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RESEARCH ARTICLE

In-vitro activity of β-lactams/trimethoprim-sulfamethoxazole combinations against different strains of *Burkholderia pseudomallei*

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ABSTRACT

Trimethoprim-sulfamethoxazole is an active agent against *Burkholderia pseudomallei* and is being used in intensive and maintenance phases of melioidosis therapy. In this study, we evaluated the bactericidal activities of β-lactams (imipenem, ceftazidime and amoxicillin-clavulanate) alone and in combinations with trimethoprim-sulfamethoxazole against *B. pseudomallei*. Four clinical strains of *B. pseudomallei* were selected based on different genotypes that are frequently found in Malaysia. The minimum inhibitory concentrations of trimethoprim-sulfamethoxazole, ceftazidime, imipenem and amoxicillin-clavulanate were determined using microdilution broth method. The bactericidal activities and synergy effects of β-lactams and/or trimethoprim-sulfamethoxazole were evaluated by checkerboard and static time-kill analyses at 1×MIC concentration of each antibiotic. Using checkerboard method, the β-lactam/trimethoprim-sulfamethoxazole combinations exhibited ΣFIC of 0.75-4.00. In time-kill analysis, ceftazidime/trimethoprim-sulfamethoxazole combination demonstrated synergy against three strains (less 2.25-2.41 $\log_{10}$CFU/mL compared to the most active antibiotic monotherapy) whereas imipenem/trimethoprim-sulfamethoxazole combination regimen showed synergy against one strain (less 3.32 $\log_{10}$CFU/mL). No antagonist effect or major re-growth was observed in all combination regimens, whereas 11 out of 12 of β-lactam monotherapy regimens were associated with re-growth of bacteria. However, all β-lactam monotherapy regimens exhibited rapid and stronger killing activities against BUPS/07/14, in the initial 12 hours compared to β-lactam/ trimethoprim-sulfamethoxazole combination regimens. The combination of β-lactams with trimethoprim-sulfamethoxazole demonstrated better killing effect at 24 hours compared to monotherapy and no major bacterial regrowth was observed. Nevertheless, delay in killing activities of β-lactam/trimethoprim-sulfamethoxazole combination regimens against BUPS/07/14 need further examination because this phenomenon can lead to treatment failure in some patients.

Keywords: Melioidosis; *Burkholderia pseudomallei*; β-lactam antibiotics; trimethoprim-sulfamethoxazole; drug combinations.

INTRODUCTION

Melioidosis has been recognised as the most neglected tropical disease (Currie & Kaestli, 2016). The disease was traditionally endemic in Southeast Asia and Northern Australia, but it is now spread to the Indian subcontinent, China, Caribbean, Africa and Middle East (Dance, 2000). The host can acquire melioidosis agent, *Burkholderia pseudomallei* through ingestion, direct inoculation or inhalation (Ong *et al*., 2016). Melioidosis has a broad range of symptoms and signs which increasethe possibility of misdiagnosis, thus resulting in treatment delay (Deris *et al*., 2010). In Malaysia, cases of melioidosis are relatively high in hyper-endemic areas especially in states where agriculture is the main economic activity. Recently, melioidosis cases in Kedah and Pahang states have been reported at 16.35 per 100 000 populations and 4.3 per 100 000 populations per year, respectively (Abu Hassan *et al*., 2019).

Current recommended regimens for the intensive phase of melioidosis therapy are ceftazidime or carbapenem for at least 10-14 days and followed by the eradication phase using oral trimethoprim-sulfamethoxazole (SXT) or doxycycline (Lipsitz *et al*., 2010; Ministry of Health, 2014). SXT is recommended to be added to ceftazidime or carbapenem in

the intensive phase of therapy only in specific clinical presentations with focal infections or abscess (Lipsitz *et al*., 2010; Dance, 2014; Currie, 2015).

