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# Differences between *Pseudomonas aeruginosa* in a clinical sample and in a colony isolated from it: Comparison of virulence capacity and susceptibility of biofilm to inhibitors

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**Abstract**

We study the differences between *Pseudomonas aeruginosa* from an infected wound (clinical strain) and a colony isolated from it. We assessed the *in vitro* inhibition of these *P. aeruginosa* biofilms by DNase and filtrate of *Lactobacillus plantarum* cultures (acid = AF and neutralize = NF) with crystal violet technique. Inhibition by AF was greatest than DNase for clinical and isolated strain ($p < 0.001$) and greatest than NF for clinical ($p < 0.05$) and isolated strain ($p < 0.001$).

Using a burn model in mice, we compared the infection producing by clinical and isolated strains in planktonic and biofilm form. Deaths were quantified and the infection was assessed by determining CFU/g of tissue in the lesion, spleen and liver.

The infections with planktonic bacteria tended to become systemic and more deadly than biofilm infections. All infected wounds required the same healing period (30 days). These findings were independent of the origin of the bacteria (clinical or colony isolated strain).
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*Keywords:* *Pseudomonas aeruginosa*; *Lactobacillus plantarum*; Biofilm inhibition; Bacterial interference; Burns

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**Résumé**

Nous avons étudié les différences entre *Pseudomonas aeruginosa* d'une blessure infectée (souche clinique) et d' une colonie purifiée à partir de cette source. On a évalué l'inhibition *in vitro* des biofilms de *P. aeruginosa* par la DNAse et par le filtrat d'une culture de *Lactobacillus plantarum* (acide = AF et neutralisé = NF), en utilisant la technique du cristal violet. L'inhibition par AF était plus grande que celle produite par la DNAse pour les souches clinique et isolée ($p < 0.001$), et elle était aussi plus grande que celle produite par NF pour les souches clinique ($p < 0.05$) et isolée ($p < 0.001$). Nous avons employé un modèle de brûlure chez la souris pour comparer l'infection causée par les souches clinique et isolée tant dans la forme planctonique que dans le biofilm. Les bactéries mortes ont été quantifiées et l'infection évaluée par la détermination du CFU/g du tissue dans la lésion, la rate et le foie. Les infections causées par bactéries planctoniques avaient la tendance à devenir systémiques et plus létales que celles causées par le biofilm. Toutes les blessures infectées ont requis la même période de guérison (30 jours). Ces observations étaient indépendantes de l'origine des bactéries (souche clinique ou isolée).
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*Mots clés :* *Pseudomonas aeruginosa* ; *Lactobacillus plantarum* ; Inhibition du biofilm ; Interférence entre bactéries ; Brûlure

## 1. Introduction

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that infects primarily immunocompromised individuals and causes many recalcitrant infections. This bacterium is extremely refractory to therapy and host immune attack when it forms biofilms. Cells within a biofilm are usually enmeshed in an extracellular matrix produced by the microorganism itself [1]. This matrix is a complex mixture of exopolysaccharides, proteins and DNA. DNA is derived from lysed cells [2] and secreted through small DNA-containing vesicles located in the outer membrane [3]. Different strains of *P. aeruginosa* vary with respect to pilus-mediated DNA binding. The ability of type IV pilus of *P. aeruginosa* to bind DNA could be related to biofilm formation during the course of infection or during the colonization of abiotic surfaces [4]. Extracellular DNA is required for the establishment of *P. aeruginosa* biofilms. Certain treatments for cystic fibrosis use inhalation of nebulized recombinant human DNase I as a therapy to reduce the viscosity of purulent sputum and prevent bacterial biofilm [5].

The biofilm matrix, in which bacteria are immersed in DNA, can promote horizontal gene transfer and induce genetic changes resulting in extensive genetic diversification [6]. The phenotypic differences between biofilm subclones and free-swimming cells (planktonic) has been proposed to partially explain the heightened resistance of biofilm cells. After most of a biofilm population has been killed by an antimicrobial agent, a very small percentage remains viable despite prolonged exposure to the antibiotic or increased dosage. These cells, called ''persisters'', confer no heritable resistance to progeny once the selective pressure is removed and to repopulate the biofilm. However, the bacteria dispersed from this biofilm as planktonic bacteria remain antibiotic sensitive. Studies of DNA exchanges *in vitro* indicate 10-fold-higher rates of transformation in biofilm bacteria

than among planktonic forms. This genomic dynamism produces huge numbers of novel subclones, some of which will have a selective advantage for particular host environments. These findings were used to explain why biofilm infections persist and why it is so difficult to developed chronic bacterial infections in animal models in which researchers deliberately infect animals with single isolated bacterial clone. Unlike clinical isolates, these pathogens do not have sufficient genetic diversity to persist in otherwise healthy animals with an intact immune system [7]. However, this hypothesis does not take into consideration differences among different host responses (human versus animals) to resist colonization and disease nor the fact that there are great differences between natural human infections and animal models with regard to the infection route [8].

