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Virulence profiles of clinical and environmental *Pseudomonas aeruginosa* isolates from Central Morocco

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The pathogenic potential of *Pseudomonas aeruginosa* comes from the expression of many secreted and cell surface virulence factors, and its biofilm formation. This study aimed to investigate and compare the virulence profiles of 123 clinical and environmental *P. aeruginosa* isolated in Meknes (Morocco). Using suitable culture media, phenotypic screening evaluated the production of β-haemolysin, caseinase, lipase, lecithinase, pyocyanin and pyoverdin, as well as the ability to swim, swarm and twitch. Biofilm formation kinetics was assessed using microtiter test plates. Data analysis was performed using Statistic Package of the Social Science software (version 21.0). High percentages of strains expressed caseinase (99.2%), β-heamolysin (95.1%), lipase (100%) and lecithinase (100%). 95.9% of isolates produced either pigment. All strains were able to swim, warm and twitch, at different levels. All strains were biofilm producers, and the evolution of adherent biomass over time varies greatly from strain to strain. Significant positive correlations were observed between proteolytic and hemolytic activities; biofilm formation and twitching; as well as swimming, swarming and twitching motilities. Twitching and swimming were significantly higher in environmental strains, which were also quickly adhered and formed denser biofilms. Clinical strains showing significantly higher proteolytic activity were isolated from cardiology ward, and those with higher twitching and denser biofilm were from the thoracic service. Inpatient strains were significantly earlier producer of denser biofilm than outpatient ones. *P. aeruginosa* strains tested have a collection of virulence markers required to cause disease in different tissues. Such bacteria present a serious therapeutic challenge for treatment of both community-acquired and nosocomial infections.

Key words: Biofilm, clinical, environmental, *Pseudomonas aeruginosa*, virulence factors.

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is an important human opportunistic pathogen that causes serious nosocomial infections. It is associated with significant morbidity and mortality, particularly in immuno-compromised hosts and vulnerable patients (Kerr et al., 2009). In fact, *P. aeruginosa* infections are especially

difficult to treat because of its versatility (Lister et al., 2009) and intrinsic/acquired antibiotic resistance (Breidenstein et al., 2011), as well as its capacity to form a protective biofilm (Breidenstein et al., 2011) and to express many secreted and cell-associated virulence factors (Goodman et al., 2004).

P. aeruginosa is armed with a large collection of virulence factors that allow it to survive in both different hosts and the environment.These factors are involved in the various stages of the infection process thereby allowing *P. aeruginosa* to colonize the host (Lau et al., 2005; Lyczak et al., 2000). Membrane factors are involved in the adhesion and motility of *P. aeruginosa* and allow the host colonization. They include flagellum, type IV pili, lipopolysaccharide, a type III secretion system, and alginate (Pier, 2002).Throughout its growth, this bacterium releases several metabolites including mainly, exotoxins, exoproteases, heamolysins and chromophores. These factors cause extensive tissue damage, as well as facilitate bacterial multiplication and the spread in the host tissues (Van Delden and Iglewski, 1998). Biofilm production is also known as an important determinant of pathogenicity in *P. aeruginosa* infections, it has been recognized as the principal mechanism associated with prolonged and recurrent infections (Wareham and Curtis, 2007).

The purpose of the present study was to investigate and compare the production, *in vitro*, of some enzymes (β-haemolysin, caseinase, lipase and lecithinase) and pigments (pyocyanin and pyoverdin) involved in the virulence of 123 *P. aeruginosa* isolates obtained from environment and clinical samples. Their ability to swim, swarm and twitch, as well as their biofilm formation kinetics were also examined. Possible correlations between these parameters were equally sought.

MATERIALS AND METHODS

Bacterial strains

A total of one hundred and twenty three environmental and clinical isolates of *P. aeruginosa* from Meknes city (Morocco), which had been identified and tested for antimicrobial susceptibility in a previous study (Maroui et al., 2016), were included in this work. These strains were collected from June 2012 to June 2014.

