

Expression Levels of Virulence Factors with Up-regulation of Hemolytic Phospholipase C in Biofilm-forming *Pseudomonas aeruginosa* at a Tertiary Care Center

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen known to be a leading cause of nosocomial and community-acquired infections worldwide. Here, the role of biofilm formation and its possibility in enhancing the colonization and spread of *P. aeruginosa* in a medical center has been studied. More specifically, this study measures the in vitro production of biofilm in nosocomial and community acquired *P. aeruginosa* isolates representing genotypes with various prevalence rates. It also assesses the possible

effect of biofilm formation on the transcription levels of virulence encoding genes.

Methodology

Forty six *P. aeruginosa* isolates recovered at a medical center in Lebanon were used in this study. Antimicrobial susceptibility testing was performed by the disc diffusion method. Biofilm production was confirmed using adherence testing to polystyrene microplates. PCR and qRT-PCR for selected biofilm forming and virulence factor encoding genes was performed. Transcription levels were determined prior to and post biofilm formation.

Results

Thirty five of 40 nosocomial and five of six community acquired isolates were adherent to polystyrene plates. Percent adherence was comparable among prevalent and less prevalent genotypes in the medical center. Biofilm forming and virulence factor encoding genes were detected in all isolates with the exception of *pilA*, detected in only 68.1% of isolates. qRT-PCR showed variations in transcription levels of certain tested genes between biofilm populations and their planktonic counterparts.

Conclusions

The up-regulation of hemolytic phospholipase C virulence encoding gene *plcH* in the biofilm cultures of selected isolates indicates a possible role for this gene in the progression of lung disease as well as in facilitating long lasting chronic infections in critically ill patients.

INTRODUCTION

Pseudomonas aeruginosa is a prototype opportunistic pathogen.¹ This organism is a leading cause of nosocomial infections that presents a major problem especially for immunocompromised patients.^{2,3} It is also a potential community-acquired pathogen causing several infections, in particular those associated with otitis externa.⁴ This bacterium is an ubiquitous pathogen involved in a broad spectrum of infections, including pulmonary infections (common in patients with cystic fibrosis), ear infections,⁵ sinusitis,⁶ and burn-wound infections, as well as urinary tract infections.⁵ *P. aeruginosa* is widely distributed in nature as well as in moist environments within the hospital.⁵⁻⁷

This organism has been intensely studied by biofilm researchers and serves as a representative model of biofilms related to Gram negative bacteria.⁸ Long lasting chronic infections associated with multidrug resistance have been linked to the ability of this pathogen to grow and form biofilms.^{9,10} Biofilms have been defined as heterogeneous and highly organized microbial communities that are attached to biotic as well as abiotic surfaces and are embedded

in a self-produced, hydrated extracellular polymeric matrix with open water channels in between.¹¹⁻¹³ Biofilms are spatially organized by the interaction of various factors, including metabolic interactions, signaling molecules, and environmental influences.¹⁴ The process of transition from a planktonic state to a biofilm state is a complex and highly regulated process, recruiting multiple genetic pathways to control biofilm development and formation which in turn is triggered by various types of signals.¹⁵

The *P. aeruginosa* biofilm developmental growth cycle consists of the following distinct steps: environmental signals, initiation of biofilm production (which includes monolayer, and microcolony formation), maturation into an exopolysaccharide (EPS)-enclosed mature biofilm, and finally, cell dispersal from the biofilm and transition to the planktonic mode of growth.^{11,14,15} Bacterial cells grown in biofilms are markedly different from their free-living counterparts. Sessile bacterial cells are more resistant to antimicrobial treatment and to host defense mechanisms.^{9,16}

Several studies have documented changes in gene expression following contact of bacteria with surfaces. Davies and colleagues showed that *algC*, an alginate biosynthesis gene encoding a phosphomannomutase, was up-regulated in attached cells compared to their planktonic cultures.¹⁷ In addition to up-regulation of the alginate synthesis gene, flagellar synthesis down-regulation was also documented following attachment to a surface.¹⁴ Moreover, it has been documented that gene expression might differ in each distinct stage of the biofilm developmental process.