On the basis of these data, we investigated the differences in the behavior of *P. aeruginosa* obtained from a human chronic infections, a clinical strain sample containing multiple genetic variants (polyclonal) [7,9], compared to a single clone of *P. aeruginosa* (monoclonal) isolated from the polyclonal sample. We also studied *in vitro* differences in biofilm formation as well as biofilm susceptibility to DNase inhibition. We compared the DNase inhibition activity with the inhibition exerted by a filtrate of *Lactobacillus plantarum* supernatants. In a previous work we demonstrated the inhibitory capacity of *L. plantarum* on *P. aeruginosa* biofilm formation [10]. In this work we compared *in vivo* the virulence of polyclonal with that of monoclonal bacteria, in both biofilm and planktonic form, in a burned-mouse model.

## 2. Materials and methods

### 2.1. Bacterial strains

The *P. aeruginosa* clinical strains (C) used in this study were obtained from a chronic burn wound of one month of evolution. The infection was resistant to several kinds of treatment, even with antibiotics. The biopsy sample was homogenized and cultured in Luria–Bertani (LB) medium (Gibco, Rockville, MD, USA) once. Aliquots were kept in glycerol 40% at −80 °C until use. We assumed that this procedure allowed us to preserve the original *P. aeruginosa* strain mixture (polyclonal) present in the wound [7,9].

For single clone isolation, one CFU was picked from the plate original sample-biopsy culture in LB agar and cultured again in the same medium. The passage and isolation of the selected CFU was repeated three times. Then the CFU was cultured in LB broth for 4 h at 37 °C. This isolated strain (I) was aliquoted and kept in glycerol 40% at −80 °C until use.

*Lactobacillus plantarum* ATCC 10241 was grown in MRS broth (Oxoid, Basingstoke, UK) at 37 °C. Culture supernatants were recovered following centrifugation and filtration through 0.22 μm filters (AF = acid filtrate); aliquots of AF were neutralized with 8 M NaOH to pH 7 (NF = neutralized filtrate).

### 2.2. Biofilm assays

A static biofilm assay using both the clinical strains and the isolated colony was performed as described previously [11], with measurements after 1 h (when bacterial

growth was negligible) and 6 h. *P. aeruginosa* was cultured in 96-well polystyrene microtiter plates. For the 6 h assay, one overnight *P. aeruginosa* culture was diluted 1:7 in LB and grown for 6 h (this biofilm is bacterial growth dependent). The biofilm inhibitors (DNase, AF, and NF) were added respective and simultaneously with the *P. aeruginosa*.

For biofilm disruption, *P. aeruginosa* was grown for 6 h in the microtiter plate and then inhibitors were added.

After each time period, the wells were stained with crystal violet and cell-attached dye was solubilized with ethanol 95% (v/v); absorbance was then measured at 540 nm.

### 2.3. DNase activity

We determined that AF and NF exerted an inhibitory activity on biofilm formation. In order to determine whether AF and NF had DNase activity we filled wells (6 mm in diameter) performed on DNA-agar (Oxoid) plates with AF and NF solutions, using as positive control DNase type I (Sigma) solutions of 1 mg% (D1) and 100 mg% (D100). After 24 h at room temperature the diameters of DNase activity, indicated by purple pigmentation around the wells, were measured.

In order to detect DNase activity in an *L. plantarum* culture, 5-ml volumes of molten semi-solid MRS agar were seeded with 50 μl of an overnight MRS culture of *L. plantarum* and poured over the DNA-agar surface. The Petri dishes were incubated at 37 °C for 24 h and DNase activity was observed.

