**Virulence factor expression patterns in *Pseudomonas aeruginosa* strains from infants with cystic fibrosis**

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Running Title: Phenotype of *P. aeruginosa* from infants with cystic fibrosis

1    **ABSTRACT**

2    *Pseudomonas aeruginosa* is the leading cause of morbidity and mortality in cystic fibrosis (CF).

3    This study examines the role of organism-specific factors in the pathogenesis of very early *P.*

4    *aeruginosa* infection in the CF airway. One-hundred-and-sixty-eight longitudinally collected *P.*

5    *aeruginosa* isolates from children diagnosed with CF following newborn screening were genotyped

6    by pulsed-field gel electrophoresis and phenotyped for 13 virulence factors. Associations between

7    virulence factors and gender, exacerbation, persistence, timing of infection and infection site were

8    assessed using multivariate regression analysis. Ninety-two strains were identified. Persistent

9    strains showed significantly lower levels for pyoverdine, rhamnolipid, haemolysin, total protease,

10   and swimming and twitching motility, than strains eradicated by aggressive antibiotic treatments.

11   Initial strains had higher levels of virulence factors, and significantly higher phospholipase C, than

12   subsequent genotypically-different strains at their initial isolation. Strains from males had

13   significantly lower pyoverdine and swimming motility than females. Colony size was significantly

14   smaller in strains isolated during exacerbation than those isolated during non-exacerbation periods.

15   All virulence factors were higher and swimming motility significantly higher, in strains from

16   bronchoalveolar-lavage (BAL) and oropharyngeal sites than BAL-alone. Using unadjusted

17   regression modelling, age at initial infection and age at strain isolation showed U-shaped profiles

18   for most virulence factors. Among subsequent strains, the longer the time since initial infection, the

19   lower the level of most virulence factors. This study provides new insight into virulence factors

20   underpinning impaired airway clearance seen in CF infants despite aggressive antibiotic therapy.

21   This information will be important in the development of new strategies to reduce the impact of *P.*

22   *aeruginosa* in CF.

23

1 **INTRODUCTION**

2 *Pseudomonas aeruginosa* airway infection of young children with cystic fibrosis (CF) is typically

3 intermittent, involving multiple non-mucoid strains that are sensitive to anti-pseudomonal

4 antibiotics. However, one strain eventually becomes established and by adolescence almost 80% of

5 CF patients are chronically-infected, resulting in increased morbidity and mortality [1].

6

7 Many host factors affect the course of infection in the CF lung [2], but there is limited

8 understanding about organism-specific traits affecting infectivity, severity of infection and

9 persistence. The environment is the main source for acquiring *P. aeruginosa*, suggesting that

10 environmental isolates have all the virulence determinants to initiate infection and cause disease [3].

11 *P. aeruginosa* adapts to long-term survival in the CF lung by overproducing alginate, forming

12 biofilms and down-regulating virulence factors [4-5]. Tracking of adaptive mutations in isolates

13 from chronically-infected adults showed defects in swimming and twitching motility, pyocyanin

14 secretion and biofilm formation [6]. However, *P. aeruginosa* strains infecting infants and young

15 children have not been systematically examined to establish the characteristics underpinning

16 pathogenesis of early infection in the CF lung.

17

18 Here we present the virulence factor phenotype of 168 *P. aeruginosa* isolates collected

19 longitudinally over the first five-years of life from infants diagnosed with CF after newborn

20 screening and classified by genotyping as 92 distinct strains. The Type 3 Secretion System profiles

21 of this cohort were published previously [7]. Relationships were sought between virulence factor

22 expression and clinical factors.

