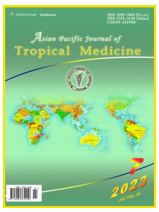

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Biofilm analyses and exoproduct release by clinical and environmental isolates of *Burkholderia pseudomallei* from Brazil

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ABSTRACT

Objective: To characterize biofilm production by clinical (n=21) and environmental (n=11) isolates of *Burkholderia pseudomallei* and evaluate the production of proteases, hemolysins and siderophores.

Methods: Initially, the 32 strains were evaluated for biofilm production in Müller-Hinton broth-1% glucose (MH-1% glucose) and BHI broth-1% glucose, using the crystal violet staining technique. Subsequently, growing (48 h) and mature (72 h) biofilms were evaluated by confocal microscopy. Finally, the production of proteases, hemolysins and siderophores by planktonic aggregates, growing biofilms and mature biofilms was evaluated.

Results: All isolates produced biofilms, but clinical isolates had significantly higher biomass in both MH-1% glucose (P<0.001) and BHI-glucose 1% (P=0.005). The structural analyses by confocal microscopy showed thick biofilms, composed of multiple layers of cells, homogeneously arranged, with mature biofilms of clinical isolates presenting higher biomass (P=0.019) and thickness of the entire area (P=0.029), and lower roughness coefficient (P=0.007) than those of environmental isolates. Protease production by growing biofilms was significantly greater than that of planktonic (P<0.001) and mature biofilms (P<0.001). Hemolysin release by planktonic aggregates was higher than that of biofilms (P<0.001). Regarding siderophores, mature biofilms presented higher production than growing biofilms (P<0.001) and planktonic aggregates (P<0.001).

Conclusions: Clinical isolates have higher production of biofilms than their environmental counterparts; protease and siderophores seem important for growth and maintenance of *Burkholderia pseudomallei* biofilms.

KEYWORDS: Mangosteen; *Burkholderia pseudomallei*; Biofilms; Proteases; Hemolysins; Siderophores

Significance

Much is known about the relevance of biofilm formation in the pathogenesis of melioidosis, however little is known about the production of other virulence factors such as enzymes and siderophores by *Burkholderia pseudomallei* biofilms. This study brings results that can help understanding the production of exoproducts by planktonic *Burkholderia pseudomallei* and their biofilms.

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1. Introduction

Burkholderia (*B.*) *pseudomallei* is a Gram-negative bacteremia that causes the infectious disease melioidosis. Melioidosis is highly endemic in Southeast Asia and northern Australia[1,2], and in South America, northeast Brazil is also an endemic area[3].

The infection is acquired mainly by contact with contaminated water and soil and has several clinical manifestations, with abscesses and pneumonia as the most common ones, which may rapidly evolve to sepsis[4]. Various virulence factors may contribute to the pathogenicity of *B. pseudomallei*, such as the biofilm formation and the production of proteases, hemolysins and siderophores.

Biofilm production gives the bacteria greater resistance to the immune system and to antimicrobial agents, and it is frequently associated with the chronic characters of melioidosis. Moreover, there might be an association between the *in vitro* biofilm-forming ability of a *B. pseudomallei* strain and the occurrence of clinical relapse of melioidosis[5]. Hemolysins lyse erythrocytes and hinder phagocytosis by the host's immune system[6], while proteases contribute to tissue invasion[7-9]. Siderophores are important for the survival of *B. pseudomallei* strains, as they promote bacterial iron acquisition from the environment, a vital element for bacterial metabolic processes[10].

Despite the knowledge on virulence of *B. pseudomallei*[10], studies on the production of enzymes and siderophores by this bacterium in the form of biofilms are scarce. Thus, this study aimed to characterize biofilm production by clinical and environmental isolates of *B. pseudomallei* from Ceará state, Brazil, and to evaluate the production of proteases, hemolysins and siderophores by these isolates as planktonic aggregates, growing and mature biofilms.