### 2.4. In vivo experiments

In order to determine the infective capacity and virulence of the clinical and isolated strains (biofilm and planktonic forms), the burned-mouse model of Stieritz and Holder, modified by Rumbaugh et al. [12], was used. The protocol was approved by the Animal Care and Use Committee at Texas Tech University Health Science Center. Briefly, mice were anaesthetized their backs were shaved, and then were placed in water at a temperature of 90 °C for 10 s to burn 15% of the body surface. Immediately following the burn, the mice were randomized into four groups and injected subcutaneously directly under the burn with *P. aeruginosa* with polyclonal and monoclonal sample, in both planktonic and biofilm forms.

To prepare the injection samples, the biofilm was isolated from planktonic cells from a 4-h culture, allowing biofilm to adhere to the wall surface of flask culture. The suspension of biofilm bacteria was performed by disruption using a vortex and adjusting the $DO^{600}$ to the same $DO^{600}$ of the planktonic suspension. We assumed that the number of bacteria in the biofilm suspension is the same in polyclonal and monoclonal biofilms and similar to that in the planktonic suspension.

The injected groups were: (1) BPaCp group, with 200–300 CFU of planktonic *P. aeruginosa* from clinical strains; (2) BPaCb group, injected with a suspension of *P. aeruginosa* biofilm from clinical strains equivalent to 200–300 CFU of planktonic cells; (3) BPaIp group, injected with 200–300 CFU of planktonic *P. aeruginosa* from the isolated strain and (4) BPaIb group, injected with a suspension of *P. aeruginosa* biofilm from the

isolated strain equivalent to 200–300 CFU of planktonic cells. The control group was injected with phosphate buffered saline (PBS).

Forty-eight hours after the initial infection, mice were killed with ether. In order to determine the bacterial load, the burnt skins, livers and spleens were aseptically removed and homogenized in sterile PBS by using a hand-held tissue grinder. The tissue homogenates were serially diluted in PBS and plated for growth in Mac Conkey agar. The inoculated plates were incubated at 37 °C overnight. The number of CFU was determined.

### 2.5. Statistics

The *t*-test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Comparative biofilm inhibition efficacy of AF, NF and DNase

A significant inhibition of biofilm formation profiles was observed for *L. plantarun* filtrates and DNase solutions in all assays in both clinical and isolated strains of *P. aeruginosa*. However, *L. plantarun* filtrates (AF and NF) were significantly greater than DNase solutions in the clinical strain assay ($p < 0.001$) (Fig. 1A). This difference was only for AF in the isolated strain assay ($p < 0.0001$); NF was significant lower than DNase 100 mg% ($p < 0.001$) (Fig. 1B). The inhibitory capacity of all agents was independent of the growth rate of *P. aeruginosa* biofilm (formation at 1 and 6 h) and of the established biofilm (biofilm disruption assays) in both the clinical and the isolated strain (Fig. 1).

A marked difference was observed between the DNase 100 mg% and DNase 1 mg% solutions ($p < 0.0001$). The inhibition activity decreased with the dilution for both the clinical and the isolated colony (Fig. 1).

NF showed reduced inhibitory activity on biofilm formation compared to AF in all assays. This effect was more remarkable in the isolated strain ($p < 0.0001$).

Inhibition of biofilm formation was lower for the isolated strain than for the clinical strain in all assays ($p < 0.01$) except for DNase 100 mg% (Fig. 1).

### 3.2. DNase activity

No DNase was found in either the supernatants (AF and NF) or in the *L. plantarum* colonies. A diameter of 15 and 2 mm for D100 and D1, respectively, was obtained.

### 3.3. Comparative infective capacity of different P. aeruginosa forms

The survival pattern of the mice injected with the different samples is shown in Fig. 2. Burn wound infection with planktonic cells proved to be more lethal than with the biofilm form for both the clinical and the isolated strain ($p < 0.05$). There were no significant