Environmental strains (n = 55) were from soil (n = 20), aliments (n = 3), amurca olive (n = 6), rivers water (n = 16), wells (n = 5) and public swimming pools (n = 5). Clinical strains (n=68) were isolated from male (n = 51) and female (n = 17), the distribution of these isolates depending on the levy type was as follows: distal bronchial levy protected (n = 6), pus (n = 35), urine (n = 18), pleural fluid (n = 4), biopsy (n = 2), blood cultures (n = 1), bronchial aspirate (n = 1) and vaginal levy (n = 1). A third of these isolates were from outpatients and two thirds from inpatients of various hospital wards mainly intensive care (12/45) and burn ward (12/45).

Screening for some virulence factors

Few colonies grown overnight on nutrient agar at 37°C were suspended in 4 ml of Luria Bertani (LB) broth. The density of this suspension was adjusted to 0.5 of the McFarland standard. 3 or 10 µl of this suspension were added to several media containing the appropriate substrates as described below. All experiments were performed in triplicate. Pigments production was assessed qualitatively by observing the dyes excreted into the culture medium surrounding the colonies.

Hemolytic activity

The strains were tested for β-hemolytic activity on blood agar plates prepared with Columbia agar supplemented with 5 % human blood. Ten microliters of each bacterial suspension was placed in 5-mm-diameter well cut into the agar and incubated at 35°C for 24 h. The presence of a clear colourless zone surrounding the well indicated β-hemolytic activity (Gerhardt et al., 1981).

Proteolytic activity

Casein hydrolysis was tested on Mueller Hinton agar containing 10% (w/v) skimmed milk (Gudmundsdóttir, 1996).Ten microliters of each bacterial suspension was placed in 5 mm-diameter well cut into the agar and incubated at 35°C for 24 h. The presence of a clearing zone around the well indicated proteolysis of casein, and the diameter of the clearing zone reflects the intensity of the exoenzyme released. Reference strain *P. aeruginosa* ATCC 27853 was used as a control in this test.

Lecithinase and lipase activities

Lipase and lecithinase activities were tested on egg yolk agar plate prepared with phosphate buffered saline, containing egg yolk to 5 and 1.5% bacto-agar. Three microliters of each bacterial suspension was placed onto the agar and incubated at 35°C for up to five days. The appearance of an opaque precipitate that spreads beyond the edge of the colony indicated lecithinase activity and the formation of an iridescent sheen that can be seen when the plate is held at an angle to a light source indicated lipase production (Forbes et al., 2007; Koneman et al., 2006). Reference strain *P. aeruginosa* ATCC 27853 was used as a control in this test.

Pyocyanin and pyoverdin production

The strains were streaked on Cetrimide agar and king B agar, and incubated for 24 h at 37°C for visual analysis of pigment production. Blue pigment was interpreted as pyocyanin, yellow-green and fluorescent indicated the pyoverdin production, and red-brown pigment was recorded as pyorubin.

Motility assays

In the following 3 tests, 90 mm plates were filled with 30 ml of medium, dried before use, and inoculated in triplicate experiments. The plates were incubated at 30°C for 24 h. Swimming and

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swarming motilities were assessed qualitatively by measuring the turbid zone formed by the bacterial cells migrating around the point of inoculation. For twitching, the zone of motility at the agar/Petri dish interface was visualized by gently removing the agar, the Petri dish was air dried and cells were stained with crystal violet (1%). Then the Petri dish was rinsed with distilled water, and the crystal violet-stained twitching pattern was measured. Strains were divided into four groups: no motile (no growth spreading), less motile (< 1 cm), moderately motile (1 cm ≤ and < 2 cm) and highly motile (≥ 2 cm).

Swimming

Swimming assays were done on 0.3% LB agar plates (Murray and Kazmierczak, 2006). Swim plates were inoculated by sterile toothpick with single colonies picked from a fresh nutrient agar plate.

Swarming

Swarm media were composed of 0.8% nutrient broth, 0.5% glucose and 0.5% bacto-agar (Rashid and Kornberg, 2000). Fresh isolated colonies were point-inoculated with sterile toothpick on the surface of the agar.

Twitching

Twitch plates were composed of 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1% bacto-agar. Bacterial colonies were inoculated with a sharp sterile toothpick inserted through the culture medium to the bottom of the Petri dish (Rashid and Kornberg, 2000).