2. Materials and methods

2.1. Microorganisms

In this study, a total of 32 strains were used, 21 from clinical and 11 from environmental sources (Table 1). All strains belong to the collection of the Laboratory of Emerging and Reemerging Pathogens (LAPERE) of the Federal University of Ceará (Fortaleza,

Table 1. Analyzed isolates of *Burkholderia pseudomallei* from the state of Ceará, Brazil.

Origin	Accession number (CEMM)	Sequence type	Isolation source	Isolation place
Clinical (*n*=21)	03-6-034	ST-1355	blood	Brazil: Tejuçuoca (Ceará State)
	03-6-035	ST-1355	blood	Brazil: Tejuçuoca (Ceará State)
	03-6-036	ST-95	broncho-alveolar aspirate	Brazil: Ipu (Ceará State)
	03-6-037	ST-1462	blood	Brazil: Aracoiaba (Ceará State)
	03-6-038	ST-95	blood	Brazil: Granja (Ceará State)
	05-3-008	ST-1355	lymphnode biopsy	Brazil: Itapajé (Ceará State)
	05-3-009	ST-1454	splenic abscess	Brazil: Ipu (Ceará State)
	05-3-010	ST-95	blood	Brazil: Pacoti (Ceará State)
	05-3-011	ST-95	blood	Brazil: Ocara (Ceará State)
	05-5-065	ST-92	blood	Brazil: São João do Jaguaribe (Ceará State)
	05-5-096	ST-92	synovial fluid	Brazil: Caridade (Ceará State)
	05-5-066	ST-1455	blood	Brazil: Tauá (Ceará State)
	05-6-089	ST-95	blood	Brazil: São Gonçalo do Amarante (Ceará State)
	05-6-090	ST-1456	blood	Brazil: Solonópole (Ceará State)
	05-6-091	ST-92	blood	Brazil: Fortaleza (Ceará State)
	05-6-092	ST-1457	urine	Brazil: Amontada (Ceará State)
	05-6-093	ST-92	blood	Brazil: Granja (Ceará State)
	05-2-059	ST-297	blood	Brazil: Ipu (Ceará State)
	JFS	NT	blood	NK
	L964	NT	NK	NK
	JSR	NT	blood	NK
Environmental (*n*=11)	03-6-039	ST-1460	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-040	ST-95	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-041	ST-95	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-042	missing narK gene	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-043	ST-1463	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-044	ST-95	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-045	ST-95	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-046	ST-1463	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-047	ST-1463	soil	Brazil: Tejuçuoca (Ceará State)
	03-6-048	ST-95	soil	Brazil: Tejuçuoca (Ceará State)
	05-6-107	ST-1461	water	Brazil: Banabuiú (Ceará State)

NT: not typed. NK: not known. Strains were typed in previous study (Gee *et al*., 2021-doi: 10.1128/mSphere.01259.)

Brazil). All experimental stages were carried out in the biosafety level 3 laboratory (BSL-3) of LAPERE.

2.2. Biofilm formation by B. pseudomallei

Initially, the 32 strains were evaluated for their ability to form biofilms using two different media: brain and heart infusion (BHI) broth supplemented with 1% glucose or Mueller Hinton (MH) broth (Kasvi, Brazil) supplemented with 1% glucose. To promote biofilm production, the strains were sub-cultured in BHI broth for 48 hours. Then bacterial cells were transferred to tubes with sterile saline, adjusting the suspension to turbidity 6 on McFarland scale, which is equivalent to a concentration of 1.8×10^9 cfu/mL. Subsequently, 25 μL of the bacterial suspension was inoculated in a 96-well plate filled with 175 μL of BHI-1% glucose or MH-1% glucose and the plates were incubated at 37 ºC for 48 hours[11]. All strains were grown in triplicate and evaluated at four different moments, and each culture medium were tested. Bacterium-free wells were included as sterility control.

