

## Comparison of Virulence Factors in *Pseudomonas aeruginosa* Strains Isolated from Cystic Fibrosis Patients

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### Abstract

**Background:** *Pseudomonas aeruginosa*, is a Gram-negative opportunistic bacterium which establishes itself in vulnerable patients, such as those with cystic fibrosis or hospitalized in intensive care units.

**Methods:** Forty-five *P. aeruginosa* strains isolated from 26 cystic fibrosis patients were obtained. Both qualitative and quantitative assays were conducted to determine a number of virulence factors such as elastase, alkaline proteases and pyocyanin. Elastase gene expression profiling was conducted using RT-PCR. Spirometry was used to measure lung function and this was correlated to the severity of *P. aeruginosa* infection. Spirometry measurements i.e. forced expiratory volume (FEV) and forced vital capacity (FVC) were made to measure lung function and to see if there was any correlation with the production of virulence factors.

**Results:** Virulence factors profiling revealed that about 30% of the isolates were of clinical significance having expressed a number of virulence factors, in particular elastase (lasB).

**Conclusion:** Some strains of *P. aeruginosa* produce greater quantities of virulence factors and are more damaging to the lungs of patients with cystic fibrosis, although statistical analysis revealed no correlation between the virulence factors tested and the level of lung function. In addition, other factors such as biofilm formation may play a larger role for CF lung infections.

**Keywords** *Pseudomonas aeruginosa*; Virulence factors; Elastase; Cystic fibrosis

### Introduction

The pathogenesis of *P. aeruginosa* is considered to be multifactorial. This is due to its wide array of virulence factors such as its flagella, pili, alginate, and extracellular proteases [1,2]. Pyocyanin, a redox-active toxin, and secreted proteases such as elastase (LasB), alkaline protease (AprA) and protease IV, have all been characterized in many clinical settings, including in the lungs of cystic fibrosis (CF) patients [3,4]. These factors assist in the invasion, and colonisation of *P. aeruginosa*, in the host lung. Proteases have been shown to be involved in pulmonary tissue degradation and inactivation of immune system components [5]. Elastase is thought to be the main contributor to the pathogen's virulence, with Galloway et al. [6] reporting its proteolytic activity to be approximately 10 times that of alkaline protease.

Amongst the *P. aeruginosa* virulence factors pyocyanin (PCN) is of particular importance since it is shown to induce oxidative stress through the oxidation of glutathione, subsequently leading to the inactivation of catalase [7,8]. In a study conducted by Caldwell et al. [9] it was shown that PCN is a significant contributor to lung

destruction during chronic *P. aeruginosa* infection of bronchiectasis airways.

During infection of a CF affected lung, *P. aeruginosa* can also secrete large amounts of pyocyanin (PCN), and as such, can interfere with many cellular processes in cultured lung epithelial cells [10]. It can promote the secretion of airway mucus and stimulate the production of interleukin 8 (IL-8) by neutrophils, thereby leading to inflammation in the lung [11,12].

*P. aeruginosa* is also recognised as a model biofilm forming pathogenic bacterium. As such a number of approaches have been taken to inhibit its biofilm formation. For example Vitexin, a polyphenolic group of phytochemical with antimicrobial property, has been studied for its anti-biofilm potential against *P. aeruginosa* in combination with azithromycin and gentamicin [13].

The main aim of this study was to investigate, and profile, a range of virulence factors produced by the clinical isolates of *P. aeruginosa* infections and study the impact they have on the lung functions of CF patients.

## Materials and Methods

### Sampling

Forty-five *P. aeruginosa* strains isolated from 26 cystic fibrosis patients were kindly provided by the laboratory at Monash Medical Centre (Victoria, Australia). These were numbered from PA1-PA 45. *P. aeruginosa* wild-type strain PAO1 was used as control. Patients from whom the isolates were collected were treated with Timentin<sup>®</sup> or Cefazidime combined with Tobramycin (TOB). All bacteria were stored as frozen stocks in 20% glycerol at -80°C (Nalge Nunc Int., Rochester, NY). A fresh culture was obtained by subculturing isolates on horse blood agar (Media Preparation Unit, University of Melbourne, Parkville, Australia) for 24 h at 35°C prior to each experiment.