In Malaysia, with this treatment guideline, the mortality rate can be as high as 65%, especially in cases associated with septicaemia (Deris *et al*., 2010) with more than 50% of the deaths occurred within 48 hours after hospital admission (Yazid *et al*., 2017). The mortality rate is particularly high even though with the use of antibiotics combination (Ganesan *et al*., 2020). The role of combination therapy needs serious attention and deeper investigation to improve the treatment outcome of melioidosis in future. Here, we investigated the bactericidal effects of β-lactams and SXT combinations against *B. pseudomallei* strains from Malaysia.

METHODOLOGY

Institutional Approval

This study has been approved by the Universiti Sains Malaysia Research Ethics Committee (Ref: USM/JEPeM/16110493). All safety trainings and precautions were carried out in accordance with the safety standard ruled by Department of Medical Microbiology and Parasitology, School of Medical Science, Health Campus, Universiti Sains Malaysia while working with *B. pseudomallei*.

Bacterial strains

Four clinical strains were selected based on the genotypes that are frequently found in Malaysia. BUPS/12/14, BUPS/07/14, BUPS/07/13 and BUPS/91/08 from sequences type 54, 376, 1322 and 1326 of previous study respectively (Zueter *et al*., 2015). The isolates were kept at -80°C before use.

Determination of minimum inhibitory concentrations (MICs) of antibiotics

Minimal inhibitory concentrations (MICs) of SXT (Sigma-Aldrich, St. Louis, MO), amoxicillin-clavulanate (GlaxoSmithKline, Middlesex, UK; AMC), ceftazidime (GlaxoSmithKline, Middlesex, UK) and imipenem (Merck Sharp & Dohme, Kenilworth, NJ) were performed by microdilution broth method using U-bottomed 96-wells plates, according to Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2015). The stock solution of antibiotics was prepared by diluting ~5.12 mg of antibiotics powder in solvent to obtain a final solution of 5.12 mg/mL. The concentrations of antibiotics used in this study ranged from 0.125 mg/L to 128 mg/L. The antibiotic solution was two-fold diluted in Mueller Hinton broth (MHB) and 100 µL of antibiotic solution was transferred into 96 wells plate accordingly. About 2 to 3 colonies of *B. pseudomallei* were suspended into normal saline solution until value of 0.5 McFarland turbidity was achieved. A 1:100 dilution of bacterial culture was performed by adding 100 µL of bacterial suspension into 9.9 mL Mueller Hinton broth. Then, 100 µL of the bacterial suspension from the dilution tube was inoculated into 96-wells plate. The plate was incubated for 24 hours at 37°C. The MIC of antibiotics was observed by turbidity visualization by unaided eye. MIC is defined as the lowest concentration of the antibiotics showing inhibition of visible growth turbidity (Andrews, 2001).

Based on standard interpretation of MIC from CLSI (2015), the breakpoint of antibiotics used in this study is amoxicillin-clavulanate: susceptible ≤ 8/4 µg/mL; resistance ≥ 32/16 µg/mL, ceftazidime: susceptible ≤ 8 µg/mL; resistance ≥ 16 µg/mL, imipenem: susceptible ≤ 4 µg/mL; resistance ≥ 16 µg/mL and trimethoprim-sulphamethoxazole: susceptible ≤ 2/38 µg/mL; resistance ≥ 4/76 µg/mL.

Checkerboard method

Fractional inhibitory concentrations (FICs) of β-lactam and SXT combinations were examined by broth microdilution checkerboard method in the same manner as the susceptibility tests. The concentrations of antibiotics were reduced to four or five of two-fold dilution below the MICs. The combination was considered as synergy when the fractional inhibitory concentration index (ΣFIC) was equal to or less than 0.5 and antagonism when the ΣFIC was greater than 4. Indifference was indicated by ΣFIC value more than 0.5 or equal to or less than 4 (White *et al*., 1996).

