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Time-kill and post-antibiotic effect of colistin at different static concentrations in *in vitro Acinetobacter baumannii*

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Abstract. Nosocomial infection caused by *Acinetobacter baumannii* is common among immunocompromised patients. Treatment strategy is limited due to rapid resistance development and lack of novel antibiotic. Colistin has been the last line therapy with good *in vitro* activity against infections caused by multi-drug resistance *A. baumannii*. However, pharmacological updates are required to support dosing optimisation. This study aimed to determine the time-kill kinetic and resistance development after antibiotic exposure as well as post-antibiotic effect of colistin at different static concentrations in *in vitro A. baumannii* system. The static *in vitro* time-kill and post-antibiotic effect experiments were conducted against two clinical isolates as well as one reference isolate ATCC 19606. Time-kill and post-antibiotic effect were studied at colistin concentrations ranging from 0.25MIC to 16.0MIC and 0.5MIC to 4.0MIC, respectively. Post-exposure resistance development was examined in time-kill study. Killing activity and post-antibiotic effect were in a concentration-dependent manner. However, delayed killing activity indicates colistin tolerance. Development of resistance after exposure was not detected except for the ATCC 19606 strain. Dosing suggestion based on the observations include administration of supplemental dose 3 MIU at 12 hours after loading dose, administration of maintenance dose 9 MIU in two divided doses and application of extended interval in renal adjustment dose. However, the information is applicable for non-colistin-heteroresistance *A. baumannii* with colistin MIC $\leq$ 1.0 mg/L. As for heteroresistance and strain with colistin MIC > 1.0 mg/L, combination therapy would be the more appropriate treatment strategy.

INTRODUCTION

The *Acinetobacter baumannii* group are commonly associated with nosocomial infection especially among immunocompromised patients (Peleg *et al.*, 2008). These nosocomial infections include ventilator-associated pneumonia, endocarditis, secondary meningitis, urinary tract infection (UTI) as well as blood stream infections. Mostly, nosocomial *A. baumannii* isolates exerting multidrug resistant (MDR) characters (i.e., resistance towards more than three classes of antibiotics used (Magiorakos *et al.*, 2012). However, emergence of extensive drug resistant (XDR) isolates which shows resistant to all but one or two classes of antibiotics and even pandrug resistant (PDR) isolates with resistant to all classes of antibiotics are increasing at an alarming rate (Lean *et al.*, 2014; Magiorakos *et al.*, 2012; Peleg *et al.*, 2008). Thus, treatment strategy is threatened by fast emergence of XDR strain and decline of

antibiotic development (Bassetti *et al.*, 2013; Manchanda *et al.*, 2010; Peleg *et al.*, 2012). Consequently, this prompted the revival of older antibiotics from polymyxins group as these antibiotics were the only drugs that retain *in vitro* activity against XDR gram negatives (Landman *et al.*, 2008; Zavascki *et al.*, 2010).

Nowadays, colistin (polymixin E) is one of the last therapeutic option for treatment of MDR *A. baumannii*. The reintroduction of colistin antibiotic usage, particularly in Malaysia as the last resort for the treatment of MDR *A. baumannii* infection together with new dosing regimen has lowered its toxicity as well as nephrotoxicity effects (Abdulzahra *et al.*, 2018; Rani *et al.*, 2017). The Malaysian National antibiotic guideline stated that colistin usage by mean DDD/1000 patient days in Malaysian hospitals was higher than polymyxin B and the usage has been gradually increased from 2011 to 2013 (Ministry of Health Malaysia, 2013). Meanwhile, the Malaysian National surveillance of antibiotic resistance (NSAR) reported that resistance rate of *A. baumannii* against carbapenem remains more than 50% for two consecutive years 2013 and 2014 (Ministry of Health Malaysia, 2016). These reports indicate the increasing use of colistin in Malaysian local hospitals in association with high incidence of XDR *A. baumannii*.