### Qualitative assessment of protease, and pyocyanin production

Alkaline protease, elastase and pyocyanin production were visualised by plating *P. aeruginosa* isolates on milk agar plate, elastin and *pseudomonas* F agar plates, respectively. This was followed by 48-hour incubation at 37°C. Zones of elastin and milk hydrolysis were indicative of elastase and alkaline protease, respectively. Pyocyanin producing isolates manifested deep blue colonies on PCN plates (media preparation unit (MPU), department of Microbiology, the university of Melbourne).

### Quantitative assessment of elastase

Bacterial isolates were inoculated from the glycerol stock into 5 mL LB, and the culture was grown for 24 hours at 260 rcf in a 37°C shaking incubator. A 100 µL aliquot were taken out of this 5 mL culture and subcultured in 5 mL LB medium for another 24 hours. These cultures were centrifuged at 4,000 relative centrifugal force (rcf) for 10 minutes at 4°C. The supernatant was collected for assay use, while the pellet was frozen at -80°C and further on used for RNA isolation. The elastase assay was carried out using the EnzChek<sup>®</sup> Elastase Assay Kit (Molecular Probes) in accordance with the manufacturer's instructions. Fluorescence was measured in a Cary eclipse fluorescence spectrophotometer multi-well plate reader set for excitation at 485 ± 10 nm and emission detection at 530 ± 10 nm. The concentrations of elastase in unknown samples were interpolated from a standard curve of known elastase concentration (0 U/mL to 1 U/mL).

### mRNA Expression Profiling of Elastase B gene (*lasB*)

#### RNA isolation and reverse transcription

Total RNA was extracted from each isolate using the TRIzol<sup>®</sup> MaxTM Bacterial RNA Isolation Kit (Ambion by Life Technologies) in accordance with the manufacturer's instructions. RNA was quantified using the Qubit<sup>®</sup> Fluorometer and Qubit<sup>®</sup> RNA Assay Kit (Life Technologies). Reverse transcription was carried out using the QuantiTect<sup>®</sup> Reverse Transcription Kit (QIAGEN) using 1 µg of RNA per reaction, in accordance with the manufacturer's instructions. For subsequent real-time PCR 1 µL of the resultant cDNA was used per reaction.

### Real-Time PCR

Quantitative real-time PCR was conducted using the BIO-RAD CFX96 Real-Time PCR System C1000 Thermal Cycler and SsoFast<sup>™</sup> EvaGreen<sup>®</sup> Supermix (BIO-RAD Laboratories). The sequence of the PAO1 *lasB* structural gene was obtained from GenBank and used as a DNA template for primer design. The primers for the RT-PCR amplification of cDNA were *lasB* forward primer 5'GCCCTTCTTGATGTCGTAGC-3', and *lasB* reverse primer 5'CAGAACTCCGGGCTGATCTA-3'. The data were normalised against the housekeeping gene *rpsL*. Primers for *rpsL* were *rpsL* forward primer 5'GCAAGCGCATGGTCGACAAGA-3' and *rpsL* reverse primer 5'CGCTGTGCTCTTGCAGGTGTGA-3'. The real-time PCR mixture consisted of 1x SsoFast<sup>™</sup> EvaGreen<sup>®</sup> Supermix, 500 nM of each primer, 1 µL of cDNA from the reverse transcription mixture corresponding to 50 ng of total RNA, and sterile DNase-free water made up to a total reaction volume of 20 µL. The cycling conditions used were an initial activation step at 95°C for 1 minute, followed by 40 cycles consisting of denaturation at 95°C for 5 seconds, and annealing/extension at 60°C for 30 seconds. A negative (no template) control containing sterile DNase-free water instead of the cDNA template was included in each real-time PCR run. All reactions were carried out in triplicate. Primer dimers and other artefacts were evaluated by melting curve analysis consisting of 65 to 95°C in 0.5°C increments at 5 sec/step. Comparative quantification was used with respect to the PAO1 strain (control).

### Western blots analysis

Culture supernatants in denaturing conditions were analyzed by Western blotting using standard protocols. This was applied especially for detection of protease IV (*sppA*). A rabbit polyclonal to *sppA* Protease IV (Abcam, Catalogue no. 65958) was used as the primary antibody.

