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Biofilm formation and virulence factors distribution among clinical isolates of Pseudomonas aeruginosa

Formação de biofilme e distribuição de fatores de virulência entre isolados clínicos de *Pseudomonas aeruginosa*

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Abstract

Introduction: *Pseudomonas aeruginosa is an opportunistic microorganism frequently isolated in the hospital environment. The World Health Organization has classified it as a critical pathogen due to its antibiotic resistance and varied virulence factors.* ***Objective:*** *to evaluate biofilm formation and the expression of virulence factors of 30 clinical isolates and two standard strains of P. aeruginosa. In addition, this work aims to analyze the distribution of virulence factors in the different categories of biofilm formation.* ***Methodology:*** *biofilm formation assays were performed in polystyrene microplate with crystal violet colouring, and the strains were classified. Other virulence factors were studied with specific phenotypic detection methods.* ***Results:*** *all P. aeruginosa were able to form biofilms, with 46.9% classified as strong formers, 25% as moderate and 28.12% as weak biofilm formers. Most isolates expressed the virulence factors analyzed, and among them, pyoverdine was the most prevalent, followed by the production of lipases B, hemolysin, and protease. Lipases A showed the lowest incidence.* ***Conclusion:*** *although no significant correlation was detected between the biofilm formation of P. aeruginosa clinical isolates and virulence factors, it was observed that in the strong formers group, alkaline protease and lipases B were expressed in all strains. The results suggest that the pathogenicity of P. aeruginosa is a multifactorial phenomenon and that virulence factors, although present in most isolates, occur independently of the biofilm formation category of the studied strains.*
Keywords: *Pathogenicity, adherence, motility, phenotypic features*

Resumo

Introdução: *Pseudomonas aeruginosa* é um micro-organismo oportunista frequentemente isolado no ambiente hospitalar e que foi classificado pela Organização Mundial da Saúde como patógeno crítico devido a sua resistência aos antibióticos e aos seus variados fatores de virulência. **Objetivo:** avaliar a formação de biofilme e a expressão de fatores de virulência de 30 isolados clínicos e 2 cepas padrão de *P. aeruginosa*. Além disso, este trabalho objetivou analisar a distribuição dos fatores de virulência nas diferentes categorias de formação do biofilme. **Metodologia:** ensaios de formação de biofilme foram realizados em microplacas de poliestireno com coloração cristal violeta e as cepas foram classificadas em grupos. Outros fatores de virulência foram estudados com métodos específicos de detecção fenotípica. **Resultados:** todas as *P. aeruginosa* foram capazes de formar biofilmes, com 46,9% classificadas como fortes formadoras, 25% como moderadas e 28,12% como fracas formadoras de biofilmes. A maioria dos isolados expressou os fatores de virulência analisados e dentre eles, a pioverdina foi o mais prevalente, seguido pela produção de lipases B, hemolisina e protease. As lipases A apresentaram menor incidência. **Conclusão:** embora não tenha sido detectada correlação significativa entre formação de biofilme e os fatores de virulência nos isolados clínicos, foi observado que nos formadores fortes houve expressão de protease alcalina e lipases B em todas as cepas. Os resultados sugerem que a patogenicidade de *P. aeruginosa* é um fenômeno multifatorial e que os fatores de virulência, embora presentes na maioria dos isolados, ocorrem independentemente da categoria de formação do biofilme das cepas estudadas.
Palavras-chave: Patogenicidade, aderência, motilidade, características fenotípicas

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INTRODUCTION

P. aeruginosa is an opportunistic pathogen with a cosmopolitan distribution found in nature, inhabiting water,

soil and plants. However, it is also frequently isolated in the hospital environment, on equipment surfaces, sinks, medical devices, and moist skin regions such as the outer ear, armpits, and anogenital region of humans[1].

This pathogen is a serious Public Health problem. It has been classified by the World Health Organization (WHO) with critical level 1 on the global list of antibiotic resistant bacteria with a priority need for research and development of modern and effective antibiotic treatments[2].

