RESEARCH ARTICLE OPEN ACCESS

Virulence Phenotype, Physicochemical Properties and Biofilm Formation of *Pseudomonas aeruginosa* on Polyethylene Used In Drinking Water Distribution Systems

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ABSTRACT
Potable water piping has been demonstrated to serve as a reservoir for opportunistic pathogens bacteria such as *Pseudomonas aeruginosa*. In this report, we describe the characterization of *P. aeruginosa* strains isolated from water intended for human consumption by the presence of virulence factors. These strains expressed their suitability for adhesion and the formation of biofilms on polyethylene (PE). Also In this work, we were able to elucidate the factors intervening in adhesion and biofilm formation by showing the role of the substrate, the environment and bacteria. Strong correlation was observed between physicochemical properties especially the electron donor property and the surface percentage covered by cells. These results indicate that this property plays a crucial role in *Pseudomonas aeruginosa* adherence on the PE surface. In addition, if no relationship was found between the adhesion results and hydrophobicity, it means that this property was not involved in the adhesion process of *Pseudomonas aeruginosa* on the PE surface.

Keywords **–** Biofilm, Physicochemical properties, Potable water piping, *Pseudomonas aeruginosa*, Virulence phenotype.

I. INTRODUCTION

Water is the most common surface-exposed compound on Earth, but only c.a. 2.6% is available as potential drinking water [1]. According to the World Health Organization (WHO), drinking water should be free from any organisms or chemicals that might pose a health risk to the human population [2].

In drinking water distribution systems, all surfaces in contact with water can be colonized by microorganisms [3, 4, 5]. It has been estimated that about 95% of all microbial cells present in drinking water distribution systems exist as biofilms on pipe surfaces and only 5% occur in the water phase [6]. However, drinking water biofilms have the potential to harbor opportunistic pathogens which can harm human health, especially in immunocompromised people [6, 7]. Contamination of drinking water occurs when opportunistic pathogens are released from biofilm as a consequence of physical disturbance or active detachment, which then pose a potential threat to human health [6, 1]. One of the most important opportunistic pathogen which can be involved in biofilm-associated contamination of domestic plumbing systems is *Pseudomonas aeruginosa* [8, 7]. *P. aeruginosa* appears sporadically in drinking water distribution systems, for example as a consequence of contamination during construction works [9, 10], but these bacteria seem to occur at a higher frequency in domestic plumbing systems compared to water mains [11]. The provision of microbially safe drinking water is one of the main requirements of drinking water supply infrastructure. Therefore, monitoring drinking water from source to tap is an essential step towards hygiene safety. As a result of the council directive indicated above, water authorities around world are obliged to monitor water for public use, so that the consumer is provided with safe and bacteria-free water. In Morocco, the water distributors have to ensure that the water reaches consumers meters are free from microorganisms and unlicensed chemicals. In fact the metal supports are very critical and are the subject of many discussions and petitions based on the economic and health plan [12]. Therefore, organic materials such as polyethylene (which will be the subject of our study) have been proposed as an easy alternative and have gained widespread acceptance in the pipe system for drinking water. This abundance has been granted because of their advantageous properties such as high corrosion resistance, non-electrical conductivity, flexibility of handling and

cost competitiveness [13].

The aim of this study is to monitor the presence of *P. aeruginosa* in water intended for human consumption, and characterization of their virulence traits. These virulence factors have been limited to qualitative research of protease, lipase, hemolysis and motility. To gain a better understanding of the mechanisms involved in the process of adhesion and biofilm formation on the widely used drinking water pipe polyethylene system, we further extended the study to the physicochemical properties and adhesion kinetics of *P. aeruginosa* isolates.

II. MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Condition

The *P. aeruginosa* strains used in this study (forty two strains) were isolated from water samples analysed by the microbiology laboratory of food and water hygiene (from Institute Pasteur, Casablanca, Morocco) in order to judge their potability. After analysis by the technique of membrane filtration isolation was performed on cetrimide agar. The suspicious strains are identified by oxidase test (Bio-Merieux) and the gallery system "API 20NE" (Bio-merieux). Stock cultures of each strain were maintained for short periods at room temperature on conservation agar and for longer storage they were either frozen at -20°C in Luria-Bertani broth (LB) supplemented with 20% glycerol (v/v). Transplanting and the revival of the strains are made as needed, in solid or liquid while taking precautions to keep a pure culture.