Quantification and kinetics of biofilm formation

Biofilm formation assays were carried out in microtiter plates of polystyrene according to the crystal violet method as described by Stepanovic et al. (2007). Briefly, 160 µl of sterile LB broth was placed in each well of a sterile 96-well microtiter plate, then 20 µl of each bacterial suspension from an overnight culture of stirring (150 rpm) in LB broth was added to each well. Microplates were covered and incubated without agitation at 37°C for 2 to 24 h. All the samples were prepared in duplicate for each sampling time with a standard strain (*P. aeruginosa* ATCC 27853) and two negative controls containing no strains by plate. After incubation at regular time intervals, the non-adherent bacteria were removed by three successive washes with distilled water then the duplicate plates were air dried. Biofilm formation was visualized by staining with 1% crystal violet (200 µl per well) for 15 min at room temperature followed by rinsing thrice with distilled water, and then the plates were air dried. Biomass attached cells was quantified by solubilizing the dye in 200 µl per well of 95% ethanol.

The amount of biofilm formed was evaluated by reading the absorbance (optical density) of each well using a spectrophotometer (BIO-RAD Laboratories PR 2100), at a wavelength of 490 nm. Based on the optical density (ODi) of the samples and the average of optical density of the negative control (ODc), the samples were classified as strong biofilm producers (4xODc < ODi), moderate biofilm producers (2xODc < ODi ≤ 4xODc), weak biofilm producers (ODc < ODi ≤ 2xODc) or non-biofilm producer (ODi < ODc) (Stepanovic et al., 2007).

Depending on the incubation time required to achieve maximum adhesion, the strains were classified as early producer (t ≤ 4 h), 1/2 early producer (6 h ≤ t ≤ 10 h), 1/2 Tardy (12 h ≤ t ≤ 18 h), and tardy producer (t ≥ 20 h).

Statistical analysis

The results were analyzed using the t-test, factor analysis and one-way analysis of variance (ANOVA). Data analysis was performed using Statistic Package of the Social Science software (SPSS version 21.0 software, IBM, Chicago, USA). The *p*-values of < 0.05 were considered statistically significant.

RESULTS

Secreted virulence factors

Studied *P. aeruginosa* strains synthesized many virulence factors. Table 1 shows the repartition of tested extracellular enzymes among strains from both origins. Caseinase, β-haemolysin, lipase and lecithinase were produced by almost all strains, although with different activity levels. All environmental strains produced the four enzymes, with an exception of a strain isolated from amurca olive which did not produce the β-haemolysin. Lipase and lecithinase were also synthesized by all clinical strains, and it was noted among these that only one isolate from urine of outpatient did not produce caseinase but produced β haemolysin, and five strains isolated from different wards did not produce β haemolysin but produced low levels of caseinase. The vast majority of isolates exhibited better caseinase, β haemolysin, lipase and lecithinase than *P. aeruginosa* ATCC 27853.

Results of pigment production by *P. aeruginosa* isolates are presented in Table 2. The pyocyanin, pyoverdin and pyorubin pigments were synthesized respectively by 73.2, 65 and 2.4% of isolates studied, however 3.2% of these were non-pigmented.

Cell surface virulence markers

All strains were motile, but showed variable degrees of motility. For each, type of motility, the rates recorded for the motility phenotypes are shown in Figure 1.

Quantification and kinetics of biofilm formation

All strains were able to form biofilm on the polystyrene substrate. Their distribution according to the amount of formed biofilm for each incubation time and each origin is shown in Figure 2. 70.9% of environmental strains and 50% of clinical ones were able to form biofilms denser than that of *P. aeruginosa* strain ATCC 27853, which was found to be moderate. Results of strains classification according to incubation time required to achieve maximum adherence and quantification of that adherence are presented in Figure 3.

Table 1. Number (%) of positive *P. aeruginosa* strains for tested extracellular enzymes.