### Measuring production of pyocyanin

In addition to visualising pyocyanin production on the *Pseudomonas* F agar plates growth, the concentration of pyocyanin in the culture supernatant was also determined quantitatively by a HPLC-based assay. The method was based on Fernandez and Pizarro [14] with modifications to improve run time and reduce the amount of sample required for the procedure. Briefly, samples were pre-treated with methanol and centrifuged (10 min, 16,100 g). An aliquot (50 µL) was chromatographed on a Phenosphere-NEXT<sup>®</sup> C18 column (250 × 4.6 mm, 5 mm), with a solvent program of 0.1% trifluoroacetic acid and acetonitrile/0.1% trifluoroacetic acid, at 1 mL/min. The total run time was 6.5 min. Detection was at 370 nm and 387 nm. The pyocyanin peak was confirmed using LC/MS. The amounts of pyocyanin in the samples were interpolated from a standard curve (0.20, 0.50, 2.0, 5.0, 10.0, 25.0, and 50.0 mg/L) of pyocyanin powder (Cayman Chemical, MI, USA) prepared in methanol. The limit of quantification was 0.20 mg/L.

### Spirometry

Patients lung functions; the basic forced volume vital capacity (FVC); most frequently used index for assessing airway obstruction, bronchoconstriction or bronchodilatation and forced expiratory volume (FEV) for assessing and quantifying airflow limitation, were measured using spirometry according to standard protocols.

## Statistical analysis

The distribution of elastase producing isolates with respect to typed groups or strains were compared using the chi-square test. All real-time PCR data was analysed using the GraphPad Prism v4.0 (GraphPad Software, San Diego, CA). For comparison between two groups (treated versus untreated), the unpaired t test was performed. Pyocyanin and elastase results were compared to FEV and FVC measurement using correlation coefficient and regression analysis (STATA data analysis software package). A P-value of less than 0.05 was considered to indicate statistical significance. Data in graphs were presented as mean ± standard error of the mean (S.E.M) and n represents the number of isolate cultures studied.

## Results

### Antibiotic susceptibility profile of clinical isolates

The clinical isolates exhibited different antibiotic resistance patterns. All isolates tested were found to be susceptible to colistin. It was also found that the majority of the isolates were susceptible to ceftazidime (90.7%), meropenem (71.4%), and amikacin (70.5%). However, only 47.6% and 34.9% of the isolates were susceptible to aztreonam and ciprofloxacin, respectively.

### Production of pyocyanin

Qualitative analysis of pyocyanin production was carried out using *Pseudomonas* F agar, plates where, under UV stimulation, fluorescein was demonstrated by a fluorescent yellow colour for most isolates (data not shown). For quantitative analysis HPLC was used to measure pyocyanin. The absorbance at 378 nm exhibited stronger signals, and due to the fact that some clinical isolates produced only minimal amounts of pyocyanin, concentrations were calculated based on peak heights at this wavelength. Detectable amounts of pyocyanin were observed in 29% of the isolates. Eight of these isolates were found to secrete a higher amount of pyocyanin than the wildtype strain, with the maximal concentration of 13.38 µg/mL observed in PA34 isolate (Table 1). However, regression analysis showed that no significant effects of level of PCN on the lung function ( $p > 0.05$ ). The same analysis shows significant effect of patients BMI lung function ( $p < 0.01$ ). Correlation coefficients between pyocyanin and FEV and FVC measurements were also close zero and therefore of no statistical significance.

### Western blot analysis

The primary antibody used in this study targets a tetrameric form of protease IV which is produced by the *sppA* gene and has a molecular weight of 67 kDa. Being polyclonal, however, the antibody also detects other isoforms of protease IV. According to the manufacturer of the primary antibody, a 47 kDa uncharacterised protein is also detected (Figure 1) [15]. However, instead of a 47 kDa protein, a much smaller protein with a molecular weight of approximately 29 kDa was observed in most isolates (F). An additional band of 67 kDa was also observed in some isolates (Table 1). Overall, the antibody detected protease IV in 80% of the isolates.

### Qualitative assessment of protease and elastases

In the qualitative analysis on elastin and milk agar plates, elastin was shown to be hydrolysed by most isolates, as judged by clear zones of

hydrolysis around colonies (data not shown). In the quantitative assays most isolates showed detectable, albeit varying levels of elastase activity (Figure 1).