2.2 Virulence Phenotypes Study

2.2.1 Proteolytic Activity

Casein hydrolysis was tested on a casein tryptone soy agar (TCS) (Sanofi Diagnostics Pasteur) containing 10% (w/v) skimmed milk (Difco, Barcelona, Spain) and 200 µl of a streptomycin solution at 50 g/ml. After centrifuged at 13000 g for 10 min a volume of 2.5 ml of a bacterial suspension of a night, 100 µl of each suspension was placed in 5-mm-diameter wells cut into an agar (TCS) and incubated at 37°C for 24 h. This test was carried out in triplicate. The presence of a transparent zone around the wells indicated protease activity and the diameter of the transparent zone reflects the intensity of the exo-enzyme released [14].

2.2.2 Hemolytic Activity

The virulence factor associated with hemolysis can demonstrate qualitatively the hemolytic activity. Briefly, the strains were tested for h-hemolytic activity on agar base (Oxoid) supplemented with 5% sheep erythrocytes. Five microliters of each suspension was streaked onto the plates and incubated at 22 and 37°C for 24 h. This test was carried out in triplicate. The presence of a clear colorless zone surrounding the colonies indicated h-hemolytic activity [14].

2.2.3 Lipolytic Activity

The activity of lipase was determined as follows: a volume of 100 µl of a suspension was placed in the dug wells in an agar agar prepared with PBS, containing egg yolk to 5%. After incubation at 37°C for 24 h. this test was carried out in triplicate. The presence of a transparent zone around the colonies indicated the lipase activity [15].

2.2.4 Motility Study

This test was carried out in triplicate. The degree of motility of the tested isolates was variable, a cutoff of 1 cm of motility was chosen to distinguish between highly motile (>1 cm) and less motile (<1 cm).

2.2.4.1 Test "Swimming"

Tryptone swim plates (1% tryptone, 0.5% NaCl, 0.3% agar) were inoculated with a sterile toothpick and incubated for 16 h at 25°C. Motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation [16].

2.2.4.2 Test "Swarming"

Swarm plates were composed of 0.5% Bacto Agar and 8 g of nutrient broth/liter (both from Difco, Detroit, Mich), supplemented with 5 g of dextrose/liter, and dried overnight at room temperature (39°C). Cells were point inoculated with a sterile toothpick, and the plates were incubated at 30°C for 24 h. Motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation [16].

2.2.4.3 Test "Twitching"

The *P. aeruginosa* colony to be tested was stab-inoculated (approximately 3-mm) through the agar to the underlying Petri dish. After incubation at 30 or 37°C for the specified times, the zone of motility at the agar/Petri dish interface was measured and visualized by staining with crystal violet after eliminating entirely the agar [16].

2.3 Kinetics of Biofilm Formation

Six strains were studied using this test. Four strains were selected among all isolates by their high capacity biofilm formation; while the other two strains were chosen as reference for the adhesion and biofilm formation: *E. coli* AL52 as a negative control and *P. aeruginosa* ATCC 27853 as a positive control. Briefly, the polyethylenes pipes are cut into cylindrical pieces of 1 cm high, washed, autoclaved and are then introduced aseptically in the sterile wells

of 24 well plate. This plate coated pieces of PE replaces the microtiter plate with 96 wells [17]. Experimentally, each well was filled with 1.4 ml of LB broth and then inoculated with 100 µl of overnight bacterial culture and incubated at 30°C without agitation. At regular time intervals, triplicate plates were rinsed thoroughly with water, and a 1% solution of crystal violet (1 ml) was added to stain the attached cells. After 10 to 15 min of incubation at room temperature, the plates were rinsed with water, and the biomass of attached cells (biofilm) was quantified by solubilization of the dye in 2 ml of 95% ethanol. The absorbance was measured at 600 nm with a spectrophotometer (BIORAD, PR 2100).

2.4 *Physicochemical Characterization of Cell Surfaces*

To evaluate the Lewis acid–base properties and the hydrophilic/hydrophobic nature of bacterial surfaces, a MATS test (microbial adhesion to solvents) was performed according to the methodology developed by [18]. The solvents used were: Chloroform (an acidic solvent) and hexadecane (apolar), ethyl acetate (a basic solvent) and decane (apolar). A bacterial suspension containing approximately 10^8 CFU/ml in 2.4 ml of PBS buffer and 0.8 ml of solvent was shaken by vortexing for 2 min to form an emulsion. The mixture was allowed to stand for 15 min to ensure the complete separation of both phases. The optical density of the aqueous phase was measured at 450 nm. This test was carried out in triplicate. The adhesion percentage to each solvent was calculated by the flow equation: % Adh = $(1 - A/A_0) \times 100$; where A_0 is the absorbance of the bacterial suspension before mixing and A is the absorbance after mixing.