Lequette and Greenberg pointed out that the *rhlA* gene, encoding a rhamnopolyltransferase and involved in rhamnolipid surfactant biosynthesis, was expressed mainly in the stalks rather than the caps of the mushroom-like structure biofilm.¹⁸ These sessile populations of cells are of growing clinical concern because of their ability to colonize and contaminate medical equip-

ment, thus contributing to several medical device-related infections in humans.^{11,19} Their role in enhancing virulence potential is yet to be investigated.

Previous studies conducted at our medical center revealed the predominant prevalence of a potentially virulent *P. aeruginosa* genotype I from patients with nosocomial infections²⁰ and the lack of the role of adhesions in its spread.²¹ Nevertheless, biofilm formation by this organism may enhance the colonization and spread of *P. aeruginosa* genotype I in the medical center. Moreover, biofilm formation may contribute to enhanced virulence potential of the organism.

For that purpose, this study aimed to: 1) assess the ability of in-vitro biofilm production in the most prevalent *P. aeruginosa* genotype in comparison to the less prevalent genotypes in previously studied nosocomial isolates at the medical center, in addition to the recently collected community-acquired isolates, and 2) determine the transcription levels of various virulence factors encoding genes in biofilm producing cell populations as compared to the transcription levels in their planktonic counterparts.

METHODOLOGY

Source and Identification of Bacterial Isolates

A total of 46 *Pseudomonas aeruginosa*

isolates recovered at a tertiary care center in Lebanon were included in this study. Forty of these *P. aeruginosa* isolates were previously obtained between September 2003 and May 2004 from clinical specimens of patients with nosocomial infections belonging to the predominant genotype I (n=20) and other less predominant genotypes (n=20). These nosocomial isolates were obtained from patients who were subjected to invasive procedures including mechanical ventilation, polysite catheters, Foley catheters, and different types of surgeries. Patients were distributed within ten different units in the medical center including the Respiratory Care Unit (RCU), Intensive Care Unit (ICU), Coronary Care Unit (CCU), and the Surgery Unit. Table 1 shows data on the nosocomial *P. aeruginosa* isolates. The remaining *P. aeruginosa* isolates were collected from six patients with community-acquired infections (otitis externa) between August and September 2009.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the disc diffusion method following CLSI guidelines.²² The following antimicrobial disks were chosen for susceptibility testing: imipenem (10 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), levofloxacin (5 µg), piperacillin-tazobactam (100/10

Figure 1. Absorbance values by spectrophotometry with respect to crystal-violet staining intensity in biofilm cultures of *P. aeruginosa* isolates.

All biofilm formation tests were done in triplicates and the results were averaged. The categories marked by 0, 1⁺, 2⁺, 3⁺, and 4⁺ represent the crystal-violet staining intensity of the formed biofilm. CA: Community Acquired; N: Nosocomial; NC: Negative Control; PA14: positive control strain for biofilm formation.

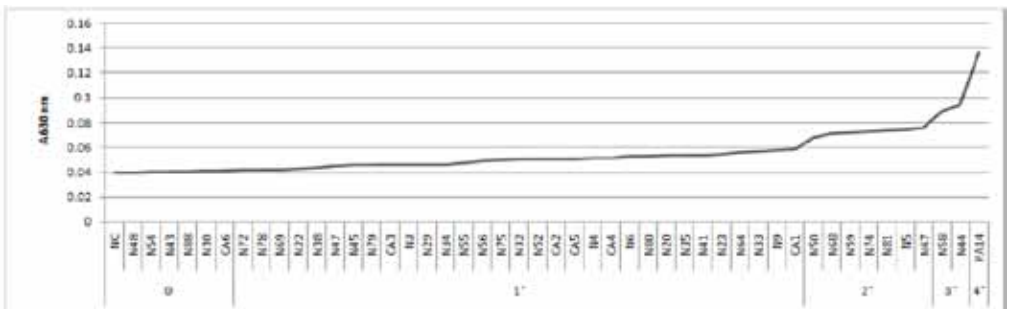


Table 1. Source and site of infection of nosocomial *P. aeruginosa* isolates (n=40), representing the various genotypes 4,5 and 9 South: Internal medicine wards, St. Jude: Pediatrics Cancer Center, CCU: Coronary Care Unit, DTA: Deep Tracheal Aspirate; ICU: Intensive Care Unit; PICU: Pediatric Intensive Care Unit; RCU: Respiratory Care Unit; n: number of isolates