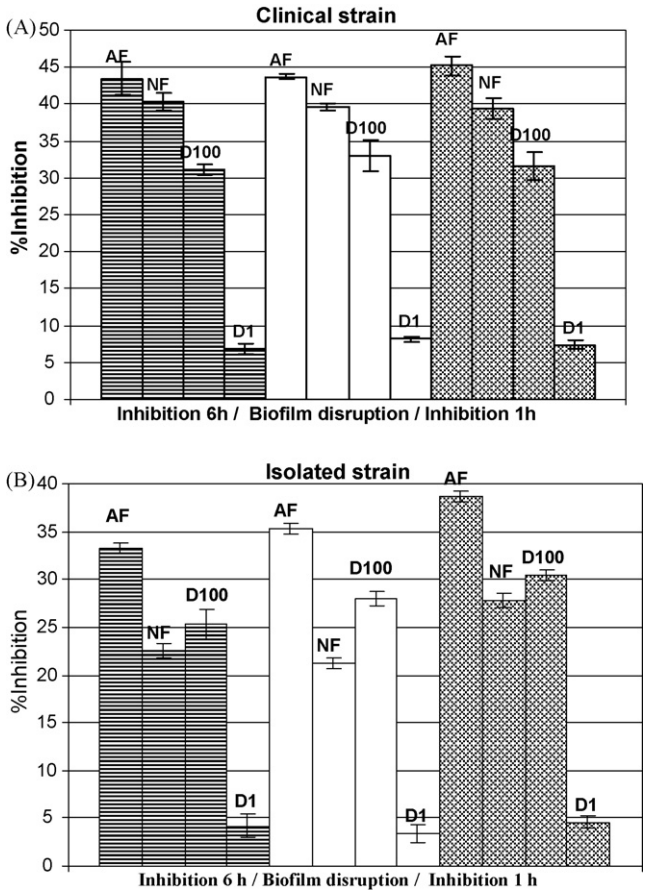


Fig. 1. Effect of different inhibitors on biofilm formation by *P. aeruginosa* clinical strains (A) and by *P. aeruginosa* isolated strain (B). Surface-attached bacteria were stained with crystal violet which was then solubilized in ethanol, and absorbance was analyzed at 600 nm. Inhibition was monitored after incubation of *P. aeruginosa* and inhibitors for 1 and 6 h. For the disruption assay, the inhibitors were added to 6 h *P. aeruginosa* biofilm. Each point is the average of three measurements of inhibition percentage. AF: *P. aeruginosa* in the presence of *L. plantarum* acid filtrate; NF: *P. aeruginosa* in the presence of *L. plantarum* neutralized filtrate; D100: *P. aeruginosa* in the presence of DNase type I 100 mg%; D1: *P. aeruginosa* in the presence of DNase type I 1 mg%.

differences in survival percentage between the planktonic form of the clinical and the isolated colony or between the biofilm of the clinical and the isolated colony.

Microbiological comparisons of the CFU in skin, livers and spleens of different groups revealed greater virulence for the planktonic than for the biofilm forms in the skin ($p < 0.001$) with strong dissemination to livers and spleens ($p < 0.0001$). No significant differences were detected between the biofilms of clinical or isolated strains. In contrast, the planktonic isolated form showed a higher number of bacteria in the organs than the planktonic clinical form ($p < 0.001$) (Table 1).

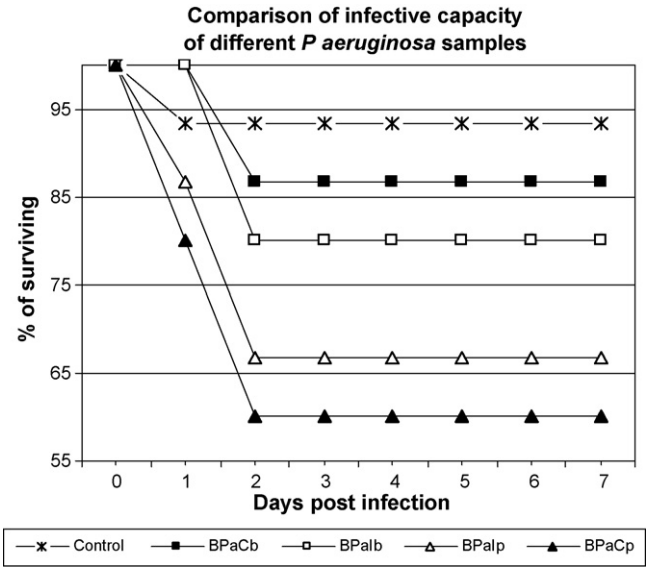


Fig. 2. Survival curves of mice for burn injected with saline (control) and different samples of *P. aeruginosa*: PaCB, burned and infected with biofilm of *P. aeruginosa* clinical strain; PaIB, burned and infected with biofilm of *P. aeruginosa* isolated strain; PaCP, burned and infected with planktonic of *P. aeruginosa* clinical strain; PaIP, burned and infected with planktonic of *P. aeruginosa* isolated strain.

Infected burn wound healed spontaneously after 30 days in the mice that survived the infection both for the clinical sample and for the colony isolated from it whether in biofilm or in planktonic form. No significant differences, was found among these groups.