After the incubation period, the biofilm-forming ability was assessed by crystal violet staining technique and the reading was performed on a spectrophotometer at 490 nm (OD_{490}). Based on the OD values of the negative control, the cutoff value (ODc) for the test to classify strains as biofilm-producing was set as three standard deviations above the mean OD observed for the negative control. In the end, the strains were classified as non-biofilm-producers ($OD_{490}\leqslant$ODc), weak biofilm-producers (ODc<$OD_{490}\leqslant2\times$ODc), moderate biofilm-producers ($2\times$ODc<$OD_{490}\leqslant4\times$ODc) or strong biofilm-producers ($OD_{490}>4\times$ODc)[12].

2.3. Confocal laser scanning microscopy of growing and mature B. pseudomallei biofilm

For the morphological evaluation of biofilms, six strong or moderate biofilm-producing isolates (3 clinical isolates: CEMM 03-6-036, CEMM 05-6-089, CEMM 05-6-093; and 3 environmental isolates: CEMM 03-6-039, CEMM 03-6-042, CEMM 03-6-048) were selected. Biofilm formation was induced in a 24-well flat-bottomed plate containing a tissue culture coverslip (Thermanox™), making a volumetric adjustment, that is, 875 μL of BHI-1% glucose and 125 μL of bacterial inoculum at turbidity 6 on the McFarland scale[11] and incubated for 48 h for the analysis of growing biofilm, and 72 h for the analysis of mature biofilm. From this stage, only BHI-1% glucose broth was used, since it showed satisfactory results of biofilm production and better results in the production of other virulence factors.

Biofilms were evaluated by confocal microscopy for the analysis of the total biomass, biomass average thickness, average thickness of the entire area, maximum thickness, roughness coefficient and surface-volume ratio at different times of growth (48 h and 72 h). After the incubation period, biofilms were washed with PBS and stained with Live/Dead™ fluorescent dye (Invitrogen, USA). Live/Dead™ dye contains SYTO9, which identifies living cells, and propidium iodide, which identifies dead or damaged cells, shown at 488 nm and 561 nm, respectively. The COMSTAT software was used for quantitative analysis of the stacked images acquired by confocal laser scanning microscope. For the analysis, 10 equidistant points were evaluated with a Nikon C2 microscope and the three-dimensional images of the biofilms were obtained and analyzed with the ImageJ software[13,14].

2.4. Evaluation of the production of proteases, hemolysins and siderophores by strains of B. pseudomallei as planktonic aggregates, growing biofilms and mature biofilms

Of the 32 strains evaluated, 16 (8 clinical and 8 environmental) strong or moderate biofilm-forming strains were selected to analyze the production of the other virulence factors. Thus, the strains were inoculated in BHI broth and incubated for 24 hours. After incubation, they were resuspended in saline until reaching turbidity of 6 on McFarland scale. For the analysis of the production of virulence factors as planktonic aggregates, 125 μL of the bacterial inoculum were added to glass tubes containing 875 μL of BHI-1% glucose, preserving the inoculum: culture medium (1: 7 v/v) ratio used to induce biofilm growth. As for the production of virulence factors by isolates in biofilm form, 25 μL of the same inoculum was added to flat-bottom 96-well polystyrene plates containing 175 μL of BHI-1% glucose per well and three wells were used for each tested isolate. The tubes were incubated at 37 ℃ for 48 h and plates were incubated at 37 ºC for 48 and 72 h for the analysis of growing and mature *B. pseudomallei* biofilms, respectively. Then, 500 μL of the culture supernatant of planktonic aggregates and the biofilm supernatant from three wells were centrifuged at $9\,408\times g$ for 10 minutes. For both forms of growth, tubes and wells containing only 1% BHI-glucose were included as a sterility control and as a blank for spectrophotometric reading. All strains were evaluated in triplicate at four different moments[15].

To evaluate the production of proteases, a 200 μL aliquot of each supernatant was collected and added to 200 μL of an azoalbumin solution, reaching a total volume of 400 μL, incubated in a water bath at 37 ºC for 3 hours. After incubation, the enzymatic reaction was stopped with the addition of 4 mL of 5% trichloroacetic acid. The aliquots were centrifuged at $9\,408\times g$ for 10 minutes and 100 μL of the supernatant were removed and added to 100 μL of 0.5 M

NaOH. After vortexing for 30 seconds, the solution was read in a spectrophotometer at 440 nm[15,16]. The result was presented as the difference between the optical density of the test samples and the optical density of the blank sample.