Patients	Isolates	Gender	BMI	FEV (%)	litres	FVC litres (%)
1	PA1	F	29.1	1.85 (70)		2.97 (92)
	PA41	F	29.4	1.86 (70)		2.92 (90)
	PA23	F	22.2	1.6 (56)		2.08 (61)
7	PA13	F	22.2	2.10 (68)		3.60 (95)
8	PA15	M	16.9	1.09 (39)		1.83 (56)
	PA43	M	19.1	1.67 (54)		2.75 (77)
9	PA19	F	21.8	1.77 (55)		2.06 (58)
	PA20	F	21.8	1.77 (55)		2.06 (58)
	PA33	F	21.4	1.99 (64)		3 (63)
	PA35	F	21.4	1.99 (64)		2.33 (63)
12	PA37	M	22	3.84 (85)		4.99 (94)
23	PA38	M	20.2	1.74 (78)		2.38 (92)
25	PA42	M	26.2	2.96 (75)		4.0 (83)
17	PA18	M	23.2	4.01 (93)		5.2 (104)
19	PA24	F	21.5	1.67 (53)		3.05 (80)
20	PA25	F	35.1	1.5 (47)		2.26 (58)
17	PA17	M	17.70	1.51 (53)		2.48 (75)
2	PA8	F	22.2	1.94 (68)		2.38 (70)
	PA22	F	22.2	1.6 (56)		2.08 (61)
	PA23	F	22.2	1.6 (56)		2.08 (61)
	PA3	F	22.2	1.39 (49)		1.77 (57)
3	PA4	M	19.2	3.16 (75)		4.17 (80)
	PA32	M	21	2.57 (63)		3.36 (66)
10	PA26	M	19.4	2.56 (66)		4.13 (87)
	PA28	M	19.4	2.56 (66)		4.13 (87)
11	PA30	M	37	2.6 (61)		3.96 (75)
	PA31	M	37	2.6 (61)		3.96 (75)
19	PA24	F	21.5	1.67 (53)		3.05 (80)
22	PA29	F	14.1	1.72(97)		2.04 (110)

BMI: Body Mass Index; FEV: forced expiratory volume; FVC: Forced vital capacity (FVC), In healthy adults this should be approximately 85%.

**Table 1:** Spirometry data showing the relationship between patient's gender, the presence of a particular isolate of *P. aeruginosa* and lung function.

Clinical isolates were normalised against the wild type PA01. Seven isolates (PA13, PA15, PA19, PA20, PA33, PA37 and PA41) showed elastase activity far greater than the wild type. Three of these isolates corresponded with the real-time PCR results, where the isolates PA1, PA15, PA17, PA33, PA35 and PA41 showed much greater elastase activity as compared to the PA01 wild type (data not shown).

### mRNA Expression Profiling of elastase B (*lasB*)

mRNA profiling of LasB has shown variations in the level of *lasB* expression (Figure 2) with some isolates; PA1, PA 15, PA17, PA 33 and PA 41 showing highest levels of *lasB* gene expression (Figure 2). However statistical analysis revealed no significant correlations between the level of elastase and lung function.

### Spirometry

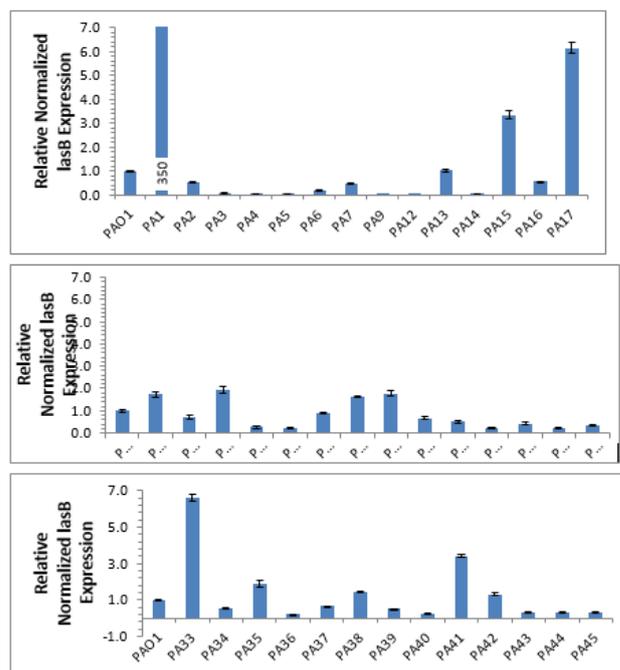
To correlate the level of proteases produced with the health status of patients, spirometer analyses were conducted. Results for isolates PA19, PA20, PA33 and PA35 with highest levels of elastase activity and how they correlate with patient FEV (forced expiratory volume) and forced vital capacity (FVC) as shown in Table 1.