1 **MATERIALS AND METHODS**

2 **Clinical isolates**

3 Investigations were conducted on 168 *P. aeruginosa* isolates from 58 children aged 3-63 (mean

4 26.8) months collected within the Australasian Cystic Fibrosis Bronchoalveolar Lavage (ACFBAL)

5 randomised controlled trial [8]. Isolates came from 150 specimens: 57 bronchoalveolar lavage

6 (BAL) fluid (35 children); 82 oropharyngeal (OP) swabs (44 children) and 11 sputa (9 children

7 post-ACFBAL study). On average, 2.5 (range 1-18) isolates were tested per child. Ethics

8 Committees of all participating hospitals approved the study.

9

10 The ACFBAL study randomised infants diagnosed with CF after newborn screening to either BAL-

11 directed therapy or standard care using clinical judgment and OP cultures [8]. BAL-directed infants

12 underwent BAL at enrolment before six-months of age, and then with exacerbations requiring

13 hospitalisation, if OP cultures grew *P. aeruginosa*, and after *P. aeruginosa* eradication therapy. All

14 subjects had OP cultures taken <u>during</u> exacerbations and after eradication therapy, while each

15 underwent BAL at age five. Children with positive *P. aeruginosa* BAL ($\geq 10^3$ colony-forming

16 units/ml) or OP (standard group only) cultures received two-weeks of intravenous tobramycin with

17 either ticarcillin-clavulanate, or ceftazidime, followed by four-weeks of oral ciprofloxacin and eight

18 weeks of nebulised tobramycin inhalation solution [8]. After treatment, BAL-directed children had

19 further BAL and OP cultures, while those receiving standard care had OP cultures alone. If *P.*

20 *aeruginosa* persisted, the eradication protocol was repeated, but if this also failed the child was

21 categorised as chronically-infected. Samples were processed by hospital laboratories where *P.*

22 *aeruginosa* identification was undertaken using standard techniques and interpretative criteria [9].

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1  **Genotypic testing**

2  Isolates were genotyped using pulsed-field gel electrophoresis (PFGE) following *Spe*I digestion

3  [10]. Analyses were performed using cluster analysis software (GelComparII[TM], Applied Maths,

4  Belgium) and the criteria of Tenover et al [11].

5  **Phenotypic tests**

6  i.      **Pyocyanin** Overnight *P. aeruginosa* grown in Cation-Adjusted Mueller-Hinton Broth

7  (CAMHB) (Oxoid Ltd, Australia) were scored (0-4) for intensity of green pigment: 0 = no pigment,

8  1 = light green, 2 = moderate green, 3 = dark green, 4 = very dark green [12].

9  ii.     **Pyoverdine** 5μl of overnight CAMHB cultures were spotted onto Kings B agar (Oxoid),

10  dried and incubated at 37°C. Plates were viewed under a transilluminator after 24h and scored on

11  the intensity of the fluorescent yellow pigment produced by pyoverdine using a graded system: 0=

12  no fluorescence, 1 = lightly fluorescent yellow, 2 = moderately fluorescent yellow, 3 = strongly

13  fluorescent yellow, 4 = very strongly fluorescent yellow [13].

14  iii.    **Haemolysin** 5μl of overnight CAMHB culture was spotted onto Columbia Horse Blood

15  Agar (Oxoid), dried and incubated for 20h at 37°C. Two perpendicular diameters of clearance zones

16  around colonies were measured and the area ($\pi r^2$) calculated ($mm^2$). This method was utilised in iv

17  to viii below.

18  iv.     **Total Protease** 5μl of overnight CAMHB culture were spotted onto a 15%v/v skim milk

19  agar plate dried and incubated for 24h at 37°C.

20  v.      **Elastase** 5μl of overnight CAMHB culture were spotted onto 10ml elastin agar plates, dried

21  and incubated for 48h at 37°C.

22  vi.     **Phospholipase C** 5μl of overnight CAMHB culture were spotted onto egg yolk agar plates,

23  dried and incubated for 20h at 37°C [14-15].

24  vii.    **Rhamnolipid** 5μl of overnight CAMHB culture were spotted onto M9-based agar plates,

25  supplemented with 0.2% (v/v) glucose, 2 mM $MgSO_4$, 0.05% (v/v) tyrosine, 0.0005% (v/v)

1  methylene blue, 0.02% (v/v) and cetyltrimethylammonium bromide dried and incubated for 48h at

2  37°C. Colonies were scored as positive by the presence of a violet halo around the colony.