To evaluate hemolysin production, 100 μL of supernatant from planktonic aggregates and growing biofilms, after centrifugation at 9 408×*g* for 10 minutes, were transferred to microtubes containing 875 μL of 1×PBS and 25 μL of defibrinated blood and incubated in a water bath at a 37 ºC for 30 minutes. After incubation, the samples were centrifuged at 5 500×*g* for 1 minute and 200 μL of each sample were transferred to a 96-well plate and read on spectrophotometry at 450 nm. The supernatants of the sterility controls (planktonic and biofilm growth) were incubated with the blood solution and used as a negative control. 2% Triton X-100 solution was incubated with the blood solution and used as a positive control, defined as 100% hemolysis[17]. Hemolytic activity was expressed as a percentage of hemolysis, which was calculated using the following equation: hemolysis (%)=[(A450 nm test strain-A450 nm negative control)/(A450 nm positive control-A450 nm blood solution)]×100[18].

To evaluate the production of siderophores, 100 μL of supernatants obtained from planktonic aggregates and biofilm growth were added to an equal volume of Chrome Azural S (CAS) solution (0.6 mM hexadecyl-trimethyl-ammonium, 0.015 mM $FeCl_3.6H_2O$, 0.15 mM Chrome Azural S, 50 mM anhydrous piperazine, 0.75 M HCl). This mixture was incubated at 28 ℃ for 15 minutes, and an increase in the intensity of the orange color was measured with the aid of a spectrophotometer at 630 nm. The supernatants from the sterility controls (planktonic and biofilm) were used as a negative control[19,10].

2.5. Statistical analysis

Student's *t* test or Mann Whitney's *U* test were used for parametric or non-parametric data, respectively, to evaluate the production of proteases, hemolysins and siderophores by clinical and environmental strains of *B. pseudomallei*, as planktonic aggregates and biofilm growth. Data obtained for the analyzed isolates were expressed as mean±standard deviation or boxplots with maximum and minimum values. Statistical analysis and graphing were performed using the GraphPad Prism 7 program.

3. Results

In BHI-glucose 1%, clinical isolates (*n*=21) were classified as strong (*n*=9), moderate (*n*=11) and weak (*n*=1) biofilm-producers and environmental isolates (*n*=11) as strong (*n*=2), moderate (*n*=6) and weak (*n*=3) biofilm-producers. In MH-1% glucose broth, clinical strains were classified as strong (*n*=20) and moderate biofilm producers (*n*=1), and environmental strains were classified as strong (*n*=7) and moderate (*n*=4) producers. In MH broth, a higher biomass production was observed (*P*<0.001). Compared with the environmental strains, a greater production by clinical strains was observed when biofilms were grown using MH-1% glucose (*P*<0.001) and BHI-glucose 1% (*P*=0.005) culture media (Figure 1). Clinical isolates presented higher biofilm formation than environmental isolates in both culture media. Therefore, we chose to use the BHI-1% glucose medium for the other analyses performed in this study, as BHI induced the production of proteases, while MH did not.

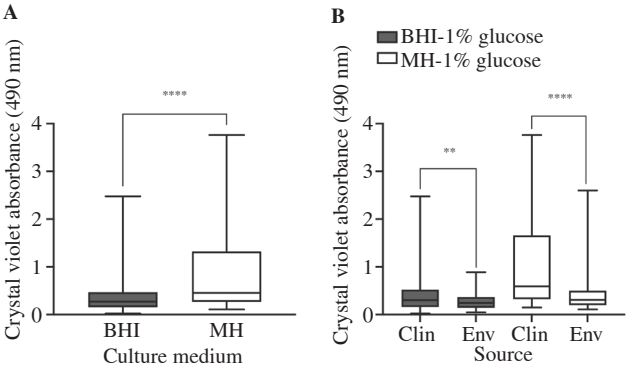

Figure 1. Biofilm formation by clinical (*n*=21) and environmental (*n*=11) Burkholderia pseudomallei in brain heart infusion (BHI) and Mueller Hinton (MH) broths. A: Biofilms grown in BHI broth supplemented with 1% glucose and MH broth supplemented with 1% glucose; B: Biofilms of clinical (Clin) and environmental (Env) isolates grown in BHI-1% glucose or MH-1% glucose broth. Boxplots with maximum and minimum values were used to express the data of crystal violet absorbance obtained for 21 clinical and 11 environmental isolates. Mann-Whitney's test was used for the analysis of the data. **indicates *P*=0.005; ****indicates *P*<0.000 1.