### Discussion

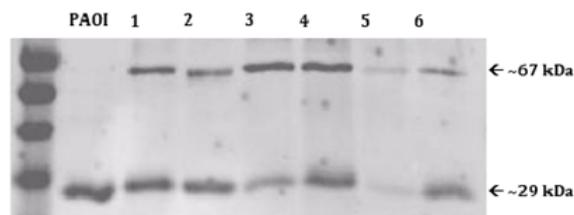
In this study, forty-five clinical isolates of *P. aeruginosa*, obtained from 26 cystic fibrosis patients, were investigated for a range of enzymatic and virulence factor activities (Table 2).

The results of the HPLC assay showed that the level of PCN production ranged from 0.2 µg/mL to 13.3 µg/mL with an average level of production of 0.453 µg/mL. Several isolates displayed no activity at all. These data were also supported by chromogenic *P. aeruginosa* - F agar plates where pyocyanin produces a blue color. However, the intensities of the colors were difficult to compare between the high pyocyanin producers (13.3. µg/mL) to those of low producers (0.2 µg/mL). It has previously been shown that the level of pyocyanin production increases with time of incubation, with no expression in the first 12 hours of cell growth and peak production in 32 hours. A study performed in 1988 involving 13 CF and other bronchiectasis patients demonstrated that *P. aeruginosa* was capable of producing PCN at concentrations as high as 27 µ/mL in their sputum [16]. It is also important to note that an extensive use of antibiotics to treat chronic CF infection by *P. aeruginosa* may affect PCN production.

Upon infection *P. aeruginosa* pyocyanin has been suggested to play key roles in lung injury. In addition, in our study there seems to be no correlation between the levels of pyocyanin produced with that of lung function. Patients lung functions were evaluated using FEV and FVC measurements using spirometry. In particular patients infected with isolates PA20, PA22, PA23, PA33 and PA35 which are high pyocyanin producers have shown poor FEV and FVC activity (Table 1). However, regression analysis has shown this to be of little statistical significance. Furthermore, because pyocyanin directly accepts electrons from NADH or NADPH with subsequent electron transfer to oxygen, generating reactive oxygen species its cytotoxicity has been strongly linked to its potential to redox cycle [15]. Pyocyanin also interferes with several cellular functions such as electron transport, cellular respiration, energy metabolism, gene expression and innate immune mechanisms [17].



**Figure 1:** Las B Transcription profiling results using Real-Time PCR. The elastase assay was carried out using the EnzChek® elastase assay kit (Molecular Probes) in accordance with the manufacturer's instructions. Fluorescence was measured in a Cary Eclipse Fluorescence Spectrophotometer multi-well plate reader set for excitation at 485 nm ± 10 nm and emission detection at 530 nm ± 10 nm. The concentrations of elastase in unknown samples were interpolated from the standard curves.



**Figure 2:** Western blot showing samples of isolates of the isoforms of protease IV. Culture supernatants in denaturing conditions were analyzed by WESTERN blotting. A rabbit polyclonal to sppA Protease IV antibody (Abcam, catalogue no. 65958) was used as the primary antibody.

In another study Das et al. report that production of pyocyanin in *P. aeruginosa* PA01 and PA14 batch cultures are responsible for promotion of extracellular DNA (eDNA) release which is responsible for biofilm formation [18].