2.5 Contact Angle Measurements

Contact angle was determined using 1 cm^2 polyethylene coupons. in order to remove all traces of organic and microbiological contamination, 1 cm^2 polystyrene coupons are cleaned by soaking in a solution of 96% ethanol for 15 minutes, followed by 6 washings with sterile distilled water and dried at room temperature, afterwards autoclave at 120°C for 15 min. The surface characteristics of the material were assessed by water contact angle measurements using three solvents (distilled water, diodomethane (Aldrich ® 99%)) and formamide (SIGMA ® ~ 100%)). To measure the contact angle, we used a simple method based on a photograph of a drop defined dimensions. For this, a unit of GBX society in Romans (Digidrop device) is used and a drop of 2 µl of a liquid sensor is made using a pipette. The image of the drop is captured by a video camera and the energy characteristics of solvents were obtained using the model of Van Oss [19].

2.6 Scanning Electron Microscopy (SEM)

Experimentally, the samples were immersed in a suspension of *P. aeruginosa* (approximately 10^8 CFU/ml maintained in a buffer solution of PBS pH= 7.2) for 3 h and 10 h at 30°C and rinsed thrice again with distilled water. Before observation the samples and analysis of adherent cells, the samples of PE are detected by a mixture of glutaraldehyde 2% for one hour, and a PBS buffer solution 0.175 M for 15 minutes three times. Afterwards the dehydration procedure was carried out by transferring the samples to ethanol/water solutions of increasing concentrations (50, 70, 90 and 100%) for 15 min in each solution. The samples were maintained desiccated until carbon sputtering and visualized by scanning electron microscope (Joël JSM). The images are scanned using the software Analysis [20].

III. RESULTS

3.1 Virulence Phenotypes

Virulence studies on *P. aeruginosa* were performed to verify the virulence factors profile. Figure 1 illustrates the production of three factors, protease, lipase and hemolysins that have been shown to be associated with the virulence of the different drinking water-isolated. The expression of these virulence factors are heterogeneously expressed and produced by 54.7, 85.7 and 80.9% of the isolates, respectively. Only 40.5% of the strains tested were able to produce all of the three virulence factors. While figure 2 shows the motility-type swimming, swarming and twitching, those were expressed respectively by 71, 43, 59.5% and were weakly expressed respectively by 9.5, 35, 26% in strains tested. In contrast, only 14% of the *Pseudomonas* isolates retained all the motility phenotypes.

3.2 Adhesion Kinetic

Because of its importance in the drinking water pipe system, PE surfaces were selected to study the kinetic of adhesion of *Pseudomonas* isolates. To despite of the fact that all the *P. aeruginosa* isolates were able to adhere to the surface of PE and form a biofilm, they were marked by the differences in their kinetic to adhere and by time required for maximal adhesion ranging between 8 and 12 hours (Fig 3). Depending on the time of their adherence the isolates can be divided into two groups: i) One

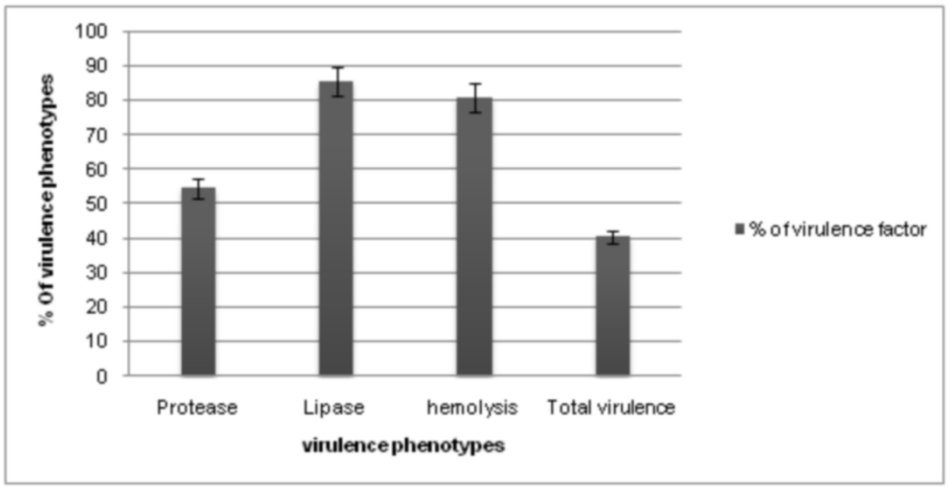

Figure 1: Virulence phenotypes of *P. aeruginosa* strains isolated (forty two strains) (% Of total virulence = % of strains possessing the three phenotypes at a time). The data are mean ±SEM of experiments carried out in triplicate.