It is a microorganism that can cause acute infections with tissue damage and systemic dissemination due to toxin production and can cause chronic infections by the ability to form biofilm. Moreover, its ability to cause a wide range of infections lies in the variety of pathogenicity factors it expresses. These factors are grouped into bacterial cell-associated and secreted pathogenicity factors[3].

Biofilm formation is a multifactorial and complex phenomenon that may be related to the drug used to treat the infectious process. Due to this ability, P. aeruginosa shows resistance and tolerance to treatments, and this complex combination is called biofilm recalcitrance[4,5].

Biofilm plays a central role in several persistent and chronic infections; moreover, associated with biofilms, bacteria are protected from the actions of the host immune system[6,7]. Furthermore, biofilm acts as a physical barrier, preventing the penetration and action of chemical compounds and the recognition of the bacteria by the host immune system[8].

The biofilm structure acts as a protective barrier that allows survival in hostile environments while promoting cell dispersal to colonize new niches under appropriate conditions[9]. This mode of microbial growth hinders antimicrobial therapy since, growing in the sessile form, microorganisms tend to be more resistant to antimicrobial treatment than planktonic forms[10].

In addition to sessile growth, P. aeruginosa exhibits various virulence factors on the cell surface or secreted into the extracellular environment. The predominance of these bacteria in environments with high selective pressure, such as hospitals, can be explained by the expression of virulence factors associated with biofilm formation and the consequent increased resistance to antimicrobials commonly employed in these environments[8].

The expression of different resistance mechanisms has a clinical impact, especially by compromising the efficacy of almost all antimicrobials due to their flexible metabolic capacity and genome encoding resistance genes[11]. In this context, the present work aimed to evaluate the biofilm formation pattern by clinical isolates of Pseudomonas aeruginosa and the distribution of virulence factors according to biofilm formation categories.

METHODOLOGY

Bacterial maintenance

In this experimental analytical study, 32 strains of Pseudomonas aeruginosa were evaluated. Thirty strains were clinical isolates isolated from secretions of tracheostomized children and two from corneal secretions of hospitalized patients (Approval of the Ethics in Research Committee no. 1.411.90000) from the Laboratory of Microorganism Biotechnology of the Tropical Pathology and Public Health Institute of the Federal University of Goiás and two standard American Type Culture Collection (ATCC) P. aeruginosa ATCC 27853 and ATCC 9027 from the Bioassay Laboratory of the State University of Goiás. Initially, the frozen aliquots of microorganisms were reactivated in cetrimide agar at 35.5ºC for 24h to perform the assays.

Biofilm Formation Assay

Biofilm formation assays were performed following the methodology described by Stepanović et al.[12] (2007) with modifications. Briefly, bacterial suspensions were prepared with previously grown isolated and typical colonies transferred to polystyrene microplate wells containing 100µL of TSB culture medium, making an initial inoculum of $1x10^5$ cfu.mL^{-1}. Sterility controls were included with non-inoculated wells, and the microplates were incubated in an oven at 35.5°C for 24h. After this period, a visual reading was performed, confirming bacterial growth and non-contamination of the controls. Then, the broths were removed from the wells and washed three times with 200 µL of sterile saline solution to remove non-adhered cells.

The plates were treated with the addition of 110µL of 1% crystal violet and incubated at room temperature for 10 minutes; then, the dye was discarded, and the wells were washed five times with 200µL of distilled water. The plates were dried in an oven at 35.5ºC for 20 minutes. After this period, 110 µL of absolute ethanol was added to each well for solubilization of the adhered bacteria stain and the plate was incubated for 10 minutes. Subsequently, each well's optical density (OD) readings were performed by the BioTek Epoch™ spectrophotometer at 492 nm[13].

The biofilm formation index was calculated by the values of the optical densities obtained from the wells using the formula: BF=AB-CW, where: BF – biofilm formation, AB – optical density of the bacteria adhered to the wells and CW – optical density of the non-inoculated wells. For the classification of biofilm formation, the following parameters were considered: Non-forming = AB ≤ CW, weakly forming = CW ≤ AB ≤ 2.CW, moderately forming = 2.CW < AB ≤ 3.CW and strongly forming = 3.CW < AB[12].

