain specific. Despite the onslaught of available literature, clinicians are still at a loss when required to select an empirical combination from the large number of potential permutations. In Singapore, we are facing a trend of an increasing number of clinicians requesting guidance for the strain-specific selection of antibiotic combinations to maximize the likelihood of therapeutic success. This is due to the fact that across most *in vitro* studies, antibiotics are often combined at a fixed concentration, which is useful for identifying potential synergistic/bactericidal activity, but they are unrealistic exposures in humans. In our HFIM experiment, we sought to determine if the addition of human simulated exposures of tigecycline in the tissue to meropenem added significant antimicrobial activity against the XDR *K. pneumoniae* isolates tested. We did not attempt to achieve human simulated exposures of tigecycline in serum in our study, as tigecycline has no role in the treatment of bacteremia in clinical practice. Another potential limitation of our approach is in the clinical setting, as its labor-intensive nature is unable to provide critical information for the large numbers of patients who require antibiotic combination testing. Moving forward, a reliable and fast-turnaround methodology will be needed to identify the optimal antibiotic combinations needed to treat patients with XDR bacterial infections.

In summary, various antibiotic combinations against XDR *K. pneumoniae* isolates were tested with varied efficacies. It is clear that the antibacterial effects can differ even if they have fairly similar resistance mechanisms. As clinical studies of this nature are difficult to design and execute to identify optimal antibiotic combination therapies, more *in vitro* work needs to be done to identify useful combinations in an accurate and timely fashion, and further *in vivo* work needs to be conducted to examine the virulence of isolates and validate this approach.

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