Enzyme	No. of positive strains (%)	
	Environmental strains (n=55)	Clinical strains (n=68)
β Haemolysin	54 (98.2%)	63 (92.6%)
Protease (Caseinase)	55 (100%)	67 (98.53%)
Lipase	55 (100%)	68 (100%)
Lecithinase	55 (100%)	68 (100%)

Table 2. Number (%) of positive *P. aeruginosa* strains for pigment production.

Pigment	No. of positive strains (%)	
	Environmental strains (n=55)	Clinical strains (n=68)
Pyocyanin	21(38.2%)	17 (25%)
Pyoverdin	7(12.7%)	19 (27.9%)
Pyorubin	0	1 (1.5%)
Both pyocyanin and pyoverdin	27 (49.1%)	25 (36.8%)
Both pyoverdin and pyorubin	0	2 (2.9%)
No pigment	0	4 (5.9%)

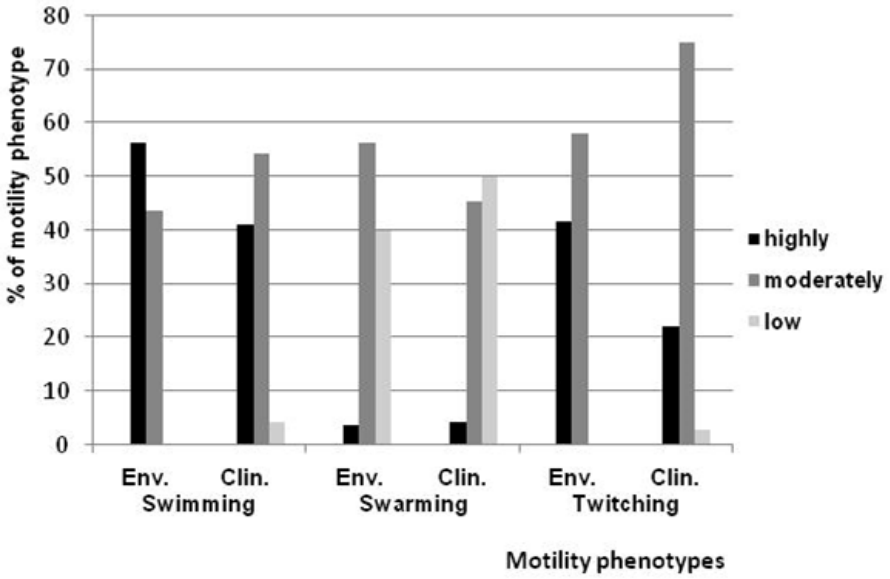

Figure 1. Motility phenotypes of *Pseudomonas aeruginosa* strains studied: highly motile (≥ 2 cm), moderately motile (1 ≤ and < 2) and low motile (< 1 cm). Env., Environmental; Clin., Clinical.

Data analysis

Comparing quantitative virulence factors studied according to the strains origin indicated that the difference observed among environmental and clinical strains was not statistically significant for caseinase, β haemolysin and swarming.

Considering all *P. aeruginosa* strains, irrespective of their origin and the isolation site, analysis of possible correlations between these virulence factors showed very significant positive correlations between β-haemolysin and protease, and also between swimming and swarming. Significant positive correlation was found between biofilm formation and twitching and between twitching and other two types of motility as well. A relatively weak positive correlation was found between