3  viii.  **Twitching and swimming motilities** Bacterial patches from 1.5%w/v Luria plates were

4  scraped with a toothpick and stab inoculated into 10ml (1%w/v Luria-broth) agar. Twitching

5  motility (light haze of growth at the agar/plate interface) was measured after 24h at 37°C. For

6  swimming, toothpick scrapings were spotted onto centre of a swim (0.3%w/v Luria-broth) plate and

7  swim diameters measured after overnight at 30°C.

8  ix.  **Swarming motility** Pre-warmed swarm plates (0.8%w/v nutrient broth, 0.5%w/v agar and

9  0.5%w/v glucose) were inoculated with a colony tooth-picked from a swim plate (above). After 24h

10  at 37°C, swarming motility was identified as spreading growth from the inoculation site and graded

11  0-4 where; 0 = no motility, 1= very limited motility. 2 = fair motility, 3 = strongly motile, 4 = very

12  strongly motile.

13  x.  **Biofilm mass** $1^{07}$ cells were inoculated into wells of a microtitre plate containing 100μl

14  CAMHB and plates incubated for 24h at 37°C. Biofilm mass was stained with 1%v/v crystal violet,

15  ethanol-extracted and absorbance read at $O_{D595}$. Readings were then normalised as a percentage of

16  *P. aeruginosa* PAO1 biofilm grown concurrently.

17  xii.  **Colony size** 100μl aliquots of a $10^{-7}$ dilution of overnight *P. aeruginosa* culture were spread

18  onto CAMHB plates and incubated at 37°C. Colony diameter was measured in mm after 24h.

19  xiii.  **Mucoidy** Cells were scraped from 1.5%w/v Luria-broth patch plate, then patched onto

20  5%v/v glycerol MacConkey plates (Oxoid) and scored (+/-) for mucoidy after 48h at 37°C.

21

22  **Statistical analysis**

23  Patients were followed longitudinally. Each isolate was tested (×3) for each outcome of interest.

24  Nine continuous and three categorical measures of virulence (measured using semi-quantitative

25  scores from 0-to-4) were analysed. These scores were categorised into binary variables to assess the

likelihood of having a score of 3 or 4 versus any score below 3. As only four isolates were mucoid, statistical analysis was not conducted on the fourth categorical measure (mucoidy).

The associations between a set of independent factors and each of the virulence measures were assessed using multivariate regression analysis. The independent factors were gender, exacerbation at sample collection time, initial vs subsequent strain infection, persistent strain (defined as one detected continuously or intermittently for at least three-months) and site of sample isolation (BAL only, BAL+OP, OP-only).

We further investigated whether: i) the child's age at acquisition of first infection, ii) the age of the child at the collection of a strain and iii) time since acquisition of the first strain, had any effect on the outcomes of interest. The associations between these three time-related variables and each of the virulence measures were estimated separately using unadjusted regression analysis.

Population-averaged panel-data regression models and generalized estimation equations (GEE) were used to assess the associations of interest. Linear or logistic regression was used depending on the measure of the outcome of interest. Regression analyses were executed using STATA 12.0 (StataCorp, College Station, TX, USA).

1 **RESULTS**

2 The 58 children fell into three categories. Twenty-eight had only one strain detected on a single

3 occasion; 10 had one strain detected on multiple occasions and the remaining 20 had multiple

4 strains detected on multiple occasions.

5 **Genotype**

6 Ninety-two strains were identified by PFGE. Of these, 90 strains had unique pulsotypes, while the

7 remaining two strains had macrorestriction patterns indistinguishable from Aust-01 and Aust-02,

8 which are highly-prevalent strains found frequently in older, chronically infected patients attending

9 Australian CF clinics [16]. Only one patient had Aust-01, and another two had the Aust-02 strain

10 identified. Since transmissibility and other properties of these clonal strains may have biased

11 results, all analyses were repeated without them and results remained comparable (data not shown).

12

13 Of the 92 strains, 52 (56.5%) were initial infecting strains detected in children at an average age of

14 26.8 (range 3-63, standard deviation 14.6) months. Seventy-three strains (79.3%) came from an

15 exacerbation, 22 strains (23.9%) were persistent (14 were persistent from the initial infection and

16 eight from a subsequent infection), 59 (64.1%) cleared, while the persistence of 11 (12.0%) strains

17 was unknown as genotyping was limited to the available sample set. Thirty-five strains were from

18 the upper airways (OP) only, 31 from the lower airways collected by BAL and 18 were found in

19 both sites (eight strains from follow-up sputa were excluded from analyses for site, as the quality of

20 these specimens was unknown and they may have contained both upper and lower airway isolates).

