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

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**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 increase the 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). The mortality rate is particularly high enough 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 meliodosis 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 μg/mL to 128 μg/mL. The antibiotic solution was two-fold concentrations of antibiotics used in this study ranged from 0.125 μg/mL to 128 μg/mL. The antibiotic solution was two-fold dilution 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 achieved. A 1:100 dilution of bacterial culture 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).

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 lowest 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).

**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 1×10^8 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 lowest 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**

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Sequence type</th>
<th>Minimum inhibitory concentrations [μg/mL]</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SXT</td>
<td>AMC</td>
</tr>
<tr>
<td>BUPS/12/14</td>
<td>54</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>BUPS/07/14</td>
<td>376</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>BUPS/07/13</td>
<td>1322</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>BUPS/91/08</td>
<td>1326</td>
<td>0.5</td>
<td>8</td>
</tr>
</tbody>
</table>

*Trimethoprim-sulphamethoxazole (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-log₁₀ 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 1A). 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 1C). The slow killing effects of the combination regimens 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 3. Interpretation of time-kill curve of β-lactam/trimethoprim-sulfamethoxazole combinations against four clinical strains of B. pseudomallei at 24 h

<table>
<thead>
<tr>
<th>Antibiotics#</th>
<th>Killing activity* (log₁₀CFU/mL)</th>
<th>Combinations of antibiotics*</th>
<th>Killing activity* (log₁₀CFU/mL)</th>
<th>Interaction at 24 h** (log₁₀CFU/mL)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUPS/12/14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SXT</td>
<td>0.03</td>
<td>AMC + SXT</td>
<td>-1.91</td>
<td>-2.01*</td>
<td>Indifference</td>
</tr>
<tr>
<td>AMC</td>
<td>0.96</td>
<td>CAZ + SXT</td>
<td>-0.55</td>
<td>-0.66</td>
<td>Indifference</td>
</tr>
<tr>
<td>IMP</td>
<td>-4.79</td>
<td>IMP + SXT</td>
<td>-4.51</td>
<td>0.40</td>
<td>Indifference</td>
</tr>
<tr>
<td>BUPS/07/13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SXT</td>
<td>-3.48</td>
<td>AMC + SXT</td>
<td>-5.60</td>
<td>-1.95</td>
<td>Indifference</td>
</tr>
<tr>
<td>AMC</td>
<td>1.64</td>
<td>CAZ + SXT</td>
<td>-5.92</td>
<td>-2.25</td>
<td>Synergy</td>
</tr>
<tr>
<td>IMP</td>
<td>-3.55</td>
<td>IMP + SXT</td>
<td>-5.15</td>
<td>-1.64</td>
<td>Indifference</td>
</tr>
<tr>
<td>BUPS/07/14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SXT</td>
<td>-0.05</td>
<td>AMC + SXT</td>
<td>-1.97</td>
<td>-1.54</td>
<td>Indifference</td>
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<tr>
<td>AMC</td>
<td>1.44</td>
<td>CAZ + SXT</td>
<td>-2.46</td>
<td>-2.41</td>
<td>Synergy</td>
</tr>
<tr>
<td>IMP</td>
<td>-0.14</td>
<td>IMP + SXT</td>
<td>-3.28</td>
<td>-3.32</td>
<td>Synergy</td>
</tr>
<tr>
<td>BUPS/91/08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SXT</td>
<td>-2.20</td>
<td>AMC + SXT</td>
<td>-3.38</td>
<td>-1.29</td>
<td>Indifference</td>
</tr>
<tr>
<td>AMC</td>
<td>1.36</td>
<td>CAZ + SXT</td>
<td>-4.41</td>
<td>-2.38</td>
<td>Synergy</td>
</tr>
<tr>
<td>IMP</td>
<td>-4.45</td>
<td>IMP + SXT</td>
<td>-6.00</td>
<td>-1.32</td>
<td>Indifference</td>
</tr>
</tbody>
</table>

# Trimethoprim-sulfamethoxazole (SXT), amoxicillin-clavulanate (AMC), ceftazidime (CAZ), imipenem (IMP).
* Killing activity-Log₁₀CFU/mL differences between at initial inoculums and at 24 h.
** Synergy > 2-log₁₀ reduction, indifference ± <2-log₁₀, antagonism ≥ 2-log₁₀ increase.
* Although the combination ≥ 2-log10, the killing activity of AMC+SXT is <2-log₁₀CFU/mL, so did not fulfill the criteria of synergy.
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 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.

**REFERENCES**