Phenotypic study of virulence factors

The virulence factors studied were swimming and swarming motility, alkaline protease, hemolysin, phospholipase C, lipases, and pyoverdin. Swimming motility was assessed by inoculating previously grown bacterial samples into LB broth using a sterile wooden toothpick and piercing the culture medium containing 5xM8 solution plus 0.3% agar, 0.2% glucose, 0.5% casamino acid solution, and 1mL of 1mM $MgSO_4$. The plates were incubated at 35.5°C

for 16 to 24h. Motility was qualitatively analyzed by the formation of a circular halo formed by cell migration from the point of inoculation. The presence of the halo was considered positive, and the absence of radial growth was considered negative. This assay measured the degree of flagellum-dependent motility of P. aeruginosa[14].

To verify swarming-type motility, 2.5µL of bacterial culture previously grown in LB broth were inoculated onto the surface of culture medium containing 5xM8 solution plus 0.8% agar, 0.2% glucose, 0.5% casamino acid solution, and 1mL of 1mM $MgSO_4$. The plates were incubated at 35.5°C for 16 to 24h, and swarming motility was considered positive when the colonies showed the appearance of growth together with the formation of tendril-like branching or as negative in the absence of such growth beyond the point of inoculation on the surface of the culture medium[15]. This assay gauged the degree of the flagellar-dependent motility of P. aeruginosa in gelatinous or viscous media[16].

For alkaline protease production, bacteria were inoculated in spots on skim milk agar (2%) and incubated at 35.5°C for 24h. A translucent halo around the colonies indicates alkaline protease production, and the absence of a halo indicates a negative result[17].

The hemolysins were evaluated with the growth of the strains on sheep blood agar incubated at 35.5°C for 24h. The isolates that presented a translucent halo around the colonies, indicating hemolysis, were considered hemolysin producers[18].

The production of phospholipase C was evaluated with the incubation of microorganisms inoculated in spots in TSA enriched with 10% (vol/vol) egg yolk and tellurite solution. The plates were incubated at 35.5°C for 24h. The presence of a black precipitate over the growth zone is considered phospholipase C production, and those that did not show the precipitate were connon-producers[19].

The lipolytic activity of P. aeruginosa was detected by the formation of halos around the colonies in three types of media to lipase A (tween 80), to lipase B (tributyrin), and to lipase C (rhodamine B)[20]. The base culture medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl and pH 7.2) was enriched with the following components: medium A: 0.01% $CaCl_2.2H_2O$ and 1% tween 80, medium B: 0.01% $CaCl_2.2H_2O$ and 1% tributyrin, and medium C: 2.5% commercial olive oil and 0.001% rhodamine B. After incubating the plates at 35.5°C for 24h, the appearance of clear zones around the bacterial colonies of media A and B indicate the production of catalytically active enzymes. Visualization of fluorescent orange halos around the colonies under UV light at 350nm indicates catalytic activity in medium C[21].

Pyoverdin production was evaluated on cetrimide agar with incubation at 35.5°C for 24h. Readings were taken by subjecting the colonies to ultraviolet light and checking for fluorescence emission[18]. The P. aeruginosa strains ATCC 27853 and ATCC 9027 were used as controls for the techniques.

Statistical analyses

All tests were carried out in independent triplicates. Quantitative variables were ordered as means and standard deviations. Qualitative variables were presented in nominal form (positive/negative). Fisher's test (QuickCalcs, GraphPad Software, LLC 2022) was used to assess the significance of differences in the distribution of virulence factors between biofilm formation groups ($p < 0.05$).