For the microscopic analysis, biofilms were grown in BHI-1% glucose broth. The structural analyses by confocal microscopy revealed that *B. pseudomallei* mature (72 h-grown) biofilms presented higher biomass (*P*=0.018), biomass thickness (*P*=0.010) and thickness of the entire area (*P*=0.036) and lower surface-volume ratio (*P*<0.001) than those of growing (48 h-grown) biofilms (Figure 2A). It was also observed that the maximum thickness in growing biofilms of clinical isolates were thicker (*P*=0.004) than those of environmental isolates (Figure 2B). Moreover, as biofilms matured (72 h), other differences became more evident between clinical and environmental isolates, with mature biofilms of clinical isolates presenting higher biomass (*P*=0.019) and thickness of the entire area (*P*=0.029), and lower roughness coefficient (*P*=0.007) than those of environmental isolates (Figure 2C). The analysis showed thick biofilms, composed of multiple layers of cells, homogeneously

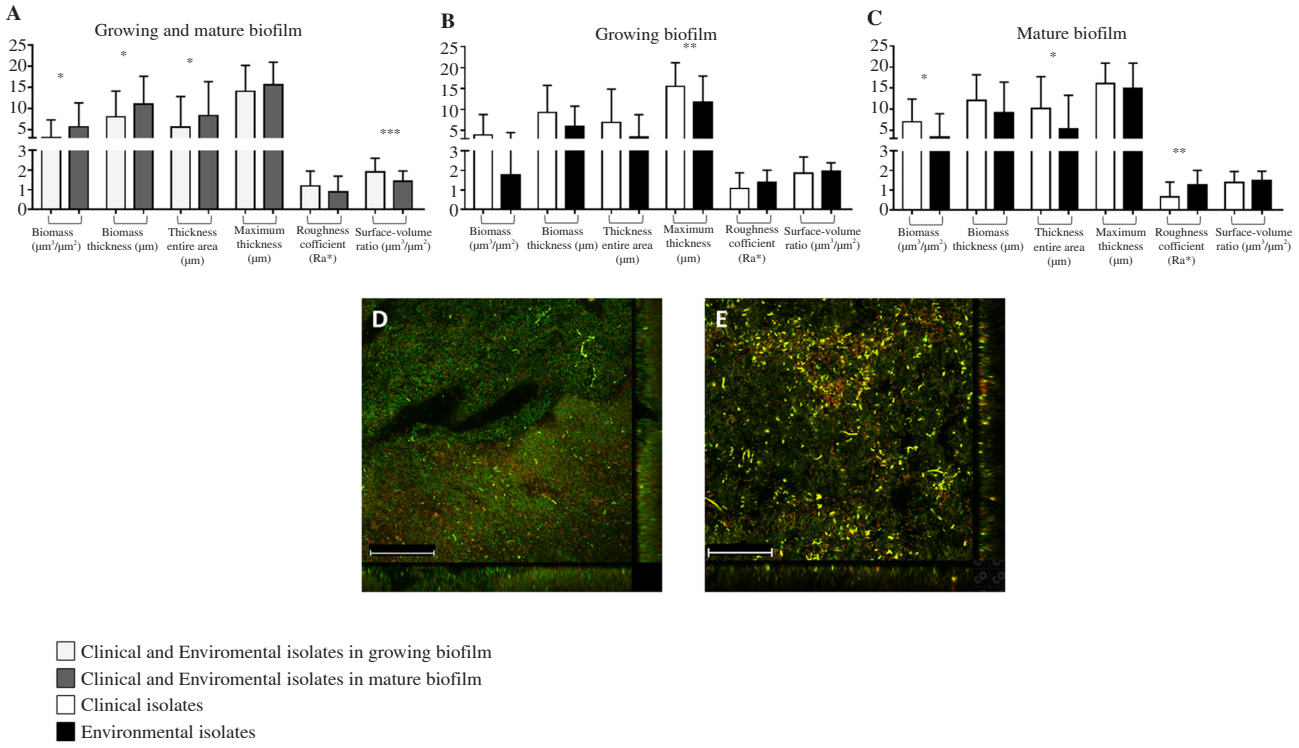

Figure 2. Confocal laser scanning microscopy of growing (48 h) and mature biofilms (72 h) of clinical (n=3) and environmental (n=3) isolates of *Burkholderia pseudomallei*. A: COMSTAT™ comparative analysis of Z-stack images acquired by confocal laser scanning microscopy between growing and mature biofilms of the tested isolates. B and C: COMSTAT™ comparative analysis of Z-stack images acquired by confocal laser scanning microscopy between clinical and environmental isolates as growing biofilms (B) and mature biofilms (C). The analyzed parameters were biomass, biomass thickness, thickness of the entire area, maximum