21 **Phenotype**

22 Pyocyanin was scored at 3 or 4 in 20.5% of the tests, pyoverdine was scored at 3 or 4 in 31.8% of

23 the tests and swarming was scored at 3 or 4 in 12.6% of the tests. The distributions of all continuous

24 measures of interest are presented in Table 1.

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1 *Persistent strains versus cleared strains*

2 Multivariate analysis (Table 2) showed that the levels of six virulence factors: pyoverdine,

3 rhamnolipid, haemolysin, total protease and swimming and twitching motilities were significantly

4 lower in persistent strains compared to cleared strains, while another six factors; pyocyanin,

5 elastase, phospholipase C, biofilm mass, colony size and swarming motility showed non-significant

6 reductions. No factor was significantly higher in persistent strains compared to cleared strains.

7

8 *Effects of gender, timing of isolation, site of infection and exacerbation on phenotype*

9 With respect to gender, pyoverdine expression and swimming motility were significantly lower in

10 strains from males compared to females (Table 2). In comparisons of initial and subsequent

11 (genotypically-different) strains at their first isolation, initial strains had higher expression of all

12 virulence factors, except pyocyanin, and this reached statistical significance for phospholipase C.

13 All virulence factors showed an increase in strains from both OP and BAL compared to BAL-only,

14 while swimming motility was significantly higher. Only smaller colony size was associated with

15 exacerbation.

16

17 *Influence of age of child and time of isolation on phenotype*

18 Table 3 shows changes in virulence factor expression and Figure 1 shows the changes for the eight

19 clearance-zone based measurements, with respect to: age at initial strain isolation, age at isolation of

20 a strain and time since initial strain isolation.

21

22 *Age at initial strain isolation*: Phospholipase C, haemolysin and total protease levels were

23 significantly lower in initial strains acquired at age 12-23 months compared with ≤12-months, but

24 were not different at age >36-months compared with ≤12-months (Table 3, part 1). Pyoverdine and

25 swarming motility were lower in initial strains acquired at 24-35 months compared with ≤12-

26 months, but were the same at ≥36 months as at ≤12 months. Biofilm mass increased with age of

initial strain isolation, becoming significant at 24-35 months. Of the eight virulence factors measured by area of clearance zone, all except biofilm mass produced a non-linear, non-monotonic U-shaped curve (Figure 1A).

*Age at isolation of a strain*: Expression of pyoverdine and haemolysin, and colony size were significantly lower in strains from children aged 24-35-months compared to children aged ≤12months. Total protease expression was significantly lower in both 12-23 and 24-35-month-old children compared with children aged ≤12months (Table 3, part 2). Of the eight virulence factors measured by area of clearance zone (Figure 1B) all except swimming and twitching motility gave a U-shaped effect.

*Time since isolation of initial strain*; The longer the time, the lower the level of most virulence factors (Table 3, part 3). Significant decreases occurred for biofilm mass and swimming motility by age 11-months, for twitching motility and colony size by 23-months and for elastase, phospholipase C, haemolysin and total protease by >36-months. Figure 1C, shows an initial fall, followed by a stable period, with a sharp decline at >36-months, for all factors except rhamnolipid. The presence of subsequent infections does not explain the sharp decline in virulence observed in the ≥36 month's group. Subsequent strains were isolated from patients between 2 and 97 months after initial strains (mean=22.8 months, median=15 months), meaning that most subsequent infections occurred before 36 months.

**DISCUSSION**

*P. aeruginosa* virulence factors are usually associated with acute infection within the host. The complex relationships between virulence factor expression and pathogenesis of airway infection in CF have been investigated mainly in chronically infected adults [17-19]. Our study is the first to comprehensively examine *P. aeruginosa* virulence factor expression profiles in CF infants and young children.

