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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 against 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 Reemerging Pathogens of the Federal University of Ceará, Brazil, were used in this study (4). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, methicillin-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, Basingstoke, 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 (OD_{570}), 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 512, 0.5 to 64, 2 to 256, and 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,

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TABLE 1 Biofilm-producing abilities of nine *B. pseudomallei* isolates[a]

Strain designation, OD_{570} value		
Weak biofilm producer (OD_{570}, 0.021 to 0.043)	Moderate biofilm producers (OD_{570}, 0.043 to 0.085)	Strong biofilm producers (OD_{570}, >0.085)
Bp096c, 0.023[b]	Bp038c, 0.052	Bp011c, 0.153
	Bp041e, 0.076	Bp040e, 0.238
	Bp044e, 0.066	Bp043e, 0.128
		Bp045e, 0.195
		Bp066c, 0.105

[a] Isolate designations ending with a c were obtained from a clinical source; those ending with an e were obtained from an environmental source.
[b] The average OD_{570}.

CAZ, DOX, IPM, and SXT, respectively. All tested *B. pseudomallei* strains were susceptible to the five antimicrobials (17) (Table 2).

MBIC and MBEC values were often the same, and the obtained MBIC/MBEC ranges were 8/4 to 64/32, 4 to <512, 1 to 8, >256, and 0.5/9.5 to 2/38 mg/liter for AMC, CAZ, DOX, IPM, and SXT, respectively. The mean inhibitory concentrations of the five tested antimicrobial agents against mature *B. pseudomallei* biofilms were higher than the obtained MICs (Table 2), especially the MBICs and MBECs for IPM ($P < 0.001$) and the MBICs for CAZ ($P < 0.001$). Many of the MBIC/MBEC values surpassed the resistance breakpoints (17), especially those for IPM, turning all strains resistant to this drug.

Biofilms are commonly associated with the occurrence of recalcitrant infections (18) and are notorious for their ability to tolerate high concentrations of antibiotics that are lethal to their planktonic counterparts (6). In general, our results showed significantly higher antimicrobial inhibitory concentrations against *B. pseudomallei* biofilms, as previously reported (5, 7), especially for β-lactams, reaching values above the resistance breakpoints. No correlation was observed between the ability to form biofilm and the antimicrobial susceptibility of each strain. Instead, biofilm susceptibility seemed to be related to the chemical characteristics of the antimicrobial. DOX and SXT, for example, showed the best inhibitory and bactericidal activities, with MBIC/MBEC values below resistance breakpoints, while *B. pseudomallei* biofilms were resistant to CAZ and IPM, which resulted in significantly higher MBIC and MBIC/MBEC values, respectively.

This resistance to CAZ and IPM may rely on a few possibilities: (i) decreased drug diffusion through the structures of *B. pseudomallei* biofilms (7); (ii) low growth rates of biofilm cells, since these drugs primarily target fast-growing bacteria (5, 7); (iii) the secretion of β-lactamases, as this is one of the main mechanisms of β-lactam resistance in *B. pseudomallei* (19, 20); and (iv) the development of persister cells (18), even though these are dormant cells with basal protein production (18) and thus low β-lactamase production, which most likely do not produce sufficient β-lactamase to hydrolyze and neutralize antibiotic molecules.

It has been shown that in biofilms of *Pseudomonas aeruginosa*, β-lactamases are secreted and maintain their activity within the biofilm matrix, and these enzymes hydrolyze β-lactam antibiotics before reaching the bacterial cells (6). In addition, the production of β-lactamases by biofilm cells can be induced by β-lactam antibiotics, such as CAZ and IPM, which increase the levels of free β-lactamases within the biofilm matrix (6). We strongly believe that a similar mechanism occurs in biofilms of *B. pseudomallei*.

