Study of Biological Characteristics of *Pseudomonas aeruginosa* Strains Isolated from Patients with Cystic Fibrosis and from Patients with Extra-Pulmonary Infections

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A total of 120 strains of *Pseudomonas aeruginosa*, isolated from cystic fibrosis (CF) patients (n = 80) and from patients having extra-pulmonary infections (n = 40) were studied regarding the presence of some virulence factors (hemolysin, gelatinase and elastase production) and presence of the *algD* and *algU* genes as detected by polymerase chain reaction-PCR. There was not a significant difference for the production of gelatinase and hemolysin between non-mucoid strains from CF patients and other isolates from extra-pulmonary infections and mucoid strains. The production of elastase was found to be significant among these strains. The *algD* gene was detected by PCR in all studied strains but the *algU* gene was detected only in 25% of the mucoid strains. Conclusion withdrawn from the results were: (i) hemolysin and gelatinase production although present in many strains of *P aeruginosa* should not be considered as general virulence factors for the mucoid phenotype but could help in the pathogenic process; (ii) elastase production could be a necessary virulence factor for the initial pathogenesis process; (iii) mucoid and non-mucoid phenotypes could also be expressed according to the host's tissues or environment, and finally, (iv) more than one regulator system for alginate production is probably present in each strain.
Key-Words: *Pseudomonas aeruginosa*, biological characterization, pathogenesis, cystic fibrosis, extra-pulmonary infections.

Pseudomonas aeruginosa is the bacterium most frequently associated with pulmonary infection in patients with cystic fibrosis (CF), being the major infectious cause of morbidity and mortality in these patients [1-5]. This bacterium remains in the airways of the CF patients despite of intensive antibiotic therapy and contributes to the pulmonary failure. Following colonization of the respiratory tract of the CF patients with *P. aeruginosa*, the mucoid variant of the original strain emerges and becomes predominant [6,7], leading to chronic pulmonary infection indicating that this bacterial colony type has virulence traits that are important for the maintenance of the infection, what leads toward a poor prognosis for the patients [2,7].

P. aeruginosa produces a variety of extracellular enzymes that contribute to its pathogenesis [8-11]. The exoenzymes: alkaline protease [12], elastase [13], exotoxin A, exoenzyme S and hemolysin are produced during the course of clinical infection and contribute for the development of infections in animal models [10,11,14]. The proteolytic enzymes help to break down physical barriers of the host [15] and hemolysin lyses cells from varied sources. The expression of these exoproducts does not occur until the late logarithmic phase of growth, when the cell density is higher. This production occurs through a phenomenon called quorum sensing that is involved in the activation of genes at high cell densities in response to chemical signals released by *P. aeruginosa* [16].

Other virulence factors include pilus expression and alginate production. The pili from *P. aeruginosa* were associated to adhesion and binding to GM_1 receptors on the surface of CF epithelial cells [17,18]. The pilus (type IV) genes responsible for these processes are all located in the bacterial chromosome and DNA sequencing revealed the presence of three open reading frames designated *pilB*, *pilC*, and *pilD* that encode proteins of 62, 38 and 32 KDa, respectively [18]. Alginate is a viscous exopolysaccharide consisting of D-mannuronic and L-guluronic acids [10,19,20]. It prevents both opsonic and non-opsonic phagocytosis, protecting the bacterium cell from the host immune response [7].

The majority of enzymes involved in synthesis and modifications of the alginate polymers are encoded by a large cluster of genes located at 34 min of the *P. aeruginosa* chromosomal map [21] that is transcriptionally regulated by the products of several genes located in the 9- to 13-min region of the chromosome [22]. It includes *algR* and *algB*, two homologous genes [23,24]; *algP*, a histone-like protein [25]; *algQ* and *algU* [6,21,26]. These genes have their effects in the expression of the critical *algD* gene encoding GDP-mannose dehydrogenase [2].

The objective of this study was to evaluate in a comparative way the expression of some virulence factors (hemolysin, gelatinase, elastase and presence of genes *algD* and *algU* as detected by polymerase chain reaction-PCR) by *P. aeruginosa* strains isolated from patients with cystic fibrosis and strains isolated from patients with extra-pulmonary infections.

Material and Methods

Bacterial Strains

The *P. aeruginosa* isolates used in this study were obtained from patients with cystic fibrosis (40 mucoid and 40 nonmucoid strains) (Table 1) and from patients suffering from several other types of infections (blood, urine and oropharynge - 40 strains) attended at the Clinical Hospital of the Campinas State University (UNICAMP), Campinas, São Paulo, Brazil. *P. aeruginosa strain* PA 1802 was used as a positive control strain

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Table 1. Frequency and percentage of produced exoenzymes and presence of the *algD* e *algU* genes in *P. aeruginosa* strains isolated from cystic fibrosis patients and from extra-pulmonary infection sites

Strains	Hemolysin	Gelatinase	Elastase	*AlgD*	*AlgU*
Mucoids	21/40	15/40	18/40	40/40	10/40
	52.5%	37.5%	45%	100%	25%
Non-mucoids	22/40	23/40	32/40	40/40	9/40
	55.0%	57.5%	80%	100%	22.5%
Extra-pulmonary	19/40	22/40	33/40	40/40	9/40
	47.5%	55.0%	82.5%	100%	22.5%

for the expression of all biological traits tested. The isolates were identified by colony pigmentation, grape-like odor, motility and biochemical tests [(carbohydrate fermentation (-), citrate assimilation (+), lysine decarboxylase (-), indol (-), oxidase (+), beta-hemolysis on blood-agar (+) and DNAse (-)].

