In Vitro Activities of Amoxicillin-Clavulanate, Doxycycline, Ceftazidime, Imipenem, and Trimethoprim-Sulfamethoxazole against Biofilm of Brazilian Strains of Burkholderia pseudomallei

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This study aimed at investigating the in vitro activities of amoxicillin-clavulanate, doxycycline, ceftazidime, imipenem, and trimethoprim-sulfamethoxazole against Burkholderia pseudomallei in planktonic and biofilm forms, through broth microdilution and resazurin-based viability staining, respectively. In planktonic growth, the strains were susceptible to the drugs, while in biofilm growth, significantly higher antimicrobial concentrations were required, especially for ceftazidime and imipenem, surpassing the resistance breakpoints. These results highlight the importance of the routine evaluation of biofilm antimicrobial susceptibility.

Burkholderia pseudomallei is the causative agent of melioidosis, a disease endemic to southeastern Asia, northern Australia, and northeastern Brazil, that presents high lethality rates (1, 2, 3). Currently, the most commonly used antimicrobials for treating melioidosis are ceftazidime (CAZ), imipenem (IPM), amoxicillin-clavulanate (AMC), doxycycline (DOX), and trimethoprim-sulfamethoxazole (SXT) (2–4), but there have been several reports of in vitro and in vivo resistance to these drugs among B. pseudomallei isolates (1–3). This resistance may be related to biofilm-associated B. pseudomallei infections (5–7), which emphasizes the importance of treating melioidosis with antimicrobials that are effective against B. pseudomallei biofilms. Thus, the aim of this study was to assess the in vitro activities of the five commonly used antimicrobial drugs in the treatment of melioidosis strains of B. pseudomallei in planktonic form and in biofilm form.

Nine strains of β-lactamase-positive B. pseudomallei, isolated from clinical and environmental sources and stored at the Laboratory of Emerging and Re-emerging Pathogens of the Federal University of Ceará, Brazil, were used in this study (4). Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, mexitilin-resistant Staphylococcus aureus (MRSA), Staphylococcus aureus ATCC 25923 (a biofilm producer), and non-biofilm-producing Staphylococcus epidermidis were used as experimental controls.

The ability of each strain to produce biofilm was quantified spectrophotometrically as previously described (8, 9). Biofilm production was induced in a flat-bottomed well of a 96-well microtiter plate, using brain heart infusion broth (Oxoid, Basing-stoke, Hampshire, England) enriched with 1% glucose. The plates were incubated for 48 h at 37°C (8, 9), and each well was dyed with 0.25% crystal violet, after washing with phosphate-buffered saline (PBS). Based on the obtained optical density values at 570 nm (OD570), the strains were classified as nonproducers, weak producers, moderate producers, or strong producers, as described by Stepanovic et al. (8).

Susceptibility testing was performed through broth microdilution with AMC, CAZ, DOX, IPM, and SXT (Sigma-Aldrich, Brazil) (4), as standardized by the Clinical and Laboratory Standards Institute (CLSI) and described in document M07-A8 (10). Plates were incubated at 37°C for 24 h, and MICs were defined as the lowest concentrations able to inhibit 100% of growth (10).

Minimum biofilm-inhibitory concentrations (MBICs) and minimum biofilm elimination concentrations (MBECs) were determined by the broth microdilution method with the use of resazurin (Sigma-Aldrich, Brazil). Briefly, 2-day adherent B. pseudomallei biofilms were grown in wells as previously described (8, 9). Then, the wells were washed with PBS, and each antimicrobial drug was added in progressive 2-fold dilutions (11). The antimicrobial concentration ranges were: 4/2 to 512/256, 4 to 256, 0.5/9.5 to 64/1,216 mg/liter for AMC, CAZ, DOX, IPM, and SXT, respectively. Plates were incubated at 37°C for 24 h. Then, 20 μl of 0.05% resazurin solution was added, and plates were incubated for 1 h (12–14). The MBIC was defined as the lowest concentration able to partially inhibit cellular activity, while the MBEC was defined as the lowest concentration with no evidence of cellular activity (15, 16).

The tests were performed in triplicate and repeated on two different occasions. Student’s t test was employed to evaluate the obtained data. Differences were considered statistically significant when P was <0.05.

The obtained cutoff for biofilm production was 0.021, and the tested strains were classified as weak (n = 1), moderate (n = 3), or strong (n = 5) producers (Table 1).

The following MIC ranges were observed: 4/2 to 16/8, 2 to 8, 0.25 to 0.5, 0.125 to 1, and 0.125/2.375 to 2/38 mg/liter for AMC,
that a similar mechanism occurs in biofilms of \textit{B. pseudomallei} -lactamases within the biofilm matrix (6). We strongly believe that these enzymes hydrolyze antibiotic molecules. In addition, the production of \textit{B. pseudomallei} -lactam resistance in biofilms was associated with the occurrence of resistances to CAZ and IPM, which resulted in significantly higher MIC, MBIC, and MBEC values for these drugs primarily targeting fast-growing bacteria (5, 7); (iii) the secretion of \textit{B. pseudomallei} -lactamase, reaching values above the resistance breakpoints. No increases in MICs were observed for AMC, CAZ, DOX, IPM, and SXT, respectively. All tested strains were susceptible to the five antimicrobials (17). (Table 2).

### Table 2: Susceptibilities to Five Antimicrobial Agents of \textit{B. pseudomallei} Isolates in planktonic and biofilm forms

| Drug Parameter | Weak producer | Moderate producer | Strong producer | Mean fold change*
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<tr>
<td></td>
<td>AMC MIC 8/4</td>
<td>AMC MBIC 8/4 (8, R)</td>
<td>AMC MBEC 8/4 (16, S)</td>
<td>256 (1, S)</td>
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<tr>
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<td>CAZ MIC 4</td>
<td>CAZ MBIC &gt;512 (64, R)</td>
<td>CAZ MBEC &gt;512 (64, R)</td>
<td>&gt;512 (&gt;256, R)</td>
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<tr>
<td></td>
<td>DOX MIC 0.25</td>
<td>DOX MBIC 1 (8, S)</td>
<td>DOX MBEC 2 (8, S)</td>
<td>16 (2, S)</td>
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<tr>
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<td>IPM MIC 0.5</td>
<td>IPM MBIC &gt;256 (&gt;512, R)</td>
<td>IPM MBEC &gt;256 (&gt;512, R)</td>
<td>&gt;512 (&gt;256, R)</td>
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<td>SXT MIC 0.254</td>
<td>SXT MBIC 0.599 (≤2, S)</td>
<td>SXT MBEC 0.599 (≤2, S)</td>
<td>&gt;0.599 (≤1, S)</td>
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*The fold change compared to the MIC. R, resistant; S, susceptible.

\(\ast , P < 0.05; **, P < 0.001.\)
The results of the present study point to the need for standardizing routine methods to evaluate biofilm antimicrobial susceptibility. Nowadays, it is a common belief that more studies should be focused on biofilm rather than planktonic growth in order to succeed in controlling bacterial infections.

ACKNOWLEDGMENTS

This work was supported by CAPES/Brazil (PNPD 2103/2009; AE10052000630100/11) and by Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico and the National Council for Scientific and Technological Development (PPSUS FUNCAP/CNPq, Brazil, process 13192409-5).

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