Genotype	Types of unit	Number of clinical isolates	Isolation site/ Number of isolates
1	RCU	4	DTA/3; Wound/1
	ICU	1	DTA/1
	5 South	4	Abscess/1; Bronchial Washing/1; Sputum/1; Urine/1
	CCU	1	Urine/1
	4 South	1	DTA/1
	PICU	2	DTA/1; Tongue/1
	Surgery	3	DTA/1; Wound/2
	St Jude	1	Wound/1
	9 South	3	DTA/1; Sputum/1; Blood/1
2	ICU	1	DTA/1
	5 South	1	Pleural fluid/1
3	RCU	2	DTA/1; Wound/1
	5 South	1	Sputum/1
4	5 South	1	Bile/1
	St Jude	1	Blood/1
5	PICU	1	DTA/1
6	ICU	1	Sinus/1
7	ICU	1	DTA/1
8	ICU	1	DTA/1
9	RCU	1	Urine/1
10	4 South	1	DTA/1
11	Oncology	1	Bronchial Washing/1
12	5 South	1	DTA/1
17	CCU	1	Urine/1
18	CCU	1	DTA/1
22	RCU	1	DTA/1
23	RCU	1	DTA/1
24	RCU	1	Wound/1

µg), tobramycin (10 µg) and aztreonam (30 µg). The ATCC *E. coli* strain #25922 was used as a control for susceptibility testing.

Planktonic and Biofilm Culture

Conditions

Bacterial isolates were cultured on MacConkey agar plates and incubated overnight at

37°C. The isolates were then grown for 22 hrs at 37°C in Luria-Bertani (LB) medium. For biofilm cultures, the latter step was followed by a 100-fold dilution with M63 medium supplemented with 0.5% casamino acid and 0.2 % glucose and incubation in 96- well polystyrene microtiter plates (150

μl/well; Costar 3788, Corning Incorporated, NY) at 37 °C for 24 hrs.

Microtiter Plate Assay for the Assessment of Biofilm Formation

Biofilm formation was assessed using a previously described technique²³ with some modifications. Biofilms were grown as described in the section above. Bacterial biofilms were then stained with 1% crystal violet, quantitated by adding 95 % ethanol, followed by absorbance measurement at 630 nm with a BIO-TEK ELx800 Automated Microplate Reader (BIO-TEK INSTRUMENTS, INC., Winooski, Vermont). Bacteria were classified into one of three categories as defined by Christensen et al.²⁴: non-adherent ($OD \leq 0.041$), weakly-adherent ($0.041 < OD \leq 0.082$), or strongly-adherent ($OD > 0.082$). The reliability of this spectrophotometric classification was confirmed by phenotypic observation of the formed biofilm (Figure 1). The biofilm assay was performed in triplicates of the same isolate; means and standard deviations were calculated. Un-inoculated M63 medium and *P. aeruginosa* PA14 strain were included as negative and positive controls, respectively.

DNA Extraction

Total DNA was extracted from all 46 isolates as well as the PA14 strain using the illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare UK Limited) according to the manufacturer’s instructions.

RNA Extraction

Total RNA was extracted from planktonic and biofilm cultures of five selected nosocomial isolates representing the various genotypes, one community-acquired isolate, and the PA14 strain using the illustra RNASpin Mini RNA Isolation Kit (GE Healthcare UK Limited) and the PowerBiofilm RNA Isolation Kit (MO BIO Laboratories, Inc., CA), respectively, according to the manufacturer’s instructions. Planktonic cells were pelleted by centrifugation. For isolation of biofilm RNA, microtiter plates containing biofilms were rinsed vigorously with sterile distilled water. Biofilm cells were then collected from the wells by swabbing and resuspension in LB broth. Each of the planktonic and biofilm suspensions was adjusted to 0.8 McFarland and centrifuged for 5 min at 13000 x g. Each formed pellet was then processed and RNA was isolated as described above. RNA concentration was determined spectrophotometrically at an absorbance of A260.

Figure 2. Percent of *P. aeruginosa* isolates (n=6) exhibiting differential transcription levels of biofilm forming and virulence encoding genes prior to and post biofilm formation.

■ Percent of isolates exhibiting lower gene transcription levels in biofilm forming cells versus planktonic cells.