Hemolysin Production

The method to detect the hemolysin production employed sheep blood agar. The strains were streaked out onto the surface of blood agar plates and incubated for 18 h at 37ºC. The formation of a clear halo around the colony was indicative of the production of hemolysin [26].

Gelatin Hydrolysis

The gelatin hydrolysis was used to detect activity of proteolytic enzyme (gelatinase). Bacterial cells were inoculated via a straight-line inside of tubes containing nutrient gelatin. The tubes were incubated for 48 h at 37ºC and after this period of time, the tubes were kept in a refrigerator (8ºC) to determine if the hydrolysis of gelatin (due to production of gelatinase) had occurred (liquefaction of the medium) [27].

Elastolytic Activity

Elastase production was observed in a Petri dish containing 1% elastin, 2% agar, and 0.03 M tris (hydroxymethyl) aminomethane (tris) buffer. Bacterial cells grew (48 h at 37ºC) onto the surface of these Petri dishes and the elastolytic activity determined by measuring the clear halo produced on elastin agar after this incubation period [10].

DNA Amplification

PCR was used to detect the presence of the *algD* and *algU genes*. Oligonucleotides were used to amplify fragments of 1.5 kb and 300 bp for *alg*U and for *alg*D, respectively. Oligonucleotides algD1 (5'-AAGGCGGAAATGCCATCTCC-3'), algD2 (5'-AGGGAAGTTCCGGGCGTTTG-3) [22], algU1 (5'- CGCGAACCGCACCATCGCTC-3') and algG2 (5'-GCCGCACGTCACGAGC-3') [28] were used to amplify the *alg*D and *alg*U genes, respectively. All amplifications were performed in a programmable thermal cycler Gene Amp PCR system 9700. The reaction mixture (50 mL) included 2 mL of DNA template, 0.1mM (each) dATP, dGTP, dCTP and dTTP, 0.75 mM MgCl2 and 1 U of Taq DNA polymerase. Each PCR reaction comprehended 30 amplification cycles, each consisting of 94ºC denaturation step (30s), a 55ºC (*algD*) and 60ºC (*algU*) annealing step (30s), a 72ºC extension step (1 min), ending with a 7 min extension at 72ºC. PCR products were visualized on 1.5% submersed agarose gel electrophoresis after ethidium bromide staining [29].

Results and Discussion

The frequency of production of the exoenzymes hemolysin, gelatinase and elastase by each bacterial group is shown in Table 1. Taking all together, 62 (51.7%) of the strains produced hemolysin, 60 (50.0%) produced gelatinase and 83 (69.1%) exhibited production of elastase and overall there was not a significant difference of exoenzymes production among strains isolated from CF patients and strains isolated from other types of infections (Table 1).

Hemolysin production is considered to be an important virulence factor of *P. aeruginosa* as indicated by Matján et al. [30] and Woods et al. [31], but our results pointed out to a different picture since there was not a significant difference among mucoid and non-mucoid strains isolated from CF patients and strains isolated from other types of infection. Based on these results we would like to suggest that hemolysin production as a virulence marker for pathogenicity should be taken carefully since a large number of strains did not present this fenotype.

Although gelatinase production was different between mucoid strains and the other two groups (non-mucoid and other types of infection) it was not statistically significant ($p > 0.01$), as observed for the hemolysin production, and in the same point of view, gelatinase production should be taken carefully as a virulence marker. Although the lack of statistical significance for both characteristics, hemolysin and gelatinase production, the expression of them must be taken in account when pathogenicity characteristics are studied since they could be necessary for the basic maintenance for the colonization process and therefore the survival of bacteria in the host's tissues.

The elastase production showed different results. There was a significant difference ($p < 0.01$) between mucoid and the other two groups of strains, with mucoid strains producing it with a lower percentage, i.e, 45% by mucoid strains and 80% and 82.5% by non mucoid and strains isolated from extra-pulmonary infections, respectively.

Since elastin is a basic component of lung tissues, as well as from other host's tissues, as those found in vases of the blood stream, elastase production could be a necessary trait for the establishment of the infectious process and maintenance of the

infection. These results partially agree with those published by Woods et al. [31] who demonstrated the production of high levels of elastase by *P. aeruginosa* strains isolated from the lung of CF patients. On the other hand, most (82.5%) of the strains isolated from extra-pulmonary infection sites also produced elastase, what was not observed by Woods et al. [31].

Alginate expression is related to the presence of an operon composed by many genes with the activation processes being regulated by many other genes (algR, algP, algB) [32] and algU, mucA and mucB, which are very important for the existence of the mucoid trait, with the algD gene being responsible for the expression of the alginate capsule. In our results, the PCR reactions indicated the presence of the algD gene in 100% of the strains but the algU was found only in 25% of the mucoid strains, what indicates that in the strains that do not have this last gene (algU) other regulating genes must be present [30]. Also many non-mucoid strains (22.5%) and strains isolated from extra-pulmonary infection sites (22.5%) presented the algU gene, in despite of them being non-mucoid.

What we can conclude from the data discussed above is: (i) hemolysin and gelatinase production although present in many strains of *P aeruginosa* should not be considered as general virulence factors for the mucoid phenotype but could help in the pathogenic process; (ii) elastase production could be a necessary virulence factor for the initial pathogenic process; (iii) mucoid and non-mucoid phenotypes could also be expressed according to the host's tissues or environment, and finally, (iv) more than one regulator system for alginate production is probably present in each strain.

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