Time-kill studies

The bactericidal activity was examined by 24 h static time-kill using 1×MIC of each antibiotic. The mid-log phase bacterial suspension of 1×10^6 CFU/mL was used as initial inoculums. All tubes containing bacterial suspension were incubated at 37°C in an incubator, shaking at 150 rpm. Quantitative culture was performed by serial dilution and spread on nutrient agar plates at time intervals of 0, 3, 6, 12 and 24 hours. The plates were then incubated at 37°C for 18-24 hours for colony count.

Synergy was defined as a reduction of viable colonies by 2-$\log_{10}$ of the most active single antibiotic in the regimen at 24 hours as well as a decrease by 2-$\log_{10}$ compared to initial inoculums. Indifference was defined as a reduction of viable colonies by 1-$\log_{10}$ whereas antagonism was defined as an increase of viable colonies by 2-$\log_{10}$ of the interaction at 24h (White *et al*., 1996). Bacteriostatic and bactericidal activities were defined as <3-$\log_{10}$ and ≥3-$\log_{10}$ CFU/mL reductions in 24 hours, respectively, in relative to the initial inoculums (CLSI, 2015; Smith *et al*., 2018).

RESULTS

The MICs of all tested antibiotics against all four strains were within the susceptibility range of CLSI breakpoints except for SXT against BUPS/07/14, which showed a MIC of 4 µg/mL (the loweest breakpoint for resistance) (Table 1).

The ΣFIC values of the β-lactam/SXT combination regimens for all four strains indicated indifference activity with values ranging from 0.75 to 4.00 (Table 2).

Table 1. Bacterial strains used in this study and their minimum inhibitory concentrations

Bacterial strains	Sequence type*	Minimum inhibitory concentrations (µg/mL)#			
		SXT	AMC	CAZ	IMP
BUPS/12/14	54	2	4	2	0.5
BUPS/07/14	376	4	8	4	0.5
BUPS/07/13	1322	1	8	2	0.5
BUPS/91/08	1326	0.5	8	2	1

Trimethoprim-sulfamethoxazole (SXT), imipenem (IMP), ceftazidime (CAZ), amoxicillin-clavulanate (AMC).
*Sequence type were based on our previous study (Zueter *et al*., 2015).

In the single antibiotic regimen, the bactericidal activities (>3-log_{10} reduction form initial inoculum) were observed in five out of sixteen regimens i.e. imipenem against BUPS/12/14, BUPS/07/13 and BUPS/91/08 and SXT against BUPS/07/13 and BUPS/91/08 (Table 3). Nevertheless, reductions of growth were observed at various time points in all single β-lactam regimens but regrowth occurred after 12 hours of interaction in eleven out of twelve regimens (Figure 1). Six of these regrowth at 24 hours were more than initial inoculum. Imipenem was the only single β-lactam antibiotic regimen not associated with regrowth against BUPS/12/14 [Figure 1(A)]. Whereas, three out of four SXT single antibiotic regimens were not associated with regrowth. The reduction was less prominent SXT single antibiotic regimen against BUPS/07/14 and regrowth occurred in BUPS/12/14.

In combination regimens, the bactericidal activities were documented in eight out of twelve regimens. All combination regimens were associated with viable bacterial count at 24 hours lower than initial inoculum. The synergy effects at 24 hours were observed in ceftazidime/SXT against BUPS/07/13, BUPS/07/14 and BUPS/91/08, and imipenem/SXT against BUPS/07/14 (Table 3). There was no major regrowth observed in combination regimens against these strains. However, there were few small regrowth at various time points; 6 hours of imipenem/SXT against BUPS/07/14, 12 hours of imipenem/SXT against BUPS/07/13 and BUPS/91/08, and 12 hours of ceftazidime/SXT and amoxicillin-clavulanate/SXT against BUPS/07/13 (Figure 1).

In this study, we found rapid and stronger killing activities against BUPS/07/14 in the early hours (3, 6 and 12 hours) for all β-lactams monotherapy regimens compared to their SXT combination regimens [Figure 1 (C)]. The slow killing effects of the combination regimes were also observed in AMC/SXT and ceftazidime/SXT regimens against BUPS/12/14, in which the bacterial killing activity was only observed after 12 hours of incubation compared to 6 and 12 hours in their single antibiotic regimens. The imipenem/SXT combination against these strains had similar pattern with the imipenem single antibiotic regimen [Figure 1 (A)].