thickness, roughness coefficient and surface-volume ratio. Data are reported as mean±standard deviation of the values obtained for each parameter, through the analysis of seven Z-stack images per evaluated isolate. Images were acquired at 488 nm for the detection of the SYTO9 fluorescent dye, which identifies live bacterial cells, and 561 nm for the detection of propidium iodide, which identifies dead/damaged cells. Mann Whitney's U test was used for the analysis of the data. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ differences between the parameters obtained. D and E: Representative images of growing (D) and mature biofilms (E) under confocal microscopy showing viable cells. Magnification: 600×. Scale: 50 μm.

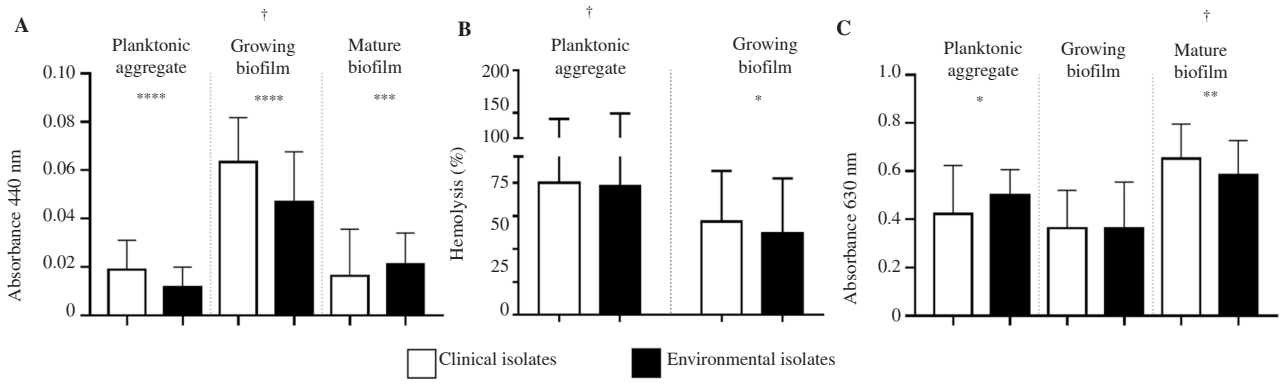

Figure 3. Release of exoproducts by clinical (n=8) and environmental (n=8) isolates of *Burkholderia pseudomallei* in planktonic growth and stages of biofilm formation. A and C: Production of protease (A) and siderophore (C) in planktonic aggregates, growing biofilms and mature biofilms, data are reported as mean±standard deviation of absorbance values. B: Production of hemolysin in planktonic form and growing biofilms, data are reported as mean±standard deviation of percentage of hemolysis (%) obtained. Mann Whitney's and unpaired t tests were used for the analysis of the data. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.000\,1$ differences between clinical and environmental isolates and †indicates statistically significant ($P<0.000\,1$) differences between the tested growth conditions (planktonic aggregates, growing and mature biofilms).

arranged (Figures 2D and 2E).