*Virulence versus persistence, initial/subsequent strain, gender, exacerbation and site of infection*

This study provides the first evidence that virulence factor expression profiles of *P aeruginosa* strains at their first isolation in the CF airway of young children may help predict the ability of the strain to persist in the face of the immune response and antibiotic therapy. Expressions of pyoverdine, rhamnolipid, haemolysin, total protease and swimming and twitching motilities were all significantly lower in persister than clearer strains and this finding was independent of potential confounders such as site of isolation (upper or lower airway) and whether the airway was *P. aeruginosa* naive or potentially damaged by a previous *P. aeruginosa* strain. Since these persister strains were not clonally related to each other or to frequent clones circulating in Australian clinics they are assumed to have been environmentally acquired. *P. aeruginosa* is known to down-regulate these virulence factors as a survival mechanism in the CF lung [17, 20]. However it seems unlikely that such adaptations begin so early in the infection process. Perhaps instead some environmental strains first colonise the sinuses where adaptation to the local microenvironment takes place before aspiration into the lower airways occurs. Studies show pyoverdine-negative mutants increase with lung colonisation time [21], which would indicate that persisters do not have a greater requirement for pyoverdine. Rhamnolipid is a biosurfactant involved in protecting cells against oxidative stress [22] and in increased swarming [23]. It is also cytotoxic to eukaryotic cells, decreases liquid surface tension and facilitates access to nutrients within biofilms [24-25]. Since these functions are important in long-term bacterial survival, the down regulation of rhamnolipid is noteworthy. Decreased haemolysin expression has been correlated with increased mucoidy [26], however all

1. persistent strains in our study were non-mucoid. Some bacterial proteases promote mucoidy [27], so

2. strains may begin to display mucoidy as persistence continues.

3.

4. The overall virulence profiles of initial strains were higher than those of subsequent strains at their

5. first isolation. In particular, phospholipase C was significantly higher in initial strains.

6. Phospholipase C is a heat-labile lecithin-degrading haemolysin. The phospholipase C/sphingo-

7. myelinase gene-pair acts as an inhibitor of lung pulmonary surfactant [28], thus phospholipase C

8. may be required to establish the initial, but not subsequent infection.

9.

10. Associations with gender proved interesting. Previous reports had indicated high virulence factor

11. expression during early CF lung disease played a role in the worse prognosis of females [29],

12. however there was no evidence of this in our analysis. The significance of the lower pyoverdine

13. expression and lower swimming motility in strains from males is unknown. There was a lack of

14. association between virulence factor expression and exacerbation other than for colony size, and

15. this was at odds with our early study of CF where higher extracellular enzyme levels were found in

16. hospitalised than non-hospitalised CF adolescent patients with chronic *P. aeruginosa* infection [30].

17. We found no significant associations between expression and site of infection, except for swimming

18. motility being greater in strains from both BAL and OP than those from BAL-alone. These

19. conflicting results could reflect differences in age, disease stage and the small number of non-

20. exacerbation samples.

21.

22. Flagella and pili-driven twitching motility are required for biofilm formation [31]. Alternatively,

23. swarming motility is inversely proportional to biofilm formation [32]. Our finding that swarming

24. motility and biofilm mass were not significantly different, while twitching and swimming motility

25. were significantly reduced in persisters highlights differences between early persisters in young

26. children and chronic infection in CF adults where biofilm formation is a distinctive feature [33].

*Influence of age of child and time of isolation on phenotype*

Particularly noteworthy were associations between age and virulence factor expression (Table 3 and Figures 1A 1B 1C). The initial reduction in virulence factor production with increasing age of initial infection (Figure 1A) could be an adaptation to the host immune response. However, the increase in almost all factors in initial strains in the older age groups was unexpected. This was particularly marked for haemolysin, which appears independent of age of acquiring the initial strain. A U-shaped curve was also evident in the analyses of virulence by age at isolation of a strain (Figure 1B), with strains from children >36-months showing a modest rise after an initial fall for all factors except swimming and twitching motilities, and haemolysin again showing a significant fall at 24-35 months followed by a rise at ≥36 months. While we are unsure of the reasons for this U-shaped effect, the fall in virulence factor expression seen in new *P. aeruginosa* isolates from older children, could offer a partial explanation. As the sinuses develop and grow in CF children, they may act as a reservoir for initial and newly acquired *P. aeruginosa* strains, where adaptation to the lung environment may already begin prior to them infecting lower airways [34-35].