Colistin use was abandoned during 1970s prior to their restoration in the following decades, therefore pharmacokinetics and pharmacodynamics appraisal is warranted to ensure optimal dosing regimen (Kassamali *et al.*, 2013). In the meantime, previously conducted population pharmacokinetics-pharmacodynamics (PK/PD) studies suggested the application of loading dose to improve colistin efficacy (Kift *et al.*, 2014; Mohamed *et al.*, 2014; Mohamed *et al.*, 2012). The National Surveillance of Antibiotic Resistance (NSAR) reported around 55% to 58% resistance rate of *A. baumannii* isolated from Malaysian hospitals against carbapenem in two consecutive years in 2013 and 2014 (Ministry of Health Malaysia, 2014). Meanwhile, the dosage of colistin used was gradually increased from 6.43 to 8.40 (DDD/1000 patients days) and higher than polymyxin B as reported by NSAR in 2011 to 2013 (Ministry of Health Malaysia, 2014). These reports indicate the resurgence use of colistin in Malaysian local hospitals in association to increase incidence of XDR *A. baumannii*. Therefore, updates on the pharmacological properties of colistin is urgently warranted especially among local population to support dosing standardisation. Therefore, this study aimed to provide pharmacodynamics information on colistin against local isolates of *A. baumannii*.

MATERIALS AND METHODS

Bacterial isolates

Two representative of *A. baumannii* isolates (PT17 and PT36) which was initially isolated from wound swabs and represented a collection of *A. baumannii* isolates of similar virulence.

Antimicrobial susceptibility test

Both isolates underwent antibiotic susceptibility testing as suggested by Magiorakos *et al.* (2012) against amikacin (30 µg), gentamicin (10 µg), ceftazidime (30 µg), ciprofloxacin (50 µg), piperacillin (100 µg), sulbactam/ampicillin (10/10 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg) and polymyxin B (300 U). The antibiotic susceptibility test was performed on Mueller-Hinton Agar (Merck, Germany) swabbed with 0.5 McFarland standard suspension of overnight cultures of *A. baumannii* and incubated at 37°C for 24 hours. Guidelines from the Clinical and Laboratory Standard Institute (CLSI, 2016) was used to interpret the diameter breakpoint of the inhibition zones produces. *Acinetobacter baumannii* ATCC 19606 (Lot 357-93-2) was used as reference strain for time-kill and post antibiotic effect whereas *Escherichia coli* ATCC 25922 (Lot 335-147-1) and *Pseudomonas aeruginosa* ATCC 27853 (Lot 353-181-1) were used as quality control strains for MIC test, oxidase test, TSI and Microgen identification kit.

Minimal Inhibitory Concentration (MIC) determination

The minimal inhibitory concentration determination of colistin (Polymixin E) was carried out by using MIC evaluator strips (Oxoid, Ltd., Basingstoke, UK). An overnight bacterial cultures of *A. baumannii* adjusted to 0.5 McFarland Standard was used to determine the MIC of colistin by using MIC Evaluator (M.I.C.E) strips (Oxoid, Basingstoke, UK). Inoculated isolates was incubated at 35°C for 16 to 20 hours. The MIC value of the respective inhibition ellipses that interact with the strips were observed, recorded, and interpret according to the guidelines provided for M.I.C.E strip.

Antibiotic preparation

Colistin sulphate (Lot A00213, AdipoGen) in powder form was used in this study. Molecular formula of the compound is $2(C_{52}H_{98}N_{16}O_{13}).5(H_2SO_4)$ whereby 12.06 mg of the powder contains 10 mg of pure colistin. Hence, 10,000 mg/L stock solution was prepared by dissolving 12.06 mg of colistin sulphate powder into 1 mL of sterile filtered reverse osmosis water. Stock solution was freshly prepared prior to each experiment being conducted.