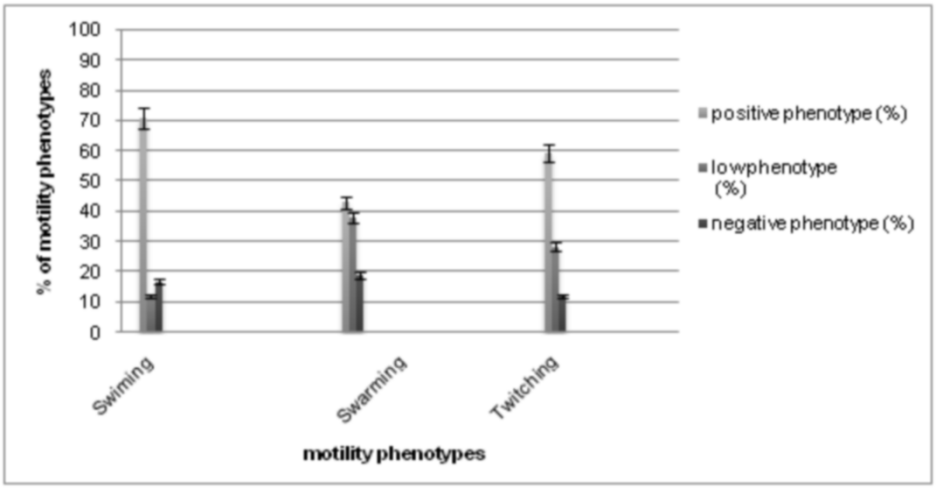

Figure 2: Study of motility phenotypes of *Pseudomonas aeruginosa* strains studied: positive phenotype: highly motile (>1 cm), low phenotype: less motile (<1 cm) and negative phenotype: no motile. The data are mean ±SEM of experiments carried out in triplicate.

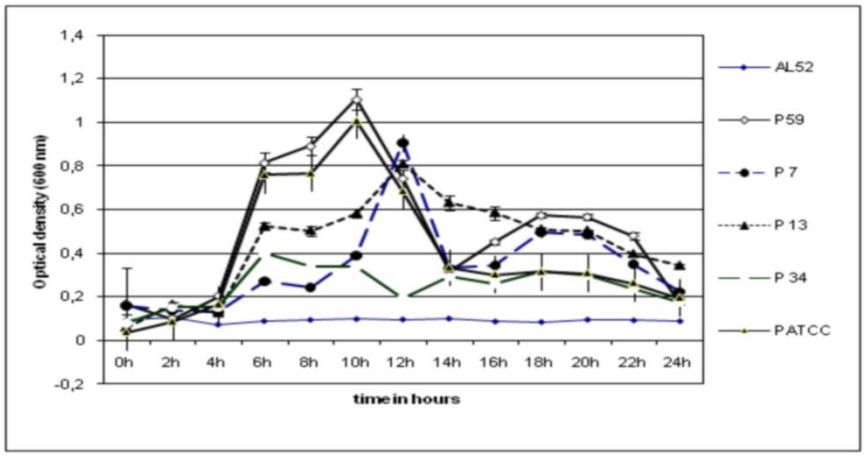

Figure 3: Adhesion kinetic of 5 *P. aeruginosa* strains, in addition to the negative control *E. coli AL52,* were cultivated in polyethylene surfaces at 30°C without agitation. At the indicated time intervals, triplicate tubes were rinsed and stained with crystal violet. The amount of stained cells was then quantified by spectrophotometry (600 nm) after solubilization of the dye in ethanol.

group of strains reached a maximum of adherence to PE substrate between 8 and 10 hours, particularly the strain P34, P59, *P. aeruginosa* ATCC 27853, ii) and another group of strains, P7 and P13 that required 12 hours for a maximum adherence.

3.3 Contact Angle Measurements

The results of measurements contact angle for the polyethylene are summarized in Table 1. These results show that the studied support is hydrophobic (θw = 79.1). Also, the estimate of the surface energy showed that the polyethylene has an electron donor property and a low relative electron acceptor property.

3.4 Physicochemical Characterization of Cell Surfaces by MATS Test

Figure 4 and Table 2 shows that the bacterial strains studied have higher affinity with chloroform than with hexadecane and with ethyl acetate when compared with decane, indicating a predominance of basic properties (electron donor) and weak acid properties (acceptor electron) on its cell surfaces. Regarding the hydrophobicity of the cell surfaces, similar characteristics were also found; all of the bacteria studied were classified as strongly hydrophilic, since the weak adhesion to apolar solvents (hexadecane and decane).