■ Percent of isolates exhibiting higher gene transcription levels in biofilm forming cells versus planktonic cells.

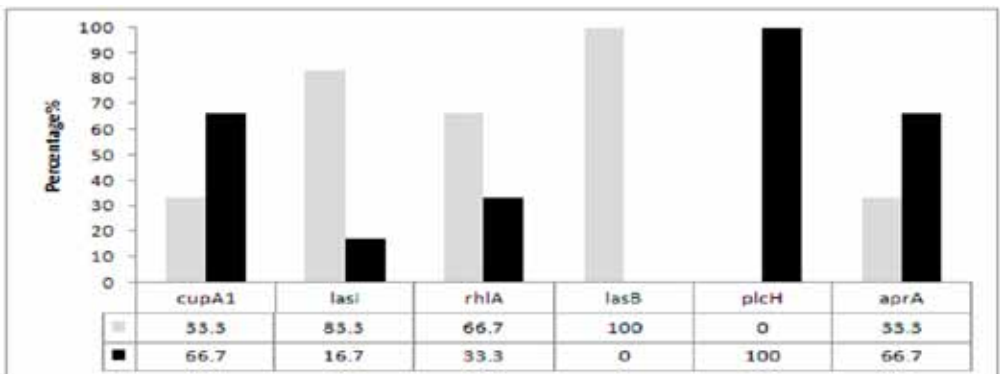


Table 2. Primers used in the study F, Forward; R, Reverse; In-house refers to primers that were designed and tested at our laboratory

Primer	Primer Sequence	Product Size(bp)	Reference
pilA-F	5'-CGAACTGATGATCGTGG-3'	199 bp	In-house
pilA-R	5'-GTTACCGGCTTGAGCATT-3'		
fliC-F	5'-CTTCCAGGTCGGTTCCAA-3'	201 bp	In-house
fliC-R	5'-GCGATGGCGATGTTCTTCA-3'		
cupA1-F	5'-TTCAGCGGCGAAGTGAC-3'	207 bp	In-house
cupA1-R	5'-GTTGGTGGCCTGGAACA-3'		
lasI-F	5'-GACGCACTCAGTCCTTAT-3'	205 bp	In-house
lasI-R	5'-GCGAGCCTTTCTGTCCA-3'		
algC-F	5'-CTACTTCAAGCAGATCCGC-3'	204 bp	In-house
algC-R	5'-AGGTCCTTCAGGTTCTCC-3'		
pelC-F	5'-TGCTCCAGCTTACCAG-3'	192 bp	In-house
pelC-R	5'-CAGTTGCAGGTCGCCTT-3'		
rhlA-F	5'-TGGCCGAACATTCAACGT -3'	107 bp	
rhlA-R	5'-GATTTCCACCTCGTCGTCCTT-3'		[31]
lasA-F	5'-GCGCGACAAGAGCGAATAC-3'	94 bp	[32]
lasA-R	5'-CGGCCCGGATTGCAT-3'		
lasB-F	5'-AGACCGAGAATGACAAAGTGAA -3'	81 bp	[31]
lasB-R	5'-GGTAGGAGACGTTGTAGACCAGTTG-3'		
plcH-F	5'-CAGTTGAACATCCGCAATCTC -3'	121 bp	[33]
plcH-R	5'-CTTCACTGATCGCCTGGTTC-3'		
aprA-F	5'-ACCCTGTCTTATTCGTTCC-3'	140 bp	[34]
aprA-R	5'-GATTGCAGCGACAACCTGG-3'		

cDNA Synthesis

cDNA was synthesized from the RNA that was extracted from planktonic and biofilm cultures of tested isolates using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's specifications.

Real time PCR

Qualitative and quantitative real-time reverse transcriptase-PCR (qRT-PCR) were performed on the total DNA and cDNA, respectively. Different sets of primers yielding an amplicon size of less than 210 bps, some of which were designed in-house using the "LightCycler Probe Design Software 2," were used for the runs (Table 2). Briefly, real-time PCR was carried out in 20µl reaction mixtures containing: 4µl of PCR grade water, 2µl forward, and 2 µl reverse

primers (0.5µM final), 10µl of SYBR Green (1x final, QuantiFast SYBR Green PCR, QIAGEN), and 2µl of DNA or cDNA. The reaction mixture was inoculated in Hard Shell 96-well PCR plates (Bio-Rad Laboratories, Inc., Hercules, CA), which were then placed in the CFX-96 Real-Time Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). The cycling conditions were as follows: Pre-incubation and enzyme activation took place at 95°C for 15 minutes. This was followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 65°C for 10 seconds, and extension at 72°C for 20 seconds. Melting curve analysis consisted of 3 segments as follows: segment 1 at 95°C for 5 seconds, segment 2 at 40°C for 30 seconds and segment 3 at 95°C for 10 seconds. This was then followed by cooling at 40°C for 30 seconds. All samples and the quantitative