## 4. Discussion

The presence of DNA from the biofilm forming bacterium is considered not only a structural component or a nutrients source. There is evidence indicating that biofilm can promote a horizontal gene transfer, originating a genetic diversity that provides the

Table 1
Microbiological analyses of tissues from mice infected with different samples of *P. aeruginosa*: PaCB, burned and infected with biofilm of *P. aeruginosa* clinical strain; PaIB, burned and infected with biofilm of *P. aeruginosa* isolated strain; PaCP, burned and infected with planktonic of *P. aeruginosa* clinical strain; PaIP, burned and infected with planktonic of *P. aeruginosa* isolated strain.

| | CFU (skin) | CFU (spleen) | CFU (liver) |
|---|---|---|---|
| PaCB | $1.32 \times 10^5$ | 6 | 4 |
| PaIB | $1.97 \times 10^5$ | 8 | 3 |
| PaCP | $1.23 \times 10^7$ | $1.13 \times 10^5$ | $1.26 \times 10^5$ |
| PaIP | $1.46 \times 10^7$ | $1.29 \times 10^6$ | $1.15 \times 10^6$ |

bacterium with adaptive advantages associated with its virulence [3,4,6,7,9]. Dénervaud et al. Collected 442 *P. aeruginosa* pulmonary isolates from 13 patients with different phenotypic characteristics [13]. Consequently, this polyclonality would be present in all clinical samples. In the present work we determined the differences between the mixture of samples from *P. aeruginosa* present in a clinical sample and a sample obtained from this mixture by successive isolations.

Our results *in vitro* indicate that there is an important contribution of DNA to the biofilm both in the clinical sample and in the isolated colony since in both cases there is a remarkable inhibition in biofilm formation and a marked biofilm disruption by DNase solutions. The supernatants from *L. plantarum* (AF) culture exhibited no DNase activity nor was it detected in the cultures of this microorganism. However, AF and NF have a stronger capacity for biofilm inhibition than DNase, which indicates that there are other elements in the microbial filtrates that are capable of inhibition and that are more important than DNase in biofilm inhibition. Moreover, the acidity of the supernatants is important to exert such inhibitory activity. Other elements of *P. aeruginosa* that are important for it pathogenicity such as acyl-homoserine lactones and ellastase are efficiently inhibited by *L. plantarum* [10].

Although in the clinical samples there would be a greater genetic diversity that would provide *P. aeruginosa* with greater resistance to inhibitors, the greater susceptibility to inhibitors of clinical sample biofilm compared to colony isolated biofilm might be due to the fact that in the clinical sample there would be a target that makes it more sensitive and that is found in smaller quantities or not at all in the isolated colony.

In the assays *in vivo* we found a greater infective capacity of horizontal (within the wound) and vertical (to the organs) dissemination for the planktonic forms than for biofilms both in the clinical simples and in the simples from isolated colonies. This would be due to the fact that in the planktonic forms bacteria are more active than in biofilm, where they are already entering a less active metabolic state. Moreover, it is possible that the bacteria in biofilm, despite their greater resistance due to their greater genetic variability, could disperse with more difficulty. However, the follow-up of scar formation in the wounds indicated the same or a longer time period required for scar formation in wounds infected with the biofilm than in those infected with planktonic forms (data not shown). It is possible that the persistent forms of biofilm are activated and present a greater resistance to the elements of the immune system and of the tissue repair system that planktonic forms, independently of whether they come from the clinical sample or from the isolated colony. This could suggest that the isolated colony requires the resistance characteristics of its precursors from the clinical sample, with no significant differences in the *in vivo* assays. The clinical sample of *P aeruginosa* comes from a chronic human wound with months of evolution that failed to heal with conventional treatments. This is a characteristic of several wounds treated in the Servicio de Cirugía Plástica y Quemados (Plastic Surgery and Burns Unit) of the Hospital Centro de Salud in San Miguel de Tucumán, Argentina. In contrast, the infected burn wound healed spontaneously in all groups of mice studied. The wounds healed after 30 days. This shows that the difference in chronicity between infected wounds in humans and in this murine model depends more on the nature of the host than on the genetic variability reached by *P. aeruginosa* in the biofilms of the wounds. It also demonstrates that an

isolated colony has in the host (mouse) the same pathogenicity as the clinical sample that would impair wound healing in humans. In consequence, we should bear in mind the fact that animal models present this limitation when extrapolating spontaneous infection to humans.

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