3.5 Scanning Electron Microscopy Analysis

The bacterial adhesion deduced by image analysis, using the Matlab ® program, is shown in Figure 5.

The adhesion results expressed as a percentage of the PE surface occupied (3 and 10 h) were important for strains of *P. aeruginosa* ATCC 27853 and P59 by 47 and 70% respectively, moderate for strains P7 and P13 and low for P34.

Table1: Measurement of contact angle and determining the surface energy of polyethylene.

Material	Contact angle θ (°)			Surface energy ($mj.m^{-2}$)		
Polyethylene	**water (□w)**	**Diodomethane (□D)**	**Formamide (□F)**	**□lw**	**□ +**	**□ -**
	79,1	39,3	65,1	40	0,2	10,4

Table 2. Percentage affinity of bacterial cells to the solvents used in MATS test and the donor / acceptor nature of electrons (acidic - basic). The data are mean ±SEM of experiments carried out in triplicat.

	Chloroforme polar	**Hexadecane apolar**	**Electron donor**	**Ethyl acetate Polar**	**Decane apolar**	**Electron acceptor**
P. aeruginosa 7	62,6 ±1,56	29,5 ±3,3	**33,14**	41,56 ±5,99	35,2 ±4,74	**6,36**
P. aeruginosa 13	55,97±1,55	34,2 ±2,62	**21,77**	38,3 ±3,54	38,06 ±5,51	**0,23**
P. aeruginosa 34	83,13±7,78	48,36 ±4,04	**32,76**	53,1 ±2,12	51,89 ±3,78	**1,20**
P. aeruginosa 59	67,83±8,68	46,16 ±8,75	**21,66**	41,53 ±4,22	49,8 ±3,51	**-8,26**
P. aeruginosa ATTC 27853	52,63 ±1,4	39,13 ±6,6	**13,5**	41,53 ±4,7	40,7 ±7,78	**0,83**

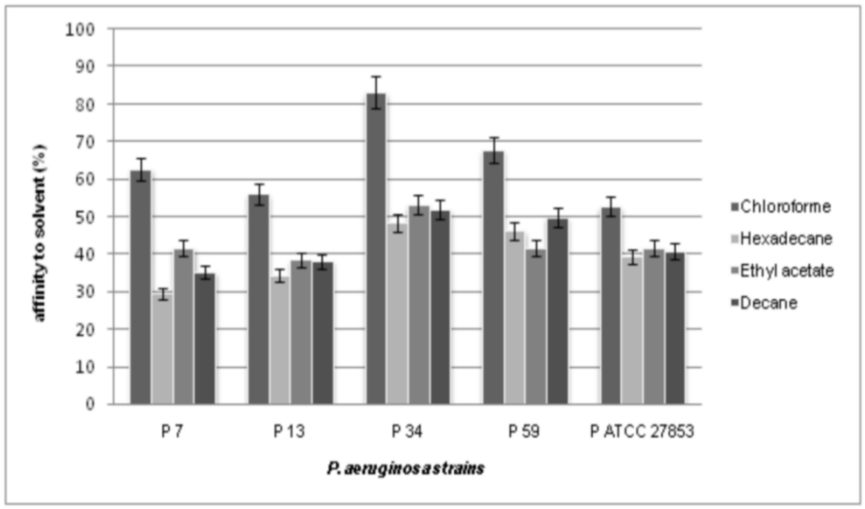

Figure 4: Solvent affinity for the strains studied (MATS test)

IV. DISCUSSION

P. aeruginosa virulence factors are numerous and are synthesized constitutively and most of them are produced in late phase of growth or under conditions of deficiency [21]. the results obtained suggest that a good part of our strains of waterborne like their counterparts isolated from the hospital environment have the means to survive and attack and colonization of host tissues, thus, it was reported that clinical strains were found in the hospital can finally imprint the system of water, and then participating in nosocomial infections [15, 22]. Let this similitude for virulence factors, it is legitimate to question the link between our aquatic virulent strains, and those of the hospital environment.

