In-Vitro Activity of Doxycycline and β -Lactam Combinations Against Different Strains of *Burkholderia pseudomallei*

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Abstract Although doxycycline is active against *Burkholderia pseudomallei* and has been used in the eradication stage of melioidosis therapy, it is not regularly used during the initial intensive phase. In order to assess its potential use in intensive phase therapy, we investigated in vitro pharmacodynamic activity of doxycycline and β -lactams alone and in combination against four Malaysian strains of *B. pseudomallei*. Using a checkerboard assay, the combinations of doxycycline and imipenem, doxycycline and ceftazidime, and doxycycline and amoxicillin–clavulanate tested against four strains showed indifferent effects with summation fractional inhibitory concentration values ranging from 0.62 to 2.12. Time-kill experiments also indicated that the combinations of doxycycline/ β -lactam antibiotics against four tested strains did not fulfil synergy criteria, in which all combinations showed indifferent effects with -1.36 to 1.26 -log CFU/mL compared to the most active monotherapy regimen in each combination. No re-growth of bacteria was detected after the early killing in doxycycline/ β -lactam combination regimens compared to β -lactam monotherapy regimens, in which 9 out of 10 were

associated with re-growth of bacteria. As no synergistic activity was observed, this in vitro study showed that doxycycline offers no additional benefit to be used in combination with β -lactams in the intensive phase of therapy.

Keywords Melioidosis · *Burkholderia pseudomallei* · Doxycycline · β -Lactams · Drug combination

American Society for Microbiology recognised melioidosis as the most neglected tropical disease [1]. Traditionally, this disease is endemic in Southeast Asia and northern Australia, however spread to Indian subcontinent, China, the Caribbean, African Countries and the Middle East. Infections have been reported [2]. The host can acquire melioidosis agent, *Burkholderia pseudomallei* through ingestion, inoculation and inhalation [1]. Melioidosis has a broad range of symptoms and signs which increases the possibility of misdiagnosis, thus resulting in treatment delay [3].

In term of treatment, current convention recommended melioidosis to be treated in two phase. The first is the acute phase with the aim to stop overwhelming sepsis and prevent mortality; the second is the eradication phase that targeting of killing any residual bacteria and reducing the risk of relapse [4]. For the acute intensive phase therapy, intravenous ceftazidime or a carbapenem (either imipenem or meropenem) is used as monotherapy or in combination with trimethoprim–sulfamethoxazole for at least 2–3 weeks for bacteremia and 4–8 weeks if severe/deep focal infection. This is followed by eradication phase therapy with oral trimethoprim–sulfamethoxazole or doxycycline for minimum of 3 months [5]. In Malaysia, with this treatment guideline, the mortality rate can be as

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high as 65%, especially in cases associated with septicæmia [3] and more than 50% of the deaths occur within 48 h after hospital admission [6].

Doxycycline is active against *B. pseudomallei* and has been used in the eradication stage of melioidosis therapy. However, it is not usually suggested for the initial intensive phase [4, 7]. In view of high mortality with current treatment recommendation, the potential use of doxycycline in combination with β -lactam antibiotics for the initial phase of melioidosis should be further evaluated. In this present study, we investigated the in vitro interactions between doxycycline and β -lactams to assess the potential for the use of doxycycline as an alternative to trimethoprim–sulfamethoxazole in the initial phase of melioidosis therapy.

This study was approved by the Universiti Sains Malaysia Review Committee (Ref: USM/JEPeM/16110493). Four strains of *B. pseudomallei*; BUPS/12/14/A, BUPS/07/14/A, BUPS/07/13/A and BUPS/91/08/A were selected based on their genotypes, which were among those frequently found in Malaysia [8].

The minimum inhibitory concentrations (MICs) of doxycycline, ceftazidime, imipenem and amoxicillin–clavulanate were determined by microdilution broth method according to Clinical and Laboratory Standard Institute (CLSI) guidelines. The fractional inhibitory concentrations (FIC) of doxycycline and β -lactam combinations were examined by the broth microdilution checkerboard method. The combination was considered as synergistic when the fractional inhibitory concentration index (Σ FIC) was equal to or less than 0.5. Indifference was indicated by Σ FIC value between 0.5 and 4, while antagonism occurred when the Σ FIC was greater than 4 [9, 10]. The MIC results indicated that all tested strains were susceptible to doxycycline, imipenem, ceftazidime and amoxicillin–clavulanate. As shown in Table 1,

doxycycline combined with imipenem, ceftazidime and amoxicillin–clavulanate against all four strains showed indifferent activity with the Σ FIC value ranging from 0.62 to 2.12.

The bactericidal activity was examined by 24 h static time-kill using $1 \times$ MIC concentration of each antibiotic. The stock solution of antibiotics was prepared by diluting antibiotic powders in solvent to obtain final solutions of 100 mg/L. Eight tubes consisting of one growth control, four single agents (doxycycline, imipenem, ceftazidime and amoxicillin/clavulanate) and three doxycycline– β -lactam combinations were prepared for each strain. The tubes were incubated at 37 °C with 150 rpm shaking. An amount of 100 μ L was taken for quantitative culture at time intervals of 0, 3, 6, 12 and 24 h. In this study, a reduction of 2-log_{10} compared to the initial inoculums was taken as active killing activity by a particular antibiotic or combination. Synergy was defined as a 2-log_{10} reduction in colony count at 24 h in combination of antibiotics compared to the most active single agent. The viable colony count of combinations of antibiotics at 24 h also must decrease 2-log_{10} compared to initial inoculums to be indicated as synergy [10]. Indifferent activity was defined when the viable colony count in the presence of a combination of antibiotics at 24 h decreased 1-log_{10} compared to most active single antibiotic, while antagonism was defined when the viable colony count of a combination of antibiotics at 24 h increased 2-log_{10} compared with the most active single antibiotic [10].