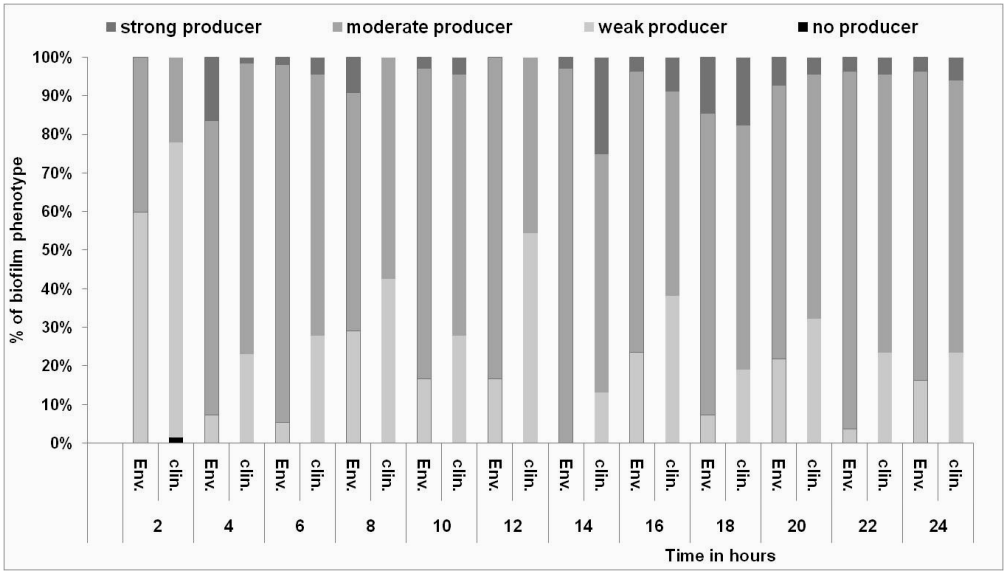

Figure 2. Comparison of the amount of biofilm formed by environmental and clinical isolates for 12 incubation times. Strong biofilm producers (4xODc <ODi), moderate biofilm producers (2xODc <ODi ≤ 4xODc), weak biofilm producers (ODc<ODi ≤ 2xODc) or non-biofilm producer (ODi<ODc). ODi: optical density of the samples, ODc the average of the negative control optical density.

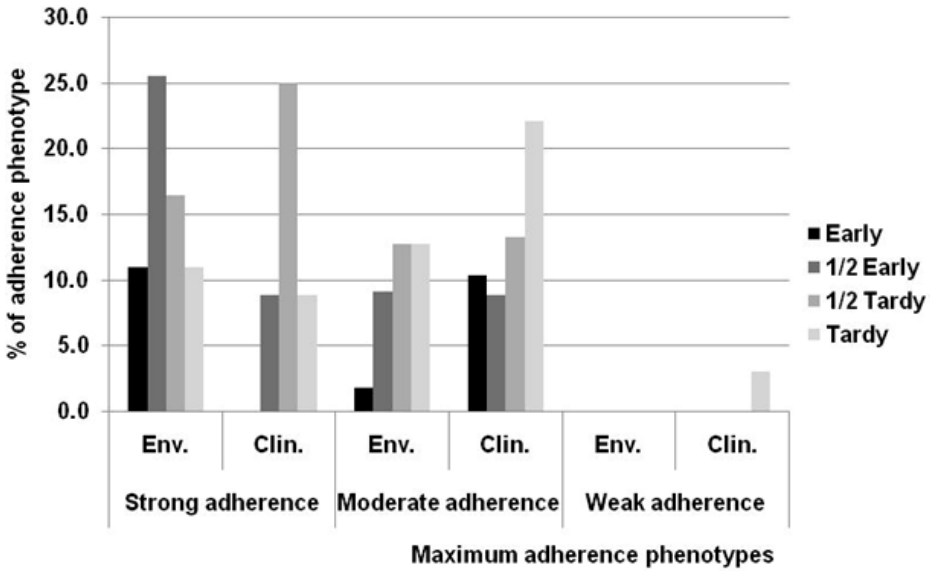

Figure 3. Adherence phenotype rates according to the incubation time required to achieve maximum adhesion. Early producer (t ≤ 4h), 1/2 early producer (6h ≤ t ≤ 10h), 1/2 tardy (12h ≤ t ≤ 18h), and tardy producer (t ≥20h). Env., Environmental; Clin., Clinical.

formed biofilm density and swimming, and swarming. Statistical significance of the effect of each categorical variable on the dependent variables studied is shown in Table 3.

The analysis of variance confirms the significant impact of the strain origin on swimming, twitching and biofilm density formed. Indeed, environmental strains had more capacity for swimming and twitching and produced denser biofilms. Significant positive impact of pyocyanin on proteolytic activity and swimming was also detected.

Significant impact of the isolation site on twitching was found. In fact, the highest twitching was observed in environmental strains especially those isolated from amurca olive. Considering clinical *P. aeruginosa* strains, patient's gender had no significant impact on virulence markers studied, but significant effects of the isolation

Table 3. ANOVA test results.