P. aeruginosa is among the bacteria that produce extracellular enzymes such as lipases [23]. These bacterial lipases receiving increasing attention because of two main reasons i) they play an important role as virulence factors produced by pathogenic bacteria against their hosts, ii) they are the largest group of biocatalysis used for a variety of biotechnological applications in various industries [24]. As the species *P. aeruginosa* is a pathogen in different persistent infections and appears to have developed strategies to survive the provocative response of the host. This is the case of phospholipase C of hemolysis secreted to 85.5%

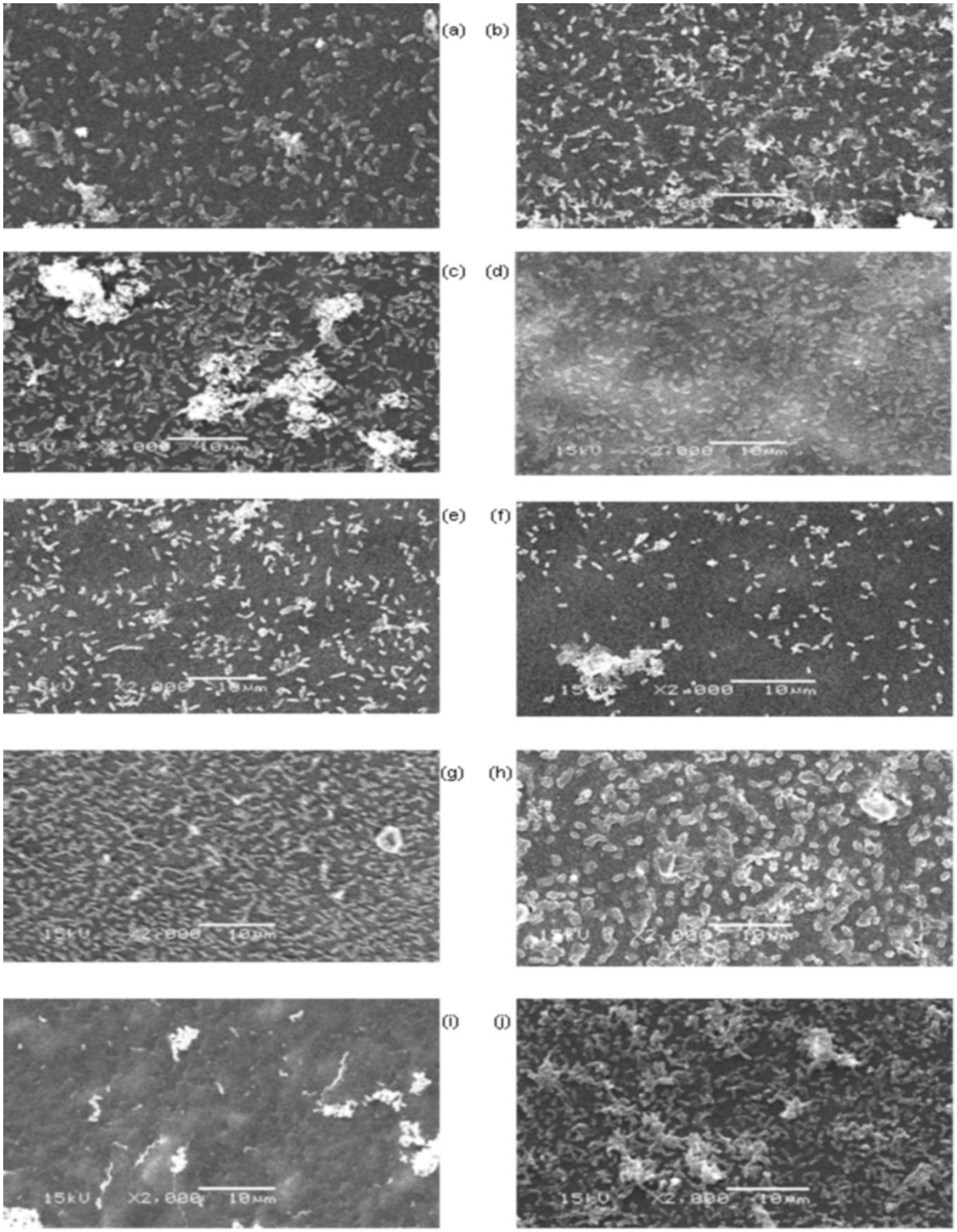

Figure 5: SEM micrographs of *P. aeruginosa* strains (isolated from drinking water) adhesion to polyethylene surfaces for 3 hours (left) and 10 hours (right) of incubation: (a), (b): *P. aeruginosa* 7; (c), (d): *P. aeruginosa* 13; (e), (f): *P. aeruginosa* 34; (g), (h): *P. aeruginosa* 59 and (i), (j): *P. aeruginosa* ATCC 27853.