Patients No.	Isolates	Morphology	Pyocyanin µg/mL	Protease IV#		Alkaline protease*	Patients No.	Isolates	Morphology	Pyocyanin µg/mL	Protease IV		Alkaline protease
				sppA 47 kDa	sppA 67 kDa						sppA 67 kDa		
WT	PA01	NM	1	-	++	+++	10	PA26	M	nd	-	++	+++
1	PA1	M	1	++	++	+++	11	PA28	NM	nd	++	-	+++
	PA40	NM	1	++	-	+++		PA30	M	1	-	++	+++
	PA41	NM	2	-	-	+++		PA31	NM	1	++	++	+++
2	PA 3	NM	1	++	++	+++	12	PA34	NM	1	++	++	+++
	PA 8	NM	1	++	-	+++	PA37	NM	3	++	++	+++	
	PA22	M	3	-	-	+++	13	PA2	NM	3	++	++	+++
	PA23	M	3	++	-	+++	14	PA7	M	1	++	++	+++
3	PA4	M	1	++	++	+++	15	PA16	NM	1	++	++	+++
	PA32	NM	1	+	+	+++	16	PA17	NM	1	++	++	+++
4	PA5	M	1	+	+	+++	17	PA17	NM	1	++	++	+
	PA6	NM	1	++	++	+++	18	PA21	NM	1	-	++	+++
5	PA9	M	1	++	++	+++	19	PA24	M	1	-	-	+++
	PA10	M	1	-	-	+++	20	PA25	NM	1	-	++	+++
	PA45	NM	1	-	++	+++	21	PA27	M	1	-	-	+
6	PA11	NM	1	+	+	+++	22	PA29	NM	1	-	++	+++
	PA12	M	1	++	-	+++	23	PA38	NM	1	++	-	+++
7	PA13	NM	1	++	-	+++	24	PA39	NM	1	++	-	+++
	PA14	NM	1	+	-	+	25	PA42	M	1	-	++	+++
8	PA15	NM	1	-	+	+++	26	PA44	NM	nd	++	-	+++
	PA43	M	2	-	-	+++							
9	PA19	NM	1	-	-	+++							
	PA20	M	1	-	-	+++							
	PA33	M	2	-	++	+++							
	PA35	NM	3	-	++	+++							

M. Mucoid, NM: Non-mucoid, +++, High level of enzyme activity, ++ Medium level of enzyme activity, + low levels of enzyme activity; nd, not done; BCG, Milk plate Assay \* Milk Agar plates # Western blot results. 1=<1 µg/mL, 2=between 2 and 5 µg/mL and 3= >5 µg/mL

**Table 2:** Protease IV (*SppA*) and pyocyanin production in the culture supernatants of isolates of *P. aeruginosa*.

In addition, they showed that A phzSH mutant of *P. aeruginosa* PAO1 that overproduces pyocyanin displayed enhanced hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) generation, cell lysis, and eDNA release in comparison to its wild-type strain [18]. They also showed that pyocyanin increases release of interleukin-8 (IL-8) by both normal and CF airway epithelial cell lines and by primary airway epithelial cells in addition to synergizing with the inflammatory cytokines tumour necrosis factor alpha (TNF-α) and IL-1α [19].

The production of pyocyanin is also found to depend both on the carbon source and on the specific strain of *P. aeruginosa* strains. For example, a cystic fibrosis lung isolate strain of *P. aeruginosa* has been shown to synthesizes and secrete pyocyanin when grown with glucose and glutamate, while the laboratory strain exhibits detectable production of pyocyanin only when grown with glutamate as the source of carbon [20].

Our results show that there is no relationship between the production of various virulence factors. That is to say one strain could be producing high levels of one virulence factors such as pyocyanin but low levels of elastase or alkaline proteases. Qualitative and quantitative elastase assays showed that most of the clinical isolates produced elastase. The resulting values were in the range from 0.08 mg/mL to 1.28 mg/mL with an average production level of 0.227 mg/mL. There are several reasons why the levels of elastase activities are low or perhaps not detected in some of the strains. In this study we have only measured the level of elastase which was secreted in the culture medium and the activity of any cell-associated elastase (vital for cell viability) has not been measured. Secondly, the isolates may produce, or secrete, elastase at suboptimal levels, possibly due to modifications in one of the elastase regulatory or secretion pathways. Thirdly, alternative isoforms of elastase may be produced which may not be active on the Elastin-Congo red substrate and finally the location of infection within the host is also shown to be a factor on the level of elastase and other virulence factors. In addition, RT-PCR was used to profile the level of elastase expression at transcription level. The results show a large variation amongst strains, with some producing a high level of elastase (Figure 2). Production of alkaline protease on the other hand, although varied in different isolates, were overall much greater.

The CF lung is a very complex ecosystem and diagnosing infections based on single isolate virulence factors alone cannot provide all the answers to the clinical presentation. There is evidence in the literature that there is loss of acute virulence in order for *P. aeruginosa* to adapt to a chronic environment. Further studies are required to explore adaptations and virulence markers in the acute and chronic CF biofilm setting [21].

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