Categorical variable	*p*-values					
	Hemolytic activity	Proteolytic activity	Swimming	Swarming	Twitching	Maximum adherence
Origin	0.172	0.706	0.010	0.066	0.01	0.01
Sample	0.178	0.790	0.05	0.246	0.02	0.233
Pyocyanin	0.339	0.018	0.024	0.588	0.850	0.927
Pyoverdin	0.052	0.538	0.070	0.051	0.469	0.141
Ward*	0.343	0.01	0.073	0.084	0.017	0.013
Gender*	0.513	0.971	0.793	0.892	0.518	0.493

*For clinical strains.

ward on proteolytic activity, twitching and biofilm density were detected. Indeed, the strains that showed higher proteolytic activity were isolated from cardiology ward, and those with higher twitching motility and denser biofilm were isolated from the thoracic service.

The evolution of adherent biomass over time varies greatly from one strain to another. Comparison of biofilm formation by clinical and environmental strains showed significant differences for the incubation times of 2, 4, 6, 8, 10 and 12 h, while for other incubation times, the differences were not significant. In fact, environmental strains were quickly adhered and formed denser biofilm within a short time.

Considering environmental *P. aeruginosa* strains, comparison of biofilm formation taking into account the isolation site showed that statistically significant differences were detected only when amurca olive and soil, amurca olive and water of wells, and amurca olive and water of swimming pools were compared. Indeed, strains isolated from amurca olive formed the densest biofilms.

Taking into consideration the clinical strains, comparison of biofilm formation depending on the anatomical isolation sample showed non-significant differences. But the density of formed biofilm was significantly higher in strains isolated from the thoracic and surgery services. When inpatient and outpatient strains were compared, the differences were significant for the incubation time 2, 4 and 6 h. Therefore, inpatient strains were earlier producer of denser biofilm.

DISCUSSION

Bacterial infectivity results from a disturbance in the balance between bacterial virulence and host defense. Virulence factors, are an important determinant of pathogenicity, they allow bacteria to invade host tissues, cause disease and escape host defense mechanisms. It has been demonstrated that *P. aeruginosa* species utilizes the same virulence determinants to infect different hosts, from plants to humans (Fajardo et al., 2008).

Taking all strains included in this work, 95.1% of them exhibited extracellular β-hemolytic activity towards human erythrocytes, 99.2% produced protease (caseinase) and 100% exhibited production of lipase and lecithinase, and overall there was no significant difference among environmental and clinical strains.

These findings suggest that the tested *P. aeruginosa* are invasive isolates, as these enzymes are of known pathogenic potential. Bacterial proteases play an important role in invasiveness, host tissue damage, and evading host-defense mechanisms (Travis et al., 1995). Haemolysins involved in invasion, are considered to be a significant virulence factor of *P. aeruginosa* as indicated previously (Majtán et al., 1991; Woods et al., 1986). The most important role of bacterial extracellular lipases may be the digestion of host cellular lipids for nutrient acquisition, which results in adhering to host tissue and neighboring cells (Stehr et al., 2003). Lecithinase enzymes modulate the host immune system (Cheng et al., 1995) and play roles in cell-to-cell spread (Vazquez-Boland et al., 1992).

Among clinical isolates, 92.6% were β haemolysin producers, this result is strongly higher if compared with that reported by Stehling et al. (2008) and Holban et al. (2013) indicating percentages of 51.7 and 36.5% respectively. For lipase and lecithinase production, our results are higher than those reported by Holban et al. (2013) which signaled respectively rates of 55.8 and 88.5%. However, for caseinase, our result remains slightly lower than that found by Holban et al. (2013) indicating a percentage of 100%.

Almost all studied strains (95.9%) produced pyocyanin and/or pyoverdin that are known to have a crucial role in *P. aeruginosa* virulence. Pyocyanin, a representative pigment produced by *P. aeruginosa*, targets multiple cellular functions and contributes to acute as well as chronic infections, as it has several effects such as stimulating IL-8 release (Look et al., 2005), depressing host-response and inducing apoptosis in neutrophils (Allen et al., 2005). Pyoverdin is also a virulence marker in this pathogen, it was found that pyoverdin regulates its own secretion and the secretion of other *P. aeruginosa* virulence factors, such as exotoxin A (Lamont et al., 2002).