Table 3. Antimicrobial susceptibility profiles of *P. aeruginosa* isolates showing resistance or intermediate resistance to at least one tested antimicrobial agent as determined by Disk Diffusion

IMP: imipenem; CIP: ciprofloxacin; CAZ: ceftazidime; LEV: levofloxacin; TZP: piperacillin-tazobactam; TOB: tobramycin; ATM: aztreonam; R: resistant; I: intermediate resistant; S: susceptible

Isolate name	Serial #	Genotype	Antimicrobial Susceptibility Profiles						
			IMP	CIP	CAZ	LEV	TZP	TOB	ATM
Pa	N3	22	S	S	S	S	S	S	I
Pa	N5	23	S	S	S	S	S	S	I
Pa	N6	24	R	S	S	S	S	S	S
Pa	N23	3	S	S	R	S	R	S	I
Pa	N29	3	R	S	S	S	S	S	S
Pa	N33	2	R	S	S	S	S	S	S
Pa	N34	8	R	S	S	S	S	S	S
Pa	N43	1	S	S	R	S	R	S	I
Pa	N44	1	S	I	S	R	S	S	S
Pa	N45	3	S	I	S	R	S	R	S
Pa	N52	12	S	S	R	S	R	S	R
Pa	N55	18	S	S	S	S	S	S	I
Pa	N67	1	S	S	S	S	S	S	I
Pa	N72	1	S	R	R	R	R	R	R
Pa	N78	1	R	S	S	S	S	S	S
Pa	N81	1	S	S	S	I	S	S	I
Pa	CA1		S	S	S	S	S	S	I
Pa	CA4		S	S	S	S	S	S	R

standards were run in duplicates.

1. Qualitative real – time PCR: Amplification of Biofilm Forming and Virulence Factor Encoding Genes

The total DNA of the 46 *P. aeruginosa* isolates was used to amplify the biofilm forming encoding genes: *pilA*, *fliC*, *cupA1*, *lasI*, *algC* and *pelC* and the virulence factor encoding genes: *rhlA*, *lasA*, *lasB*, *plcH*, *aprA* by qualitative real-time PCR using specific primers (Table 2). *P. aeruginosa* PA14 strain was used as a reference strain for gene detection.

2. Quantitative real-time reverse transcrip-

tase-PCR (qRT-PCR)

qRT-PCR was performed on the synthesized cDNA to determine the relative transcription levels of the biofilm, forming encoding genes: *cupA1* and *lasI* and virulence factor encoding genes: *rhlA*, *lasB*, *plcH*, and *aprA* in the six selected *P. aeruginosa* isolates in addition to the PA14 strain prior to and post biofilm formation using specific primers (Table 2). The housekeeping gene RNA polymerase D subunit (*rpoD*) was used as a reference gene. The *P. aeruginosa* PA14 strain, the positive control for biofilm formation, was used as the control sample for normalization. Gene transcription levels of

Table 4. Transcription levels of biofilm forming and virulence factor encoding genes
Pa: *Pseudomonas aeruginosa*, *N*: Nosocomial, *CA*: Community Acquired.