RESULTS

All P. aeruginosa (n=32) were able to form biofilms, with 15 (46.9%) classified as strong formers, 8 (25%) as moderate formers, and 9 (28.1%) as weak biofilm formers. The isolates Pa03, Pa11, Pa28, Pa22, Pa20 e Pa05 and Pa21 showed higher biofilm formation patterns. (Table 1)

Table 1 – *Biofilm formation index and categories of clinical isolates and standard strains of P. aeruginosa.*

Biofilm formation		
P. aeruginosa	**Index (mean ± SD)**	**Categories**
Pa01	0.098 ± 0.079	Moderate
Pa02	0.109 ± 0.013	Moderate
Pa03	0.278 ± 0.075	Strong
Pa04	0.134 ± 0.034	Strong
Pa05	0.175 ± 0.081	Strong
Pa06	0.184 ± 0.067	Strong
Pa07	0.087 ± 0.016	Weak
Pa08	0.128 ± 0.061	Moderate
Pa10	0.079 ± 0.049	Weak
Pa11	0.278 ± 0.092	Strong
Pa12	0.138 ± 0.047	Strong
Pa13	0.063 ± 0.013	Weak
Pa14	0.064 ± 0.027	Weak
Pa15	0.155 ± 0.080	Strong
Pa16	0.118 ± 0.054	Moderate
Pa17	0.097 ± 0.042	Moderate
Pa18	0.073 ± 0.024	Weak
Pa19	0.064 ± 0.018	Weak
Pa20	0.178 ± 0.046	Strong
Pa21	0.174 ± 0.074	Strong
Pa22	0.190 ± 0.073	Strong
Pa23	0.168 ± 0.084	Strong
Pa24	0.126 ± 0.055	Moderate
Pa26	0.101 ± 0.054	Moderate
Pa27	0.118 ± 0.061	Moderate
Pa28	0.197 ± 0.141	Strong
Pa29	0.148 ± 0.052	Strong
Pa30	0.149 ± 0.052	Strong
Pa31	0.069 ± 0.014	Weak
Pa32	0.074 ± 0.013	Weak
PaATCC 27853	0.079 ± 0.019	Weak
PaATCC 9027	0.149 ± 0.052	Strong

Pa = Pseudomonas aeruginosa, SD = standard deviation, ATCC = American Type Culture Collection

Source: research data

Table 2 shows the other virulence factors studied. Pyoverdin was the most prevalent in all 32 P. aeruginosa, followed by hemolysin production in 30 (93.7%) protease and lipases B in 29 (90.6%) each. The virulence factors with the lowest incidence were lipases A found in 13 (40.6%) and lipases C in 21 (65.6%) isolates.

Table 2 – *Virulence factors of clinical isolates and standard strains of P. aeruginosa.*

Strains	Virulence factors								
	Swi	Swa	AP	Hemo	PhosC	LipA	LipB	LipC	Pyov
Pa01	+	+	+	+	-	-	+	-	+
Pa02	+	+	+	+	+	-	+	+	+
Pa03	-	+	+	+	-	-	+	+	+
Pa04	+	+	+	+	+	+	+	-	+
Pa05	-	+	+	+	+	+	+	-	+
Pa06	+	+	+	+	+	-	+	+	+
Pa07	+	+	+	+	-	+	+	+	+
Pa08	+	+	+	+	-	-	+	+	+
Pa10	+	+	+	+	+	+	+	+	+
Pa11	+	+	+	+	-	-	+	+	+
Pa12	+	+	+	+	+	+	+	+	+
Pa13	+	-	-	+	+	-	+	+	+
Pa14	+	+	+	+	+	+	+	+	+
Pa15	+	+	+	+	+	-	+	+	+
Pa16	+	+	-	+	+	+	+	-	+
Pa17	-	-	-	+	-	-	+	-	+
Pa18	-	+	+	+	-	-	-	-	+
Pa19	-	-	+	+	+	-	+	-	+
Pa20	+	+	+	+	+	+	+	+	+
Pa21	+	+	+	+	+	-	+	+	+
Pa22	+	-	+	+	+	-	+	-	+
Pa23	+	-	+	+	+	+	+	+	+
Pa24	+	-	+	+	+	-	+	+	+
Pa26	-	+	+	+	+	-	+	-	+
Pa27	+	-	+	-	+	-	+	+	+
Pa28	+	+	+	+	+	-	+	+	+
Pa29	+	+	+	-	+	-	+	+	+
Pa30	+	+	+	+	+	+	+	+	+
Pa31	+	-	+	+	+	+	+	+	+
Pa32	+	-	+	+	+	-	+	+	+
PaATCC 27853	+	+	+	+	+	+	+	+	+
PaATCC 9027	+	+	+	+	-	+	+	+	+