Static time-kill analysis

The clinical isolates as well as reference isolate ATCC 19606 were studied in static time-kill kinetics analysis against colistin at multiple concentrations as previously described (Mohamed *et al*., 2014; Owen *et al*., 2007). Stationary phase bacterial suspension was prepared by 6 hours incubation of bacterial inoculum in 4 mL Mueller Hinton broth at 37°C. Logarithmic growth phase was obtained by dilution of the stationary suspension in Mueller Hinton broth containing colistin solution. Serial dilution of colistin sulphate was performed from stock solution to obtain the intended concentrations ranging from concentrations with sub-minimal and minimal antibacterial activity (0.25XMIC, 0.5XMIC, 1.0XMIC), concentrations with efficient bacterial killing (2.0XMIC, 4.0XMIC) to concentrations with maximum killing activity (8.0XMIC, 16.0XMIC). Time-kill experiment was conducted by incubating the logarithmic growth bacterial suspension with each of the colistin concentration at 37°C for up to 24 hours in polypropylene tube. At pre-dose, 0.5, 1, 2, 3, 4, 6, 8 and 24 hours of incubation, bacterial concentration was enumerated using 10-fold serial dilution agar plate method (viable colony count). Colony count enumeration is in cfu/mL and the lower limit of counting is 10 cfu/mL. In the meantime, control experiment was carried out simultaneously with same procedure without colistin addition. Three independent experiments were conducted to ensure reproducibility and variability.

Observation of resistance development after colistin exposure

Development of resistance was studied by determination of the isolates MIC at 8 hours and 24 hours incubation of the time-kill experiment. MIC was determined by strip method provided that growth is present at the defined incubation time. Control analysis was performed simultaneously without colistin exposure.

Post-antibiotic effect

Post-antibiotic effect experiment was performed against the same isolates used in time-kill study. The procedure of the experiment is as previously described (Gaibani *et al*., 2014; Özbek and Şentürk, 2010). Bacterial at logarithmic growth phase was exposed to colistin in Mueller Hinton broth for 1 hour incubation at 37°C. The concentrations of colistin used are at multiple MIC (0.5MIC, 1.0MIC, 2.0MIC and 4.0MIC), justified based on the achievable plasma level after parenteral administration (Mohamed *et al*., 2012). After exposure, colistin was removed by centrifugation at 3000g for 10 minutes followed by 3 times washing steps. Bacterial pellet was then re-suspended in 4 mL Mueller Hinton broth and incubated at 37°C.

Viable colony count was performed at time 0 (immediately after addition of colistin), immediately before and after colistin removal and every hour after colistin removal for up to 8 hours and finally at 24 hours after colistin removal. Growth curve was plotted starting at 0 hour which is the time immediately after

antibiotic removal. Control growth was simultaneously conducted without colistin addition. PAE was determined using formula: PAE= T – C, where T is the time required for treated culture colony count to increase by 10-fold from the count obtained immediately after colistin removal and C is the corresponding time required for control culture. Three independent experiments were conducted for each isolate to ensure reproducibility and variability.

RESULT

Antibiogram profile of clinical isolates

Based on the international standard definition, the clinical isolates used in this study is considered extensively drug resistance. The isolates are susceptible to polymyxin B while non-susceptible to aminoglycosides, antipseudomonal carbapenems, antipseudomonal fluoroquinolones, antipseudomonal penicillins, antipseudomonal penicillins + β-lactamase inhibitors (tazobactam), penicillins + β-lactamase inhibitors (sulbactam) and extended-spectrum cephalosporins antibiotic categories as listed in Table 1.

Static time-kill analysis

The typical static time-kill curves of colistin at multiple concentrations against the studied isolates are shown in Figure 1 to Figure 3. From the observation, killing activity of colistin was concentration dependent and bactericidal activity ($\geq 3 \log_{10}$ cfu/mL bacterial count reduction) was observed between 2 hours to 6 hours incubation. Bactericidal activity was evident at least at 1.0 MIC for ATCC 19606 and PT36 isolates; while at least at 0.5 MIC for PT17 isolate. Apparently, ATCC 19606 showed 24 hours regrowth at up to 4.0 MIC, PT17 exhibited incomplete killing at 24 hours for up to 8.0 MIC while complete killing at 24 hours was observed at least at 1.0 MIC against PT36 isolate.