Isolate Name	Serial#	Genotype	Biofilm Forming Encoding Genes				Virulence Factor Encoding Genes							
			Real Time RT-PCR: Gene Expression Level (Quantitative)				Real Time RT-PCR: Gene Expression Level (Quantitative)							
			Planktonic Cultures		Biofilm Cultures		Planktonic Cultures				Biofilm Cultures			
			<i>cupA1</i>	<i>lasI</i>	<i>cupA1</i>	<i>lasI</i>	<i>rhIA</i>	<i>lasB</i>	<i>plcH</i>	<i>aprA</i>	<i>rhIA</i>	<i>lasB</i>	<i>plcH</i>	<i>aprA</i>
Pa	PA14		1	1	1	1	1	1	1	1	1	1	1	1
Pa	N5	23	0.93	0.4	0.94	0.1	0.6	0.2	1.004	0.3	0.2	0.02	3.5	0.4
Pa	N44	1	1.31	0.1	1.27	0.2	0.4	0.2	0.2	0.7	0.1	0.01	2.4	0.1
Pa	N58	10	1.1	0.1	1.4	0.01	0.3	0.05	0.6	0.5	0.1	0.04	0.7	2.03
Pa	N59	1	0.5	2.0	0.8	0.03	2.5	2.4	0.2	2.3	1.7	0.3	3.2	3.1
Pa	N68	5	0.4	0.5	0.3	0.2	1.9	1.1	1.2	3.7	3.2	0.4	1.5	5.01
Pa	CA1		0.6	1.3	1.2	0.1	2.4	2.5	0.6	4.9	5.3	0.3	3.7	2.3

each of the planktonic cultures and biofilm cultures of tested isolates were normalized to those detected in PA14-planktonic culture and PA14-biofilm culture, respectively. Amplification curves, melting curves and relative quantification were generated using the Bio-Rad CFX Manager software (Bio-Rad Laboratories, Inc., Hercules, CA).

RESULTS

Biofilm Formation by *P. aeruginosa* Isolates

Among the 40 nosocomial isolates representing the various genotypes, 12.5% were characterized as non-adherent, 82.5% as weakly adherent, and 5% as strongly adherent. Percent adherence was almost similar among prevalent and less prevalent genotypes. Among the six community-acquired isolates, one isolate was characterized as non-adherent and five as weakly adherent. Phenotypic observation of the formed biofilm as determined by the intensity of the crystal violet- stained rings was concordant with the absorbance (A630 nm) values obtained (Figure 1).

Antimicrobial Susceptibility Testing

Two community-acquired isolates and 16 nosocomial isolates were resistant or intermediate resistant to at least one of the

tested antimicrobial agents. Table 3 shows susceptibility profiles for only the resistant or intermediate resistant isolates. All remaining isolates were susceptible to all tested antimicrobial agents.

PCR Amplification of Biofilm Forming and Virulence Factor Encoding: Genes

PCR detection of the biofilm forming encoding genes showed that the four genes *fliC*, *cupA1*, *algC* and *pelC* were present in 100%, *pilA* in 68.1%, and *lasI* in 97.9% of isolates. The virulence encoding genes: *rhIA*, *lasA*, *lasB*, *plcH* and *aprA* were present in 100% of isolates. All genes involved in biofilm formation were equally distributed among the predominant genotype I and the other less predominant genotypes.

Gene Transcription by qRT-PCR

Relative expression using qRT-PCR of biofilm forming encoding genes: *cupA1* and *lasI* and virulence factor encoding genes: *rhIA*, *lasB*, *plcH*, and *aprA* showed that the transcription levels of the mRNA transcripts among biofilm forming populations differed from that in their planktonic counterparts for a number of genes, especially the hemolytic phospholipase C encoding gene *plcH*, which was up-regulated in the biofilm cultures of all selected isolates (Table 4, Figure 2).

DISCUSSION

Biofilm formation was assessed in nosocomial and community-acquired isolates of *P. aeruginosa*. It appears that the nosocomial isolates were more capable of forming biofilms than the community-acquired isolates. However, results are not conclusive since six community-acquired isolates were only tested and hence may not be representative of all community-acquired isolates. Several mechanical devices were associated with nosocomial *P. aeruginosa* infections in our patients including mechanical ventilators, polysite catheters, and Foley catheters. The use of these intrusive devices renders the patients more susceptible to nosocomial infection.

The increased ability to form biofilms may reflect acquisition of additional factors by these nosocomial strains in the hospital setting rendering them more capable of producing biofilms. This is reflected by their ability to colonize the gastrointestinal and respiratory tracts of sick patients who get exposed to invasive devices and respirators. This colonization is exacerbated by exposure of patients to a broad spectrum of antimicrobial agents,⁵ rendering the bacteria more resistant to these agents. It is expected in this respect that biofilm formation confers a physical barrier, inhibiting the entry of antimicrobials into bacterial cells.⁹ In the case of our isolates, the majority were susceptible to all tested antimicrobial agents, suggesting that biofilm formation was not associated to increased antimicrobial resistance.