In contrast, results of the analyses examining time since isolation of initial strain (Figure 1C), which showed significant decreased expression for eight of the twelve virulence factors by the ≥36 month time period. The significant decreases start from the earliest time point (1-11 months) in the case of biofilm mass and swimming. These results are comparable to those found by a study of *P. aeruginosa* isolates from CF children of undisclosed ages [36], except that this trend was evident at 24 months compared with over >36-months in our study. Interestingly, four virulence factors, (pyocyanin, pyoverdine, swarming and rhamnolipid) remained virtually unchanged over the entire period.

This study provides important new information on factors influencing virulence factor expression of *P aeruginosa* infecting the CF airway in the first years of life. While it is generally accepted that *P.*

*aeruginosa* loses its virulence in the transition to chronic infection in CF, our results which show a

a diverse range of up and downregulation as well as maintenance of expression amongst the twelve

virulence factors over time, challenge this view. It seems likely that there is considerable diversity

within microbial population evolution, with some strains retaining or enhancing virulence and

others losing virulence over time. Our data suggest that this process begins within 36 or so months

after initial infection.


Our findings may help in understanding which virulence factors are important in establishing

infection in CF infants where, despite aggressive antibiotic therapy, impaired airway clearance

mechanisms exist. This is turn will be important in developing new more effective strategies to

reduce the impact of *P. aeruginosa* on morbidity and mortality in CF.

1 **ACKNOWLEDGEMENTS**

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1 **CONFLICT OF INTEREST**

2 All authors declare that they have no conflict of interest.

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1 **TABLES AND FIGURES**

2 **Table 1: Statistical distributions of all continuous measures of interest**

3

| Variable | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|
| Elastase (mm$^2$) | 82.8 | 40.5 | 0 | 144.0 |
| Rhamnolipid (mm$^2$) | 60.7 | 28.0 | 0 | 125.3 |
| Haemolysin (mm$^2$) | 84.8 | 63.2 | 0 | 233.1 |
| Total Protease (mm$^2$) | 87.1 | 30.2 | 0 | 134.4 |
| Phospholipase C (mm$^2$) | 90.4 | 39.0 | 0 | 155.0 |
| Biofilm mass (% of PAO1 biomass) | 88.6 | 49.0 | 11.3 | 358.3 |
| Swimming (mm$^2$) | 69.6 | 35.5 | 0 | 198.8 |
| Twitching (mm$^2$) | 60.6 | 43.2 | 0 | 136.5 |
| Colony Size (diameter in mm) | 2.6 | 1.3 | 0.5 | 6.0 |

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**Table 2: Multivariate analysis of associations between a set of independent variables and individual virulence factors**

| Independent variables | Pyoverdine OR[a] (95% CI) | Pyocyanin OR[a] (95% CI) | Swarming OR[a] (95% CI) | Elastase AEC[b] (95% CI) |
|---|---|---|---|---|
| **Gender:** Male vs Female | **0.48 (0.23-0.99)*** | 0.87 (0.36-2.11) | 0.50 (0.18-1.41) | -5.55 (-19.6-8.85) |
| **Exacerbation**: Yes vs No | 0.53 (0.20-1.42) | 1.48 (0.42-5.13) | 0.65 (0.21-2.02) | -8.9 (-26.58-8.79) |
| **Strain**: Initial vs Subsequent | 1.64 (0.55-4.89) | 0.55 (0.16-1.88) | 1.62 (0.28-9.34) | 17.84 (-3.84-39.52) |
| **Strain**: Persister vs clearer | **0.21 (0.08-0.56)**** | 0.77 (0.27-2.17) | 0.50 (0.16-1.55) | -11.03 (-28.40-6.34) |
| **Sample**: OP vs BAL | 1.34 (0.43-4.16) | 1.53 (0.39-5.99) | 0.40 (0.07-2.20) | 3.7 (-17.88-25.27) |
| **Sample:** (BAL+OP) vs BAL | 3.28 (0.83-12.88) | 3.27 (0.05-12.9) | 1.48 (0.31-7.05) | 8.0 (-16.89-32.90) |