Pa = P. aeruginosa, Swi = swimming, Swa = swarming, AP = Alkaline protease, Hemo = hemolysin, PhosC = phospholipase C, LipA = lipase A, LipB = lipase B, LipC = lipase C, Pyov = pyoverdine, + = positive, – = negative, ATCC = American Type Culture Collection

Source: research data

The prevalence of virulence factors in biofilm formation categories was evaluated among the strong, moderate, and weak categories. The significance of the differences was checked by Fisher's paired two-tailed test ($p < 0.05$) (Table 3).

Pyoverdine, protease and lipase B were detected in all P. aeruginosa strong biofilm formers, hemolysin in 93.3%, swarming motility in 86.7% and phospholipase C and lipase C in 80% of the bacteria in this category. The moderate biofilm formers group showed the predominance of pyoverdin and lipase medium B production in all isolates and hemolysin detection in 87.5%. The most prevalent virulence factors in the weak biofilm formers category were pyoverdine and hemolysin in 100% of the isolates and protease and lipase B in 88.9%. Lipase A was the least detected phenotypic characteristic in all groups.

Table 3 – *Distribution of virulence factors according to the biofilm formation groups of clinical isolates and standard strains of P. aeruginosa.*

VF	Categories of biofilm formation				p		
	Total n=32	Strong n=15	Moderate n=8	Weak n=9	SF vs WF	MF vs WF	SF vs MF
Swi	26 (81.2%)	13 (86.7%)	6 (75%)	7 (77.8%)	0.6146	1.0000	0.5889
Swa	23 (71.9%)	13 (86.7%)	5 (62.5%)	5 (55.5%)	0.1501	0.2969	0.1501
AP	29 (90.6%)	15 (100%)	6 (75%)	8 (88.9%)	0.3750	5.765	0.1107
Hemo	30 (93.7%)	14 (93.3%)	7 (87.5%)	9 (100%)	1.0000	0.4706	1.0000
PhosC	24 (75%)	12 (80%)	5 (62.5%)	7 (77.8%)	1.0000	0.6199	0.6214
LipA	13 (40.6%)	7 (46.7%)	1 (12.5%)	5 (55.5%)	1.0000	0.1312	0.1763
LipB	31 (96.9%)	15 (100%)	8 (100%)	8 (88.9%)	0.3750	1.0000	1.0000
LipC	23 (71.9%)	12 (80%)	4 (50%)	7 (77.8%)	1.0000	0.3348	0.1819
Pyov	32 (100%)	15 (100%)	8 (100%)	9 (100%)	1.0000	1.0000	1.0000

p = significance level of differences (p < 0.05), FV = Virulence Factors, Swi = swimming, Swa = swarming, AP = Alkaline protease, Hemo = hemolysin, PhosC = phospholipase C, LipA = lipase A, LipB = lipase B, LipC = lipase C, Pyov = pyoverdine, SF = Strong formers of biofilm, MF = Moderate formers of biofilm, vs = versus, WF = Weak formers of biofilm.

Source: research data

DISCUSSION

The pathogenicity of P. aeruginosa is a multifactorial process determined by a wide range of virulence factors associated with the ability to resist the host immune response and the presence of antibacterials[22,23].

Over 70% of the isolates analyzed could form strong or moderate biofilms. In recent works, the ability of Pseudomonas aeruginosa to form biofilms was found to vary from 34 to 59%[23-25].