Concerning protease production, growing biofilms presented stronger production than planktonic aggregates (P<0.001) and mature biofilms (P<0.001). In addition, production by clinical isolates was higher than that of environmental isolates as planktonic aggregates (P<0.001) and growing biofilms (P<0.001), whereas the opposite was observed for mature biofilms (Figure 3A).

As for hemolysin production, planktonic aggregates showed hemolytic activity 1.8 times greater than growing biofilms (P<0.001). Planktonic aggregates showed a percentage of hemolysis that ranged from 89% to 100%, with an average of 100% for clinical strains and 97.51% for environmental strains. Isolates in growing biofilm also produced hemolysins, with a percentage of hemolysis ranging from 44% to 96%, with an average of 70.76% for clinical strains and 61.66% for environmental strains (P=0.014) (Figure 3B).

Regarding siderophores, mature biofilms presented higher production than growing biofilms (P<0.001) and planktonic aggregates (P<0.001). As for the origin of the isolates, environmental isolates had a higher production than clinical ones (P=0.017) as planktonic aggregates, whereas as mature biofilm, clinical isolates had a stronger production (P=0.008) (Figure 3C).

4. Discussion

The present study evaluated the difference in behavior of clinical and environmental isolates of *B. pseudomallei*, regarding the production of virulence factors, which is important for understanding the pathogenesis of melioidosis. It was observed that both clinical and environmental strains show good biofilm-forming ability in MH-1% glucose broth. BHI and MH media have different composition: BHI is an enrichment medium that has more complex proteins, peptone and salts, while MH contains an infusion of dehydrated beef, acid-hydrolyzed casein and corn starch[20]. Biofilm biomass was more abundant in MH broth, as shown by the crystal violet staining method, possibly because it contains smaller and simpler protein molecules that are more easily uptake by bacterial cells. Although the production of biomass in MH-1% glucose broth was superior, it did not favor the production of proteases (unpublished data), probably because it is not composed of complex proteins. Thus, as the aim of the study was to analyze the biofilm of clinical and environmental strains and greater formation was observed by clinical strains in both culture media, BHI-1% glucose broth was chosen for further analyses, as it induced the production of proteases and satisfactory biofilm growth.

Biofilm confocal laser scanning microscope analyses showed a robust and homogenous three-dimensional biofilm structure, demonstrating higher biomass, thickness of the entire area and biomass thickness in mature (72 h) than in growing (48 h) biofilms. This fact may be associated with the accumulation of the biofilm extracellular polymeric matrix, a sine qua non component of biofilms. Moreover, an overall tendency of more robust and homogenous biofilm was observed for clinical isolates compared to environmental isolates. Growing biofilms of clinical isolates were thicker than those of environmental isolates and mature biofilms of clinical isolates were denser, thicker and more homogenous than those of environmental isolates, as shown by biomass, thickness of the entire area and roughness coefficient values, respectively. Thus, these findings suggest that mature biofilms of clinical isolates are more homogeneous and robust than those of environmental isolates[14].

Biofilm formation is a natural strategy of survival of bacteria, providing greater resistance to adverse environmental conditions. These biofilms are important for the maintenance of microorganisms in harsh environments and demand favorable conditions for their development, such as temperature and osmolarity, nutrient availability, including iron and DNA, important factors for the first stages of biofilm formation by *B. pseudomallei*[22,23]. When bacterial biofilms are formed in a clinical context, it can hamper the therapeutic efficacy of antimicrobials, leading to disease relapse. Previous studies suggest that there might be an association of the *in vitro* biofilm-forming ability of a *B. pseudomallei* strain with the occurrence of clinical relapse of melioidosis[5,6].