| Independent variables | Total Protease AEC[b] (95% CI) | Rhamnolipid AEC[b] (95% CI) | PLC[c] AEC[b] (95% CI) | Haemolysin AEC[b] (95% CI) |
|---|---|---|---|---|
| **Gender**: Male vs Female | 2.75 (-7.23-12.7) | -6.99 (-16.40-2.42) | 7.0 (-6.12-20.12) | -2.32 (-23.2-18.56) |
| **Exacerbation**: Yes vs No | -3.49 (-16.1-9.11) | -1.6 (-13.48-10.28) | -8.05 (-24.61-8.51) | -12.13 (-38.48-14.22) |
| **Strain**: Initial vs Subsequent | 11.37 (-4.08-26.82) | 9.25 (-5.31-23.83) | **26.08 (5.78-46.39)*** | 15.99 (-16.31-48.3) |
| **Strain**: Persister vs clearer | **-12.92 (-25.30--0.54)*** | **-12.32 (-23.99--0.64)*** | -9.72 (-25.99-6.55) | **-32.93 (-58.82--7.04)*** |
| **Sample**: OP vs BAL | 3.03 (-12.34-18.40) | -4.87 (-19.37-9.63) | -7.8 (28.1-12.40) | -10.1 (-42.24-22.06) |
| **Sample:** (BAL+OP) vs BAL | 10.63 (-7.10-28.37) | 8.42 (-8.31-25.15) | 2.79 (-20.52-26.10) | 19.54 (-17.55-56.63) |

| Independent variables | Swimming AEC[b] (95% CI) | Twitching AEC[b] (95% CI) | Biofilm Mass AEC[b] (95% CI) | Colony Size AEC[b] (95% CI) |
|---|---|---|---|---|
| **Gender**: Male vs Female | **-13.95 (-25.55--2.36)*** | -2.71 (-17.14-11.71) | 1.19 (-16.34-18.72) | 0.23 (-0.04-0.5) |
| **Exacerbation**: Yes vs No | 0.3 (-14.33-14.94) | 2.98 (-15.22-21.19) | -14.32 (-37.86-9.21) | **-0.4 (-0.74--0.07)*** |
| **Strain**: Initial vs Subsequent | 16.90 (-1.04-34.84) | 21.61 (-0.7-43.9) | 14.76 (-11.7-41.22) | 0.11 (-0.3-0.53) |

**Table 3: Change in virulence factor expression over time**

**Part 1: Age at initial strain isolation**

| Virulence factor | Units | <12mths | 12-23mths | pvalue[a] | 24-35mths | pvalue[a] | 36+mths | pvalue[a] |
|---|---|---|---|---|---|---|---|---|
| Pyocyanin | % | 23.08 | 26.79 | 0.720 | 12.12 | 0.271 | 4.35 | 0.069 |
| Pyoverdine | % | 42.31 | 28.57 | 0.207 | 20.20 | 0.057† | 46.38 | 0.767 |
| Swarming | % | 12.8 | 14.3 | 0.850 | 2.0 | **0.027*** | 17.4 | 0.643 |
| Elastase | mm² | 85.27 | 72.63 | 0.158 | 89.45 | 0.672 | 102.44 | 0.112 |
| Rhamnolipid | mm² | 65.23 | 59.85 | 0.412 | 59.22 | 0.407 | 68.50 | 0.679 |
| PLC[b] | mm² | 97.26 | 79.52 | **0.044*** | 92.92 | 0.656 | 114.04 | 0.115 |
| Haemolysin | mm² | 103.78 | 71.50 | **0.017*** | 69.53 | **0.008**** | 132.45 | 0.080 |
| Total Protease | mm² | 94.52 | 80.15 | **0.026*** | 85.66 | 0.213 | 102.28 | 0.317 |
| Biofilm Mass | %[c] | 73.47 