DISCUSSION

Antibiotic combination regimen is one of the strategies to improve the treatment efficacy and thus, reduce the mortality rate of infections by resistant organisms. Inhibition of different targets has been used in treating *Mycobacterium tuberculosis* infections (Worthington & Melander, 2013). While *B. pseudomallei* is similar to *M. tuberculosis* in term of

Table 2. ΣFIC index values of checkerboard assay and interpretation of activity of trimethoprim-sulfamethoxazole and its β-lactam combinations

Combinations of antibiotics[#]	Strains	ΣFIC values	Interpretations
SXT + AMC	BUPS/12/14	2.50	Indifference
	BUPS/07/13	1.00	Indifference
	BUPS/07/14	2.50	Indifference
	BUPS/91/08	0.75	Indifference
SXT + CAZ	BUPS/12/14	4.00	Indifference
	BUPS/07/13	1.00	Indifference
	BUPS/07/14	1.00	Indifference
	BUPS/91/08	1.00	Indifference
SXT + IMP	BUPS/12/14	1.00	Indifference
	BUPS/07/13	0.98	Indifference
	BUPS/07/14	1.00	Indifference
	BUPS/91/08	0.98	Indifference

[#] Trimethoprim-sulfamethoxazole (SXT), imipenem (IMP), ceftazidime (CAZ), amoxicillin-clavulanate (AMC).
Synergy - ΣFIC ≤ 0.5; Antagonism - ΣFIC > 4; Indifference - ΣFIC >0.5 and ≤ 4.

Table 3. Interpretation of time-kill curve of β-lactam/trimethoprim-sulfamethoxazole combinations against four clinical strains of *B. pseudomallei* at 24 h

Antibiotics[#]	Killing activity* (log_{10}CFU/mL)	Combinations of antibiotics[#]	Killing activity* (log_{10}CFU/mL)	Interaction at 24 h** (log_{10}CFU/mL)	Interpretation
BUPS/12/14					
SXT	0.03				
AMC	0.96	AMC + SXT	-1.91	-2.01[a]	Indifference
CAZ	0.38	CAZ + SXT	-0.55	-0.66	Indifference
IMP	-4.79	IMP + SXT	-4.51	0.40	Indifference
BUPS/07/13					
SXT	-3.48				
AMC	1.64	AMC + SXT	-5.60	-1.95	Indifference
CAZ	-0.25	CAZ + SXT	-5.92	-2.25	Synergy
IMP	-3.55	IMP + SXT	-5.15	-1.64	Indifference
BUPS/07/14					
SXT	-0.05				
AMC	1.44	AMC + SXT	-1.97	-1.54	Indifference
CAZ	-0.14	CAZ + SXT	-2.46	-2.41	synergy
IMP	0.15	IMP + SXT	-3.28	-3.32	synergy
BUPS/91/08					
SXT	-2.20				
AMC	1.36	AMC + SXT	-3.38	-1.29	Indifference
CAZ	-0.22	CAZ + SXT	-4.41	-2.38	Synergy
IMP	-4.45	IMP + SXT	-6.00	-1.32	Indifference

[#] Trimethoprim-sulfamethoxazole (SXT), amoxicillin-clavulanate (AMC), ceftazidime (CAZ), imipenem (IMP).
* Killing activity-Log_{10}CFU/mL differences between at initial inoculums and at 24 h.
** Synergy ≥ 2-log_{10} reduction, indifference ± <2-log_{10}, antagonism ≥ 2-log_{10} increase.
[a] Although the combination ≥ 2-log10, the killing activity of AMC+SXT is <2-log_{10}CFU/mL, so did not fulfill the criteria of synergy.