Resistance development after colistin exposure

The 8 hours and 24 hours exposure to 0.25 MIC, 0.5 MIC and 1.0 MIC colistin concentrations did not result in increase of colistin MIC of the ATCC 19606 strain. However, exposure of the strain to 2.0 MIC and 4.0 MIC colistin concentrations resulted in the increase of colistin MIC to 3.0 mg/L and 12.0 mg/L, respectively. Colistin MIC 3.0 mg/L and 12.0 mg/L against *Acinetobacter* sp. were considered intermediate and resistance, respectively based on CLSI interpretive criteria (CLSI, 2016). Meanwhile, exposure to 8.0MIC and 16.0 MIC colistin concentrations rendered complete bacterial killing at both 8 hours and 24 hours hence determination of resistance development is not applicable (Table 1).

With regards to clinical isolate PT17, 24 hours exposure to colistin during time-kill kinetic did not result in development of resistance. Although the isolate persists after exposure, the MIC value remained (Table 3). Meanwhile, colistin MIC of the clinical isolate PT36 increased mildly after 8 hours exposure to 0.25MIC, 0.5MIC, 1.0MIC,

Table 1. List of antibiotics resistant to both of isolates tested

Antibiotics agent	*A. baumannii* PT17	*A. baumannii* PT36
Amikacin (≤12 mm)	R	R
Ceftazidime (≤14 mm)	R	R
Gentamycin (≤12 mm)	R	R
Ciprofloxacin (≤15 mm)	R	R
Piperacillin (≤17 mm)	R	R
Sulbactam/ampicillin (≤11 mm)	R	R
Piperacillin/tazobactam (≤17 mm)	R	R
Imipenem (≤18 mm)	R	R
Meropenem (≤14 mm)	R	R

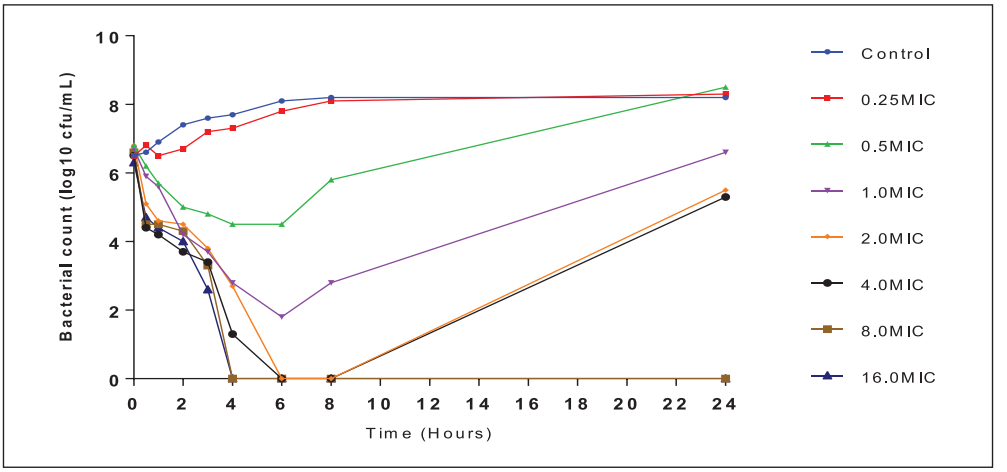

Figure 1. Static time-kill curve for colistin against *A. baumannii* reference strain ATCC 19606.

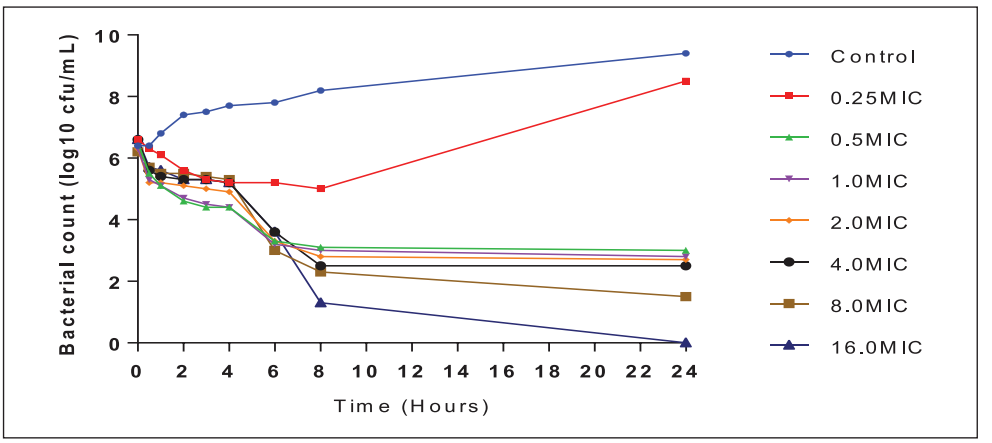

Figure 2. Static time-kill curve for colistin against *A. baumannii* clinical isolate PT17.