As for the production of virulence factors, there are no reports comparing them between planktonic and biofilm growth of *B. pseudomallei*. Considering that *B. pseudomallei* is an auto-aggregating bacterium[24] that produces exuberant extracellular polymeric substance[25], forming aggregates on the surface of the culture broth. Even when grown under agitation, planktonic aggregates were evaluated instead of free-floating individual growth. Though planktonic aggregates have been referred as unattached biofilms, it has been shown that planktonic bacterial aggregates present different metabolic and molecular profiles from those of biofilms and free-floating planktonic growth[26,27].

In this study, it was observed that protease release was greater in growing biofilms, which may indicate an influence of these proteases on biofilm formation. Corroborating this finding, there is evidence that these proteases significantly contribute to bacterial pathogenesis and biofilm formation[9,28].

Additionally, clinical strains showed higher protease production than environmental strains in planktonic form and growing biofilms. These data corroborate a study by Vellasamy *et al.*, who observed higher protease production by *B. pseudomallei* strains after an *in vivo* passage[29]. The presence of these proteases is important for the pathogenesis, as they catalyze the cleavage of structural proteins in the host tissue through the hydrolysis of peptide bonds, aiding the virulence of *B. pseudomallei*[7,8]. Furthermore, proteases can play an important role in the environment, mainly in the acquisition of an energy source and persistence in an unfavorable environment[30].

Regarding hemolysins, a higher hemolytic activity was observed for isolates in as planktonic aggregates. Yadav *et al*. compared the *in vivo* production of exoproducts by *P. aeruginosa* in planktonic and biofilm form and observed lower hemolysin production by biofilm than planktonic form[31], as observed in the present study. Thus, hemolysin production seems to be more relevant for bacteria in planktonic form, since there is a greater contact of bacterial cells with host blood cell

in planktonic form than biofilm counterpart, as biofilm cells are embedded in the exopolymeric matrix[32,33]. Considering hemolysin production by growing biofilms was weak, the productions of these enzymes by mature biofilms was not evaluated.

The results showed that clinical and environmental isolates as planktonic aggregates presented similar hemolysin production, whereas growing biofilms of clinical isolates produced more hemolysins than those of environmental isolates. It is worth mentioning that bacterial strains that produce these enzymes are generally associated with pathological processes in their hosts, and are detected in high concentrations in fatal infections caused by members of the *Burkholderia cepacia* complex[34].

B. pseudomallei also showed siderophore production under different tested forms of growth, reaching its highest levels of production as mature biofilms. Siderophores is an important enzymatic cofactor for cellular functions, necessary for the survival of bacteria and possibly for their virulence[35]. Kamjumphol *et al.* demonstrated that high iron concentrations increase the growth of *B. pseudomallei*, change bacterial morphology and increase biofilm formation[36]. In addition, the high availability of iron stimulates the production of the biofilm matrix exopolysaccharide, which chelates ferrous iron, acting as an iron deposit and guaranteeing the maintenance of *P. aeruginosa* biofilm[37]. Therefore, siderophores are considered important virulence factors for biofilm maintenance.

The main limitation of this study is the fact that all strains come from the same region and that only phenotypical methods were applied. For a better understanding of exoproduct release during biofilm growth dynamics, more accurate methods could be beneficial, including gene expression and proteomic analyses.

Finally, the investigation of the virulence attributes of *B. pseudomallei* in planktonic and biofilm forms of growth highlights differences in the pattern of production of these factors in these two morphological conformations. Such findings demonstrate the participation of these exoproducts in the process of biofilm formation and maintenance, but further studies are necessary to elucidate the role of each product in biofilm growth dynamics.

Conflict of interest statement

We declare that we have no conflict of interest.

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Authors' contributions

GMMG: supervision, formal analysis, writing-review and editing; CJO: investigation, validation; ASF: investigation, formal analysis, writing-original draft; RMP: investigation, writing-original draft; GBR: formal analysis, writing-review and editing; SPB: resources; RAC: conceptualization, funding acquisition, resources; MFGR: conceptualization, resources, funding acquisition; JJCS: conceptualization, resources, funding acquisition; DSCMCB: conceptualization, formal analysis, investigation, supervision, writing-review and editing.

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