Sessile microbial growth in the form of biofilm is present in more than 80% of microbial infections associated with catheters, implants, and urinary tract infections, among others, and usually produces persistent chronic infections[26]. Virulence factors such as biofilm formation, extracellular enzyme production, secondary metabolites, and motility, among others, are associated with phenotypic characteristics that can be regulated by quorum sensing[27].

Surface motility is a complex adaptation that leads to resistance to antibiotics and various drugs and the production of virulence factors in P. aeruginosa. This phenomenon is receiving increasing attention from researchers as it relates to the development of P. aeruginosa[1,15].

Motility is related to the type of motility, adhesion, and colonization, which are considered early stages of biofilm formation. Furthermore, P. aeruginosa has a higher motility capacity associated with strong biofilm formation when compared to other species in the genus[28].

Although the incidence of swimming and swarming type motilities was not significant in the strong formers compared to the other groups, it was possible to observe that most of the strains in this group (86.70%) showed positive swimming motility, while swarming motility was slightly lower in the group that formed less biofilm. The result is similar to a previous study29, in which no significant association was observed between motility types and biofilm formation ability (p > 0.05) in P. aeruginosa strains from environmental sources.

Surface-associated bacterial motility plays an essential role in biofilm formation and spatial arrangement by overcoming the electrostatic repulsion force of cell surfaces and participating in the initial colonization phase of the host cell surface[30] through adhesion by motility structures such as flagella and type IV pili[31].

Extracellular proteases, especially alkaline proteases, contribute to the adhesion of bacteria. Hemolysin production determines pathogenicity, as it degrades host tissues, allows for invasion and dissemination, and invades the immune response. Phospholipase C, on the other hand, preferentially hydrolyzes lipids containing quaternary ammonium groups found in eukaryotic membranes and inactivates pulmonary surfactant, which may be responsible for atelectasis associated with chronic and acute P. aeruginosa lung infections[32].

Lipases are involved in lipid signalling, influencing the virulent phenotypes of P. aeruginosa, especially LipA and LipC. LipA is associated with decreased pyoverdine production in iron-deprived media, and LipC affects motility, rhamnolipid production, and biofilm formation[33]. Thus, pyoverdine is the main siderophore produced by P. aeruginosa and is evident in colonies under iron-deficient conditions. In addition to obtaining iron by excretion, followed by incorporation after chelating ferric iron, it is associated with the formation of recurrent biofilms in the lungs[34].

Pyoverdine is also associated with its regulation and in the regulation of other virulence factors through the recognition of the pyoverdine-iron complex at the level of the cell outer membrane, promoting a cascade of cytoplasmic signals, which will regulate genes involved in the synthesis of pyoverdine, endoprotease and ToxA[32].

Similar to our work, in another study, it was found that 92% of P. aeruginosa expressed hemolysin, 86% lipases,

86% protease, and 84% formed biofilm. However, unlike our work, the authors found a relationship between biofilm production and the presence of virulence genes and antimicrobial resistance[35].

CONCLUSIONS

In this work, we identified that all P. aeruginosa were able to form biofilm, that most isolates expressed the virulence factors studied, and that there was no correlation between biofilm formation and swimming and swarming motilities, alkaline protease, hemolysin, phospholipase C, lipases in A, B and C, and pyoverdine.

Although no correlation was detected between the biofilm formation of P. aeruginosa clinical isolates and the virulence factors studied, we observed that in the strong formers group, there was expression of alkaline protease and lipase B in all strains, in the moderate formers group, lipase B was found in 100% of isolates, and all isolates in the weak formers group produced hemolysin.

In another study, the virulence factors of 302 P. aeruginosa isolates were analyzed, and no relevant correlations between biofilm formation rate and virulence factors were found either[24]. However, data on the interaction between the presence and mechanisms of biofilm formation and virulence are fundamental to addressing chronic bacterial infections and providing strategies for their daily management[28].

Our results suggest that the pathogenicity of P. aeruginosa is a multifactorial phenomenon and that virulence factors, although present in most isolates, occur independently of the biofilm formation category of the P. aeruginosa studied.

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