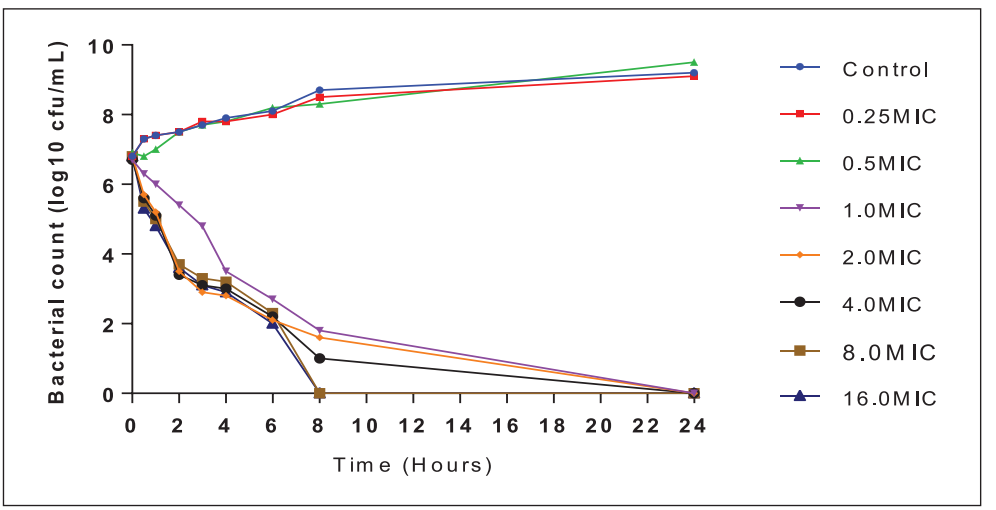

Figure 3. Static time-kill curve for colistin against *A. baumannii* clinical isolate PT36.

2.0MIC and 4.0MIC; as well as after 24 hours exposure to 0.25MIC and 0.5MIC colistin concentrations (Table 3). However, these values are still within the colistin susceptible range for *Acinetobacter* sp. (CLSI, 2016).

Post-antibiotic effect

The typical post-antibiotic effect growth curves of the studied isolates are shown in Figure 4 to Figure 6. From the observation, control experiments showed linear growth with 1 $\log_{10}$ increase in bacterial count at 3 hours, 2 hours and 1 hour for ATCC 19606, PT17 and PT36 strains respectively. With regards to the test concentrations for ATCC 19606, bacterial count increased to 1 $\log_{10}$ at 4 hours, 5 hours and after 8 hours against 0.5MIC, 1.0MIC and 2.0MIC as well as 4.0MIC, respectively. Meanwhile for PT17, bacterial count increased to 1 $\log_{10}$ after 8 hours when tested against 0.5MIC, while for other concentrations, bacterial count was not increased for up to 24 hours. Lastly for PT36, bacterial count increased to 1 $\log_{10}$ at 1 hour against 0.5MIC, at 4 hours and after 8 hours against 1.0MIC and 2.0MIC respectively; and was not increased for up to 24 hours against 4.0MIC. PAEs against the respective colistin concentrations for each of the studied isolates are tabulated in Table 4. The PAEs ranging from approximately 1.0 to 4.0 hours are considered modest. Meanwhile, PAEs more than 5 hours are considered significant (Owen *et al*., 2007; Özbek and Şentürk, 2010).

Table 2. Static time killing activity of colistin against isolates tested

Isolates	Colistin concentration (mg/L)*
A. baumannii PT17	1.0
A. baumannii PT36	0.75
A. baumannii (ATCC 19606)	1.0

* ≥3log10 cfu/mL killing at 3-6 hours and less substantial regrowth (10^6) at 24 hours.