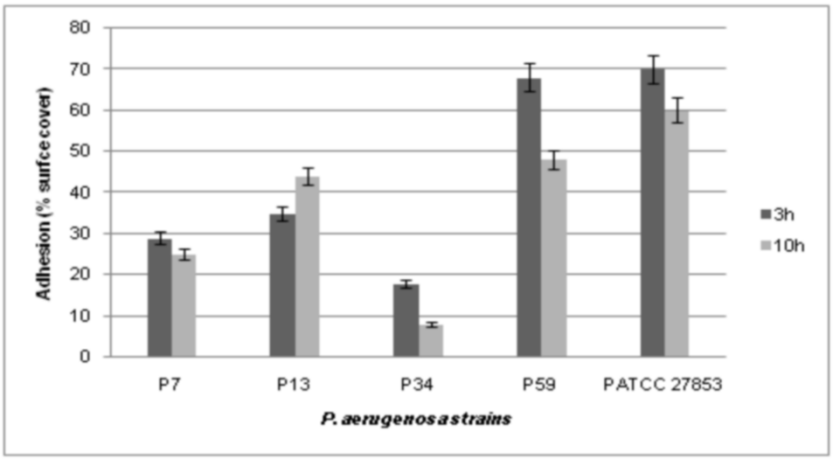

Figure 6: Percentage of PE surface covered by 5 *P. aeruginosa* strains after 3 h of incubation (left) and 10 h of incubation (Right).

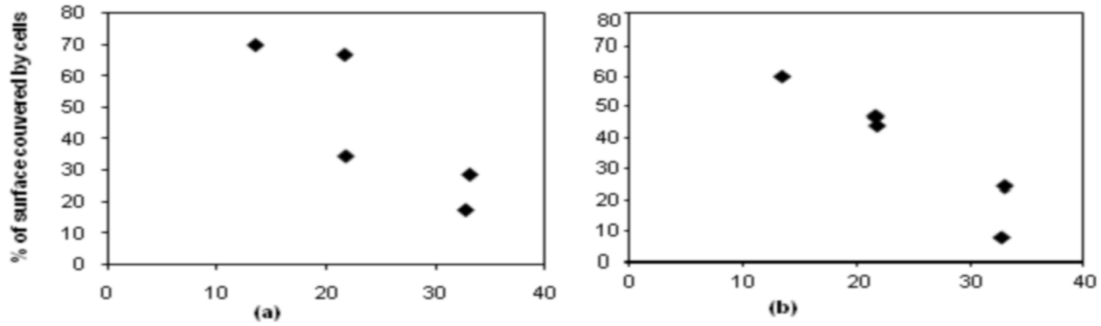

Figure 7: Correlation between the results of adhesion properties and donor / acceptor electron (a): after 3 h of adhesion (r^2 = 0,85), (b): after 10 h of adhesion (r^2 = 0.94).

among our isolates [25].

Bacterial motility is among the virulence factors that bacteria use to move closer the surfaces for initiate colonization and trigger their pathogenic [26]. Thereby, extracellular appendages such as flagella, curli and the pilis allow interactions between bacteria and surfaces and they permit the secondary minimum passage mentioned in the theory of DLVO [27, 28, 29, 30]. Active swimming is an important mechanism for transport of *P. aeruginosa* bacteria to surfaces.

The polar flagellum is the key to move the cell in aqueous environments. *P. aeruginosa* also possesses type IV pili for adherence to host tissues. This variation in mobility among the different strains can be explained by intraspecific variability and the potential for genes expression coding for the formation of flagella and pili [31]. As for the relationship mobility and establishment of biofilms, it was also shown that type Twitching motility directly influences the ability of adhesion and biofilm formation [32]. In *P. aeruginosa* the presence of genes coding for the formation of pili and flagella are important to the accelerated initiation of biofilm formation by a more efficient attachment to the surface and induction of virulence phenotypes [17, 29, 33, 34]. In contrast, non-motile mutants were unable to form a biofilm on Polyvinylchloride (PVC) surfaces. The bacteria could well probe the surface with their cellular appendages [16, 35, 36].

Adhesion and biofilm formation kinetics follows the appearance of a standard bacterial growth curve, marked by fall after a maximum adhesion. This same observation was found by Deziel [37] in finding that a *P. aeruginosa* variant rapidly forms a dense biofilm in the early incubation hours on the polyethylene surface [37]. One of the features of biofilms is to provide an environment where nutrients are continuously trapped by the exopolysaccharide matrix and available to the bacteria [37]. Obviously, cells inside a biofilm do not require extensive motility until the time they leave to colonize another available surface [27, 38].