Among clinical isolates, 92.6% produced pyocyanin and/or pyoverdin, 61.8% produced pyocyanin, and 67.6% produced pyoverdin; these results partially agree with those reported by Finlayson and Brown (2011) which signaled respectively rates of 82.5, 57.9 and 78.5%. Other studies report the production of pyocyanin in *P. aeruginosa* isolates ranging between 41.3 and 81.5% (Fothergill et al., 2007; Iwalokun et al., 2006).

Another group of virulence factors are attachment and motility organelles, including polar flagellum and type IV pili. These cell surface structures of *P. aeruginosa* are responsible for the three most known types of motility: twitching pilus-dependent movement on solid surfaces, flagellum-mediated swimming in aqueous environments, and swarming, requiring both flagella and pili, on semisolid surfaces (Köhler et al., 2000; Rashid and Kornberg, 2000; Wall and Kaiser, 1999). In this study, it was reported that all examined strains express these motility structures. It has been demonstrated that both of these cell structures bind specifically to the host cell glycosphingolipids (Gupta et al., 1994), and that this binding event is fundamental for epithelial cell invasion and cytotoxicity (Comolli et al., 1999).

The biofilm is relevant to *in vivo* growth; it contributes to increasing infections *in vivo*. Biofilm forms a protective barrier that provides increased tolerance to antibacterials and phagocytic cells (Flemming and Wingender, 2010). In the present study, 100% of the isolates were able to form biofilm on polystyrene substrate with diversity in biofilm biomass, variable times required to achieve maximum adherence, and wide variation in the adherent biomass evolution over time. Considering the maximum adherent biomass reached for each strain, it was noted that 64 isolates form strong biofilms, 57 form moderate biofilms and 2 form weak biofilms.

In the present study, significant correlation was found between the three types of motility (mainly twitching) and biofilm density. Indeed, the flagellum and type IV pili are recognized as central components in the biofilm process. The flagellum provides mobility needed to actively approach a surface, and the type IV pili intervene in surface attachment and motility. It has been reported that type IV pili and twitching motility are involved in biofilm architecture and are responsible for the formation of microcolonies on abiotic surfaces (Chiang and Burrows, 2003).

These findings are consistent with those reported by Wolska and Kot (2013) pointing out that 100% of clinical *P. aeruginosa* strains isolated from different anatomical sites formed biofilm, and that biofilm production is in correlation with twitching motility.

The impact of pyocyanin production on biofilm formation was not detected, suggesting a similar ability of pyocyanin producers and non-producers to produce denser biofilms.

Among all tested isolates, only two strains producing pyocyanin and pyoverdin, isolated from the urine of outpatients, showed a relatively low level of virulence. In fact, these isolates showed low hemolytic and proteolytic activities, the lowest motility and the weakest biofilm formation on polystyrene surfaces.

The increased virulence observed in environmental strains could be explained by the fact that bacteria encounter a myriad of stresses in their natural environments. These stresses elicit a variety of specific adaptive responses that protect bacteria, therefore their virulence is amplified. Based on these results, it is suggested that the production of the four exoenzymes tested, pyocyanin and pyoverdin, the flagellum and type IV pili expression, and the biofilm formation as virulence markers for pathogenicity should be considered as general virulence factors, since a large number of *P. aeruginosa* strains present this phenotype.

In conclusion, *P. aeruginosa* infections typify those of a pathogen with many potential virulence factors that allow it to colonize and infect humans, animals and plants. The phenotypic characterization performed in this work clearly revealed that the studied *P. aeruginosa* strains have a collection of virulence markers required to cause disease in different tissues. Such bacteria present a serious therapeutic challenge for treatment of both community-acquired and nosocomial infections. In this context, the virulence factors produced by *P. aeruginosa* could be possible therapeutic targets to reduce the establishment of severe infections in patients presenting serious health conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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