Table 3. Colistin MIC at post-exposure

Isolate	Colistin concentration (mg/L)	Post exposure MIC (mg/L)	
		8 hours exposure	24 hours exposure
ATCC 19606	Control	1.0	1.0
	0.25MIC	1.0	1.0
	0.5MIC	1.0	1.0
	1.0MIC	1.0	1.0
	2.0MIC	N/A	3.0
	4.0MIC	N/A	12.0
	8.0MIC	N/A	N/A
	16.0MIC	N/A	N/A
PT17	Control	1.0	1.0
	0.25MIC	1.0	0.75
	0.5MIC	0.75	1.0
	1.0MIC	1.0	1.0
	2.0MIC	1.0	1.0
	4.0MIC	1.0	1.0
	8.0MIC	1.0	1.0
	16.0MIC	0.75	N/A
PT36	Control	0.75	0.75
	0.25MIC	0.75	1.0
	0.5MIC	1.5	1.5
	1.0MIC	1.5	N/A
	2.0MIC	1.5	N/A
	4.0MIC	1.0	N/A

*N/A: Not applicable.

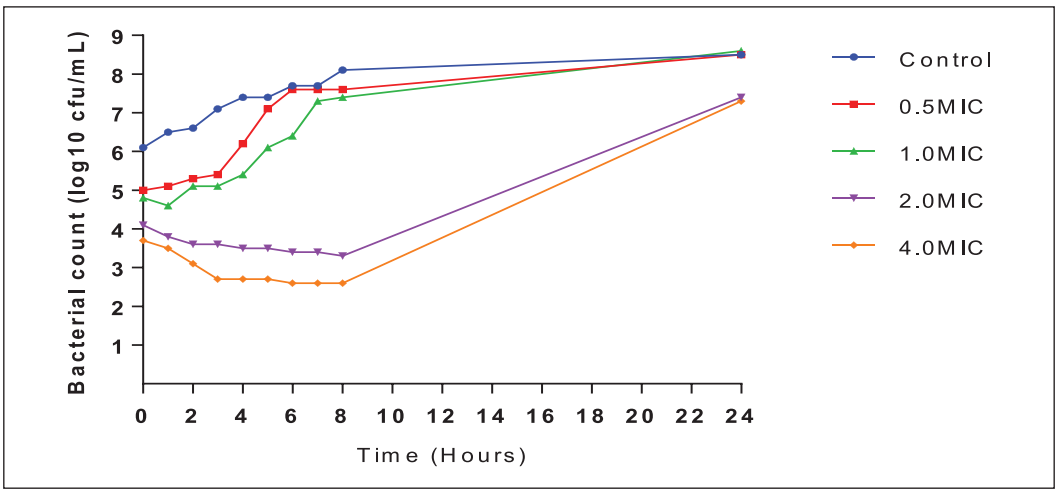

Figure 4. Post-antibiotic effect growth curve for colistin against *A. baumannii* reference strain ATCC 19606.