TABLE 2 Susceptibilities to five antimicrobial agents of *B. pseudomallei* isolates in planktonic and biofilm forms

Drug	Parameter	MIC, MBIC, and MBEC (mg/liter) for isolate in indicated biofilm-producing group (fold change[a])									Mean fold change[b]
		Weak producer	Moderate producer			Strong producer					
		Bp096	Bp038	Bp041	Bp044	Bp011	Bp040	Bp043	Bp045	Bp066	
AMC	MIC	8/4	16/8	8/4	4/2	16/8	8/4	16/8	8/4	8/4	
	MBIC	64/32 (8, R)	8/4 (0.5, S)	8/4 (1, S)	8/4 (2, S)	64/32 (4, R)	8/4 (1, S)	8/4 (0.5, S)	8/4 (1, S)	16/8 (2, S)	3
	MBEC	64/32 (8, R)	16/8 (1, S)	16/8 (2, S)	16/8 (4, S)	64/32 (4, R)	8/4 (1, S)	16/8 (1, S)	8/4 (1, S)	32/16 (4, R)	3
	Fold ratio	8	4	4	2	8	4	8	4	8	
CAZ	MIC	>512 (>64, R)	8 (2, S)	16 (4, S)	4 (2, S)	>512 (>64, R)	8 (2, S)	16 (2, S)	4 (1, S)	4 (0.5, S)	>18
	MBIC	>512 (>64, R)	64 (16, R)	>512 (>128, R)	>512 (>256, R)	>512 (>64, R)	128 (32, R)	>512 (>64, R)	>512 (>128, R)	>512 (>64, R)	>91**
	MBEC	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.25	
	Fold ratio	1 (4, S)	2 (8, S)	1 (4, S)	2 (8, S)	1 (4, S)	1 (4, S)	2 (8, S)	2 (4, S)	1 (4, S)	5**
DOX	MIC	2 (8, S)	2 (8, S)	4 (16, S)	8 (32, S)	2 (8, S)	2 (8, S)	4 (16, S)	8 (16, S)	2 (8, S)	13*
	MBIC	0.5	1	1	0.5	0.125	0.5	0.25	1	0.5	
	MBEC	>256 (>512, R)	>256 (>256, R)	>256 (>256, R)	>256 (>512, R)	256 (2,048, R)	>256 (>512, R)	256 (1,024, R)	>256 (>256, R)	>256 (>512, R)	>654**
	Fold ratio	>256 (>512, R)	>256 (>256, R)	>256 (>256, R)	>256 (>512, R)	>256 (>2,048, R)	>256 (>512, R)	>256 (>1,024, R)	>256 (>256, R)	>256 (>512, R)	>654**
IPM	MIC	0.25/4.75	0.5/9.5	2/38	0.125/2.375	0.5/9.5	0.25/4.75	2/38	0.25/4.75	0.25/4.75	
	MBIC	≤0.5/9.5 (≤2, S)	≤0.5/9.5 (≤1, S)	≤0.5/9.5 (≤0.25, S)	≤0.5/9.5 (≤4, S)	≤0.5/9.5 (≤1, S)	≤0.5/9.5 (≤2, S)	≤0.5/9.5 (≤0.25, S)	≤0.5/9.5 (≤2, S)	≤0.5/9.5 (≤2, S)	<2
	MBEC	≤0.5/9.5 (≤2, S)	≤0.5/9.5 (≤1, S)	2/38 (≤1, S)	≤0.5/9.5 (≤4, S)	≤0.5/9.5 (≤1, S)	≤0.5/9.5 (≤2, S)	2/38 (≤1, S)	1/19 (4, S)	≤0.5/9.5 (≤2, S)	≤2*
SXT	MIC	0.25/4.75	0.5/9.5	2/38	0.125/2.375	0.5/9.5	0.25/4.75	2/38	0.25/4.75	0.25/4.75	
	MBIC	≤0.5/9.5 (≤2, S)	≤0.5/9.5 (≤1, S)	≤0.5/9.5 (≤0.25, S)	≤0.5/9.5 (≤4, S)	≤0.5/9.5 (≤1, S)	≤0.5/9.5 (≤2, S)	≤0.5/9.5 (≤0.25, S)	≤0.5/9.5 (≤2, S)	≤0.5/9.5 (≤2, S)	<2
	MBEC	≤0.5/9.5 (≤2, S)	≤0.5/9.5 (≤1, S)	2/38 (≤1, S)	≤0.5/9.5 (≤4, S)	≤0.5/9.5 (≤1, S)	≤0.5/9.5 (≤2, S)	2/38 (≤1, S)	1/19 (4, S)	≤0.5/9.5 (≤2, S)	≤2*

[a] The fold change compared to the MIC. R, resistant; S, susceptible.
[b] *, $P < 0.05$; **, $P < 0.001$.

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.

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