Our data has also shown the absence of any correlation between the predominant prevalence of *P. aeruginosa* genotype I and biofilm formation. The adherence capability of the nosocomial strains among the predominant genotype I and the less predominant genotypes was almost the same. Biofilm forming encoding genes were detected by PCR in all isolates with the exception of *pilA*, which was seen in only 68.1% of the isolates. The biofilm developmental process is shown to be multi-factorial.²⁵ All genes involved in biofilm formation were evenly distributed among the prevalent genotypes.

Hence, there was no correlation between the predominant prevalence of *P. aeruginosa* genotype I and the types of biofilm forming genes present. Therefore, the prevalence of a particular genotype is due to additional factor(s) that facilitate its spread in the medical center. Hospital personnel transmission is a possible factor, since all intensive care units are open units. Thus, the epidemiology of colonization and infection related to *P. aeruginosa* at our medical center should be further investigated.

All virulence encoding genes were also detected by PCR and were found to be equally distributed among the isolates. This observation indicated that all tested isolates possess similar virulence potential and hence contributed equally to disease production. As to the presence of the *aprA*, *lasA* and *lasB* virulence encoding genes, our results were almost consistent with those obtained phenotypically, by enzymatic activity, from the same isolates in a previous study done at our medical center.²⁰

Relative expression analysis using qRT-PCR showed differential transcription levels of selected genes in biofilm populations as compared to their planktonic counterparts maintained under similar conditions, especially for the hemolytic phospholipase C encoding gene *plcH*. This result argues strongly for the proposal that growing in a biofilm mode of life results in major changes in the bacterial cells' overall activity,⁹ which might include differences in gene expression. This is also in concordance with a study carried out by Sauer et al.²⁶ who compared a particular stage of biofilm development to planktonic cells and noted that more than 50% of all detectable proteins were found to be differentially expressed. On the other hand, our findings contrast with those of Whiteley and co-workers who demonstrated that gene expression is almost similar in sessile cells as compared to planktonic growing cells, where no more than 1% of the genes tested showed variation in gene expression in the two modes of life.²⁷ However, our study assessed gene expression

in biofilms that consist of heterogeneous communities of cells possessing different metabolic activities.^{12,28,29} Therefore, the difference in transcription levels observed in our isolates might be due to the presence of certain biofilm subpopulations that exhibited differential patterns of gene expression based on the location of each individual cell in different regions of the biofilm, its distinct metabolic activity, and the particular stage of the biofilm developmental process being examined.^{18,27,30} Moreover, the expression of all genes at the same time might not be beneficial for bacteria that possess very sophisticated intracellular machinery where expression of genes is tightly controlled.

Among the virulence encoding genes tested, the hemolytic phospholipase C encoding gene *plcH* was more transcribed in all the isolates in biofilm forming cells v. planktonic cells (Table 4, Figure 2). Our data suggests that biofilm formation enhances the transcription of particular genes rendering infection more serious. For example, hemolytic phospholipase C has been shown to contribute to tissue destruction by breaking down lipids and lecithin⁵; hence it may facilitate bacterial entrance and may thus enable the organism to establish itself in the respiratory tract. Moreover, the expression of hemolytic phospholipase C by the bacterium, *P. aeruginosa*, has been shown to suppress neutrophil respiratory burst activity. This facilitates the survival of the bacterium in tissues even in the presence of neutrophils rendering it more resistant to the host inflammatory response. This eventually enables the survival of *P. aeruginosa* in the airways of patients with chronic lung disease, particularly cystic fibrosis.³¹

In this study, we conclude that: 1) the prevalence of a particular genotype is not due to biofilm formation but to additional factor(s), 2) biofilm formation might enhance or reduce the virulence potential of the organism, and 3) the observed up-regulation of hemolytic phospholipase C in all biofilm forming isolates indicates that it may constitute an important component in the progression of

lung disease in patients with cystic fibrosis. Additional testing is required to demonstrate under which conditions up-regulation of a particular virulence gene occurs in biofilm producing cells and to confirm the role of hemolytic phospholipase C in exacerbation of infection in-vivo.

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