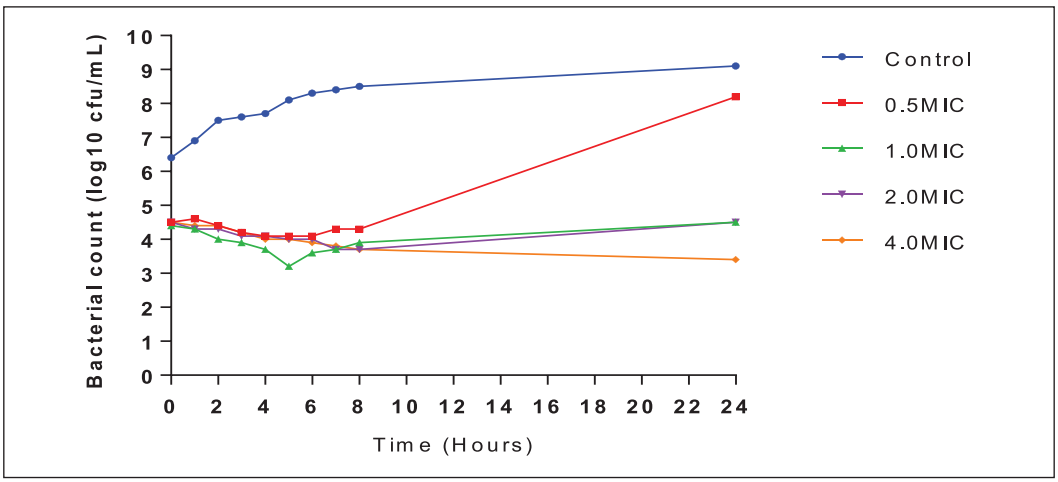

Figure 5. Post-antibiotic effect growth curve for colistin against *A. baumannii* clinical isolate PT17.

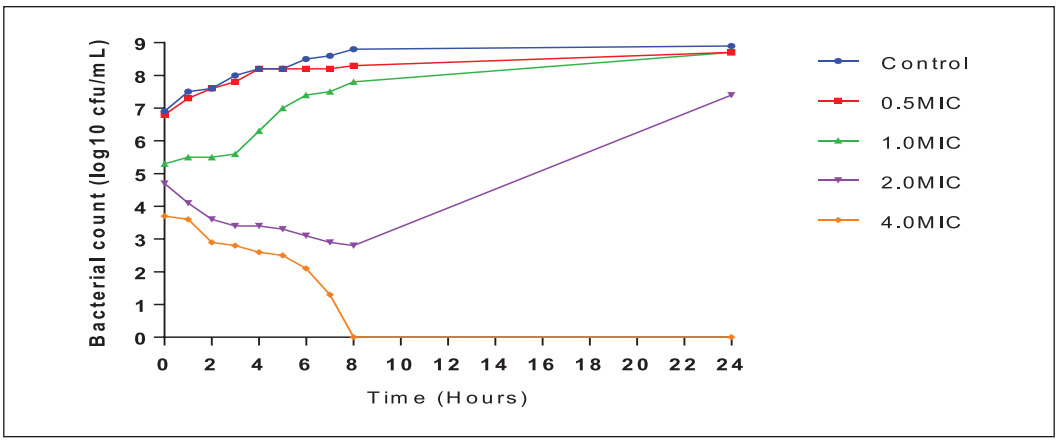

Figure 6. Post-antibiotic effect growth curve for colistin against *A. baumannii* clinical isolate PT36.

Table 4. Post-antibiotic effect of colistin at suboptimal and optimal concentration against the studied isolates

Isolate	Colistin concentration (mg/L)	Post-antibiotic effect (hour)	Interpretation
ATCC 19606	0.5MIC	1	Modest PAE
	1.0MIC	2	Modest PAE
	2.0MIC	>5	Significant PAE
	4.0MIC	>5	Significant PAE
PT17	0.5MIC	>6	Significant PAE
	1.0MIC	Prolong	Significantly prolonged PAE
	2.0MIC	Prolong	Significantly prolonged PAE
	4.0MIC	Prolong	Significantly prolonged PAE
PT36	0.5MIC	0	No PAE
	1.0MIC	3	Modest PAE
	2.0MIC	>7	Significant PAE
	4.0MIC	Prolong	Significantly prolonged PAE

DISCUSSION

The time-kill kinetic experiment in this study further supports the concentration-dependent activity of colistin against *A. baumannii*, consistent with previous reports (Li *et al.*, 2006; Owen *et al.*, 2007; Yau *et al.*, 2009). At least 1.0MIC colistin concentration is bactericidal against all tested isolates and killing activity was observed between 2 to 6 hours exposure to the antibiotic. However, the previous studies reported rapid killing activity at initial hours between 0.5 to 3 hours against clinical isolates as well as reference strain ATCC 19606. Therefore, it is hypothesised that some intrinsic mechanisms related to colistin tolerance were responsible for lack of rapid killing activity against the isolates in this study. One of the possible mechanisms is synthesis of compatible solute such as proline which reduces osmotic stress and prevents protein misfolding. Another suggested mechanism is production of glycosyltransferase enzyme which functions in structure maintenance and integrity of bacterial outer membrane (Hood *et al.*, 2013). Both mechanisms contribute to increase in the bacterial membrane integrity as well as protein stability. Hence, these mechanisms account for colistin tolerance since colistin activity involves disruption of the lipopolysacharide and membrane permeability which result in osmotic lysis of bacterial cells.