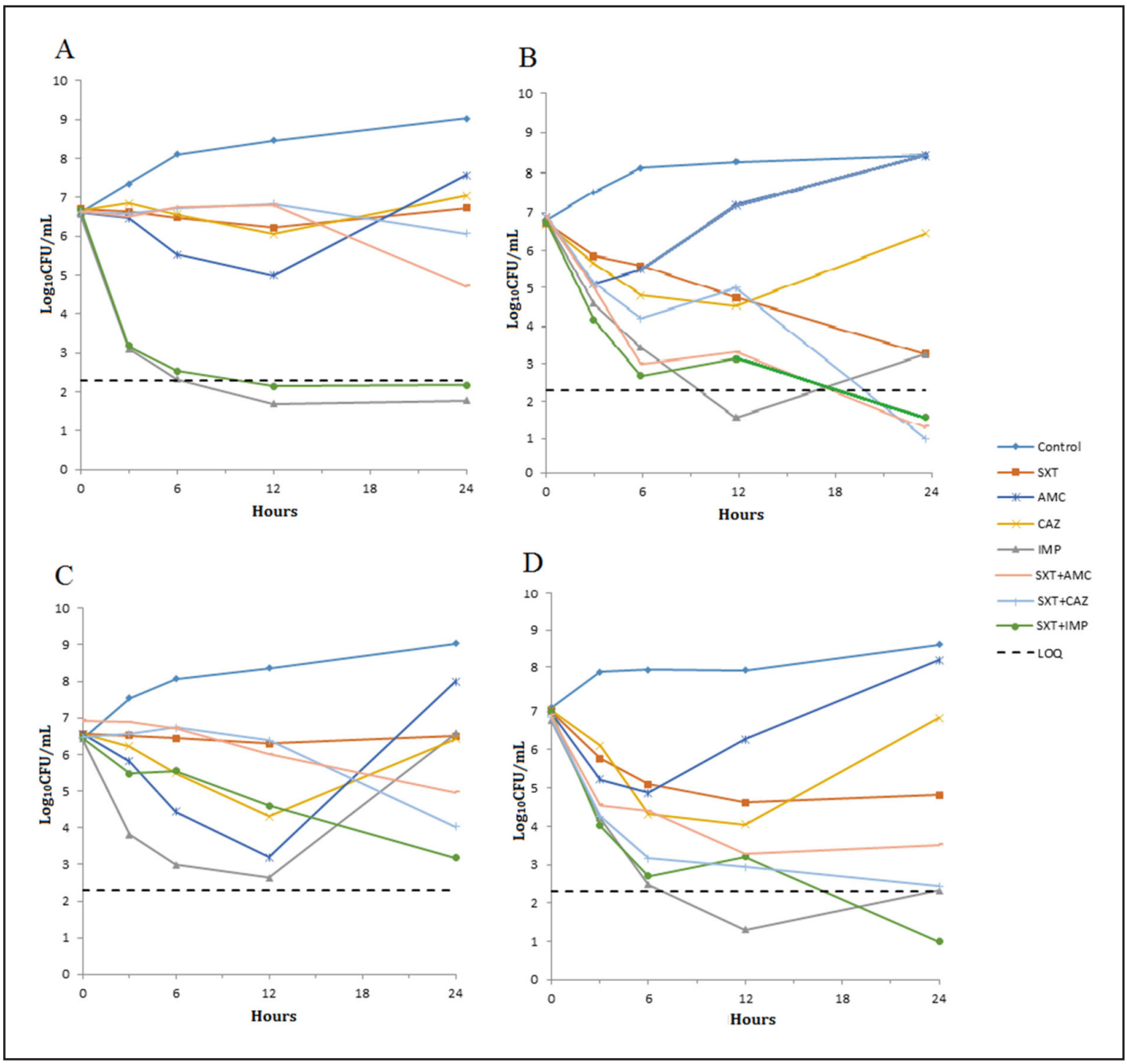

Figure 1. Time-kill curve of antibiotics in combination with trimethoprim-sulfamethoxazole against strain BUPS/12/14 (A); BUPS/07/13 (B); BUPS/07/14 (C) and BUPS/91/08 (D). Trimethoprim-sulfamethoxazole (SXT), amoxicillin-clavulanate (AMC), ceftazidime (CAZ) and imipenem (IMP). The dash horizontal line indicates limit of quantification (LOQ). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