Although the fundamental mechanisms governing bacterial adhesion are still poorly understood and have not been completely defined, it is accepted that the physicochemical properties of the bacterial membrane and that supports matrix are key determinants of initial adhesion [18]. Several theoretical models have been proposed to predict the phenomenon of adhesion of colloidal particles to surfaces, among them the DLVO approach developed by van Oss, which considers three types of

physicochemical interactions responsible for the initial adhesion: Van der Waals acid-base and electrostatic. According our results, all strains show an electron donor character. the importance of this character can be attributed to the presence of basic groups exposed on the cell surface, such as carboxyl groups (COO^-), phosphate (PO_4) phospholipids, lipoproteins and lipopolysaccharides or amine (NH_2) [39] or sulfate groups [40].

While the hydrophilic property of our strains appears to be related to the aquatic environment effect that is the original habitat of Pseudomonas strains studied (Table: 2) [41]. But it contradicts other results that were reported by the contact angle method which is only interested in the external energy to determine a hydrophobicity far from being influenced by other physicochemical interactions - chemical and hydrodynamic. In addition, these results agree with those of [42, 43], The electron acceptor character is estimated by the difference between the percentage of adhesion to diethyl ether and hexane [18]. Its importance can be attributed to the presence of acid groups exposed at the cell surface, such as R-NH-or R-OH. Several studies regarding the estimation of acid-base character of the surface of microbial cells at neutral pH showed that the cells surface exhibit a character strongly donor and weakly acceptor of electron [42, 39].

The measurement of the contact angle coupled to the Young - van oss equation provides an important factor in predicting organisms adhesion in several surfaces, so this has been considered one of the most widespread methods for assessing the hydrophobicity of the solid supports surfaces. The estimation of surface energy showed that the polyethylene has a property electron donor and a low relative electron acceptor property. Hydrophobic surfaces have shown to be particularly colonized by microorganisms, probably because these surfaces facilitate the close approach between microorganism and solid substratum, favouring the elimination of interfacial water present in the interacting surfaces [44].

DLVO theory [19] was very useful to predict the phenomena of microbial adhesion between cells and hydrophobic substrate and acid-base interactions in the sense of Lewis was the dominant factor that moderates the changes observed in the adhesion initial rates. Our results showed that all *P. aeruginosa* strains expressing a hydrophilic character adheres better to the hydrophobic polyethylene surface. This finding is not consistent with the adhesion thermodynamic properties [20]. This was also concluded in various works, to affirm that hydrophobic strains adhere preferentially to hydrophobic supports better than hydrophilic bacteria which in turn also adhere better to hydrophilic surfaces. However, our results agree with those of Cerca and colleagues [45], who found that Staphylococcus epidermidis has a hydrophilic character and expressed a high initial agglutination with hydrophobic substrates. As Van Oss [19] reported that the adhesion between hydrophobic micro-organism and hydrophilic surface may have a places and those in the presence of other interactions that will condition the approximation surface - microorganism. Thus the accession of any strain of *P. aeruginosa* on the PE surface could also be related to acid-base interactions and properties using the factor acceptor / donor electron. A significant adhesion was observed for *P. aeruginosa ATCC 27853*, according to the SEM and the study of the kinetics adhesion, this could probably be explained by the presence of EPS that are among the substances conditioning the bacterial adhesion to solid supports [46]. Also this important adhesion can be explained by the interaction between a low electron donor strain and electron donor support thus a weak repulsion occurred between the microbial surface and the PE surface low electron donor.

The correlation between physicochemical properties and the adherence results were examined, a strong correlation was observed between the electron donor property and the surface percentage covered by cells (Fig 6 and 7). These results indicate that this property plays a crucial role in Pseudomonas aeruginosa adherence on the PE surface. This finding is consistent with the work of Hamadi and colleagues [17, 42] who found that the electron donor property is one of the important factors that plays an important role in the adhesion of Staphylococcus aureus and Escherichia coli on the glass surface. Also, if no relationship was found between the adhesion results and hydrophobicity, it means that this property was not involved in the adhesion process of Pseudomonas aeruginosa on the PE surface [19, 45].

V. CONCLUSION

The drinking water supply can be a real reservoir for some opportunistic pathogenic bacteria such as *P. aeruginosa*. Special attention must be reserved to the emerging research in drinking water and also to the study of interactions with different types of supports used for water distribution systems.

It is essential to put a comprehensive water safety plan in place to protect the water from the source to the tap. This plan should address multi-barrier treatment and integrity of the water distribution system to avoid the entrance of pathogens to the system. Such plans are described in detail by the World Health Organization [2].

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