The mechanism of colistin tolerance explains bacterial persistence and delayed killing activity particularly among clinical isolates. Persistence was significantly displayed by clinical isolate PT17 with failure of bacterial eradication for up to 24 hours. This phenomenon provides reservoir of viable cells and with longer exposure to sub-lethal antibiotic concentration, it stimulates mutation, consequently promoting development of colistin-resistance (Fauvart *et al.*, 2011; Martinez and Baquero, 2000).

Our finding also described regrowth of ATCC 19606 after 24 hours exposure to colistin indicating colistin-heteroresistance, in line with previous report (Li *et al.*, 2006). The increase in colistin MIC after exposure further supports this feature. Meanwhile, identical colistin MIC between post-exposure and ancestral isolate of the PT17 supports colistin-persistence. However, mild increase in colistin MIC was observed for PT36 after 24 hours exposure to suboptimal colistin concentrations suggesting hormesis activity of the antibiotic (Couce and Blazquez, 2009; Davies *et al.*, 2006). Significant and concentration-dependent post-antibiotic effect of colistin against the studied isolates was also demonstrated in this study. Overall, significant PAEs were observed at 2.0MIC and 4.0MIC colistin concentrations.

Based on the pharmacodynamics observation in this study, some recommendations on colistin dosing strategy

against XDR *A. baumannii* were proposed. Since concentration-dependent killing activity was evident, 9 MIU daily dosing in two divided instead of three divided doses is suggested for maintenance dose. As for renal adjusted dose, extended dosing interval for up to 48 hours depending on renal status is suggested instead of adjustment by reducing the dosing amount (Kift *et al.*, 2014; Dalfino *et al.*, 2012). This is based on theoretical background that high peak concentration achieved from higher dosing would render effective bacterial killing by a concentration-dependent antibiotic. In addition, the half-life of colistin is considerably long (14.4 hours) and post-antibiotic effect imposed by the antibiotic would also render sustained bacterial suppression despite longer dosing interval (Craig, 1993; Plachouras *et al.*, 2009).

The evidence of colistin tolerance might require application of booster dosing in addition to loading dose. The purpose is to provide sustained killing activity as well as suppression of mutational events related to bacterial persistence by rapid attainment of therapeutic level. The suggested booster dosing is 3.0 MIU at 12 hours after loading dose considering prediction from previous pharmacokinetic study (Martinez and Baquero, 2000; Mohamed *et al.*, 2014).

The pharmacodynamics information from the current study is applicable for colistin monotherapy against *A. baumannii* with colistin MIC $\leq$ 1 mg/L and non-heteroresistance. This is because, to achieve the therapeutic level of colistin that exhibits maximum efficacy against heteroresistance and strain with colistin MIC > 1 mg/L would pose risk of nephrotoxicity side effect (Ortwine *et al.*, 2015). It was reported that the mutant prevention concentration (MPC) of colistin against heteroresistant *A. baumannii* strains exceeds 128 mg/L which is very much higher than colistin serum concentrations (median 2.36 mg/L) associated with approximately 50% toxicity rates (Cai *et al.*, 2010; Garonzik *et al.*, 2011). As such, combination therapy from different antibiotic classes with identical pharmacokinetics profile to promote closure of mutant selection window is the appropriate treatment strategy against these isolates (Drlica, 2003; Zhao and Drlica, 2001).

The static *in vitro* pharmacodynamics model of the current study was performed using constant drug concentrations as well as inoculum size. As such, a follow up study utilising dynamic *in vitro* and *in vivo* model is recommended to support the data obtained. Overall, this study evaluates colistin *in vitro* pharmacodynamics activity as single agent against *A. baumannii*. The finding is valuable for dosing optimisation and improvement of treatment approach upon integration with pharmacokinetics profile of targeted patient population. A well-designed study assessing treatment outcome of colistin and colistin-based combination therapy is warranted in the near future.

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