intrinsically resistant to many antibiotics, at this moment, no such recommendation available in treating melioidosis except in focal infections (Lipsitz *et al*., 2010; Dance, 2014). SXT has been recommended to be used in combination with ceftazidime or carbapenem because of excellent tissue penetration (Dance, 2014; Currie, 2015). SXT is also being used to treat other intracellular pathogens due to having different activity site from β-lactams (Zinner & Mayer, 2015). Combination of two antibiotics with different mechanisms of action is expected to enhance the bacterial killing activity because when the bacteria started to become resistant to one antibiotic, the other antibiotic is supposed to inhibit the bacterial infection successfully (Ankomah *et al*., 2013). Furthermore, SXT is active against *B. pseudomallei* and being used as monotherapy in the maintenance phase of the melioidosis therapy (Currie, 2015). With all these arguments, although there is a lack of clinical evidence to support the combination (Dance, 2014; Currie, 2015), SXT is worth to be tested again in vitro as a potential antibiotic to be used in combination with β-lactams in the intensive phase of the melioidosis therapy.

Compared to our previous study that showed no additional benefit of adding other active antibiotics against *B. pseudomallei* such as, doxycycline to β-lactams (Mohamad *et al*., 2018), in this study we found that the ceftazidime/SXT combination demonstrated synergy against three out of four tested strains whereas the imipenem/SXT combination regimen showed synergy against one out of four strains. There was no antagonist effect of the β-lactam/SXT combinations in checkerboard as well as in time-kill analysis. Furthermore, there was no major re-growth in the combination regimens compared to β-lactam monotherapy

regimens, where eleven out of twelve experiments were associated with re-growth of bacteria.

All these evidences in line with the use of the β-lactam/ SXT combinations in the treatment of melioidosis. However, we found rapid and stronger killing activities in early hours of all β-lactams monotherapy regimens, compared to their SXT combination regimens against BUPS/07/14. The similar trend was observed when using doxycycline as second antibiotic. Adding doxycycline to β-lactams regimens led to attenuation and delay in the bacterial killing activity against three out of four tested strains. This is particularly prominent on the imipenem monotherapy at 3, 6 and 12 hours, compared to the doxycycline/imipenem combination against BUPS/12/ 14, BUPS/07/14 and BUPS/91/08. AMC and ceftazidime monotherapies were also superior than their doxycycline combination regimens at 3, 6 and 12 hours against BUPS/07/ 14 and BUPS/91/08 (Mohamad *et al*., 2018).

We need to further evaluate this phenomenon in order to advice the use of combination therapy in the clinical practice. This is probably due to the activity of β-lactam antibiotics which are mainly on actively dividing cells, on the other hand, the inhibition of growth induced by SXT should result in an overall reduction of actively dividing cells (Ocampo *et al*., 2014). The secondary resistance of the same class of antibiotics with same mode of action is common and may lead to the resistance mechanism of different class of antibiotics (Zamani *et al*., 2020). This resulted in reduce efficacy especially during early part of the experiments when the β-lactam/ SXT combination was used from the beginning of the therapy. Therefore, the time of commencement of the second antibiotic probably play an important role in the bactericidal activity of the combinations.

In conclusion, this study has shown the benefits of the β-lactam/SXT combinations over the monotherapy against a few strains of *B. pseudomallei* from Malaysia. However, we also found attenuation and delay in the bactericidal activity of the combination regimens against some strains, which may lead to the treatment failure. Further study is warranted to understand this phenomenon in order to increase the efficacy of combination therapy against melioidosis. Furthermore, pharmacodynamic examination to find an optimum time to initiate the second antibiotic is also critical to improve patient's survival.

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Conflict of interest statement

The authors declare that they have no competing interests.

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