In the time-kill experiments, doxycycline alone was active against BUPS/07/13/A with no re-growth at 24 h (Fig. 1). Against BUPS/91/08/A, doxycycline showed some killing activity with reduction of 1.73-log_{10} CFU/mL, however no reduction of viable count compared to initial inoculum was observed against BUPS/12/14/A and BUPS/

Table 1 The fractional inhibitory concentrations (FICs) of four *B. pseudomallei* strains from North-Eastern Malaysia by checkerboard method

Combinations of antibiotics	Strains	Σ FICs	Interpretation ^a
Doxycycline + imipenem	BUPS/12/14/A	1.00	Indifference
	BUPS/07/13/A	0.62	Indifference
	BUPS/07/14/A	1.00	Indifference
	BUPS/91/08/A	0.62	Indifference
Doxycycline + ceftazidime	BUPS/12/14/A	1.00	Indifference
	BUPS/07/13/A	0.75	Indifference
	BUPS/07/14/A	1.00	Indifference
	BUPS/91/08/A	0.75	Indifference
Doxycycline + amoxicillin–clavulanate	BUPS/12/14/A	2.12	Indifference
	BUPS/07/13/A	1.00	Indifference
	BUPS/07/14/A	1.00	Indifference
	BUPS/91/08/A	0.62	Indifference

^aSynergy is when the fractional inhibitory concentration index (Σ FIC) was ≤ 0.5 , indifference was 0.5–4 and antagonism ≥ 4

Fig. 1 Time-kill curve of antibiotics in combination with doxycycline against strain BUPS/12/14/A (a), BUPS/07/13/A (b), BUPS/07/14/A (c) and BUPS/91/08/A (d). Doxycycline (DOX), imipenem

(IMP), ceftazidime (CAZ) and amoxicillin–clavulanate (AMC). The dash horizontal line indicates limit of quantification (LOQ)

07/14/A. Among β -lactams agents, imipenem was the most active. This antibiotic was active against all tested strains, but re-growth occurred in BUPS/12/14/A, BUPS/07/14/A and BUPS/91/08/A. Ceftazidime and amoxicillin–clavulanate were inactive against BUPS/12/14/A. There was initial bactericidal activity of ceftazidime and amoxicillin–clavulanate against BUPS/07/13/A, BUPS/07/14/A and BUPS/91/08/A, however re-growth of the bacteria occurred in all single agents (Fig. 1b–d).

The combination of doxycycline/imipenem was active against all tested strains with no indication of re-growth at 24 h. However, when compared to the most active single antibiotic in the regimens at 24 h, the definition of synergy was not fulfilled. The combinations of doxycycline/ceftazidime and doxycycline/amoxicillin–clavulanate were

active against BUPS/07/13/A and BUPS/91/08/A with no re-growth after 24 h (Fig. 1b, d). These combinations showed indifferent interactions. When tested against BUPS/12/14/A and BUPS/07/14/A, these combinations were inactive (Fig. 1a, c).

Although the international recommendation is to use β -lactam antibiotics as monotherapy during the intensive phase in the majority of situations [4, 7], a combination of two antibiotics with different mechanisms has been used to treat multidrug resistant organisms [11]. The combination therapy might enhance the antibacterial activity, partly because if the bacteria start to become resistant to one antibiotic, the other antibiotic may still successfully clear the remaining bacteria [11]. The possibility of synergy in the doxycycline with β -lactam antibiotics combination

worth investigating as doxycycline is active against *B. pseudomallei* and has been routinely used in eradication therapy [4, 5, 7]. It has also been shown that doxycycline reduced the mortality rate in animal trials [12].

In the time-kill analysis, most antibiotic monotherapy regimens tested in this study (9 out of 10) were associated with re-growth after 3–12 h of incubation. Doxycycline alone failed to demonstrate bactericidal activity against three strains (BUPS/12/14/A, BUPS/07/14/A and BUPS/91/08/A), as the reduction of doxycycline monotherapy regime at 24 h was $< 2\text{-log}_{10}\text{CFU/mL}$ compared to initial inoculum. In fact, the time kill curve of doxycycline alone against two strains (BUPS/12/14/A and BUPS/07/14/A) showed an increase in bacteria at every time-point in this 24-h experiment. These observations confirmed that doxycycline is not suitable to be used as monotherapy in the intensive phase.

Time-kill analysis is in agreement with the checkerboard method in as much as synergistic activity was not detected in doxycycline and β -lactam combination regimens. Although doxycycline alone was inactive against strains BUPS/12/14/A, BUPS/07/14/A and BUPS/91/08/A, in combination with imipenem there was some activity. The bacterial count was markedly decreased after 24 h ($> 1\text{-log}_{10}\text{CFU/mL}$ less than the count in the most active single antibiotic in the combination) but this did not meet the criteria for synergy. Against BUPS/07/13/A, doxycycline alone was active and its killing effect was boosted after combination with ceftazidime and amoxicillin–clavulanate, which were inactive when acted as single agents. Only two out of 12 combinations were associated with re-growth of bacteria, i.e. doxycycline/ceftazidime and doxycycline/amoxicillin–clavulanate against BUPS/12/14/A.

In conclusion, on the basis of in vitro results, we would predict that doxycycline would be inferior to trimethoprim–sulfamethoxazole to be used in combination with β -lactam antibiotics in the intensive phase of melioidosis treatment. Although no synergistic effects were seen in both checkerboard and time-kill analysis, there was no case of major re-growth in bacterial population observed in the doxycycline and β -lactam combinations as compared to the individual agents. The addition of doxycycline did not antagonize the activity of β -lactam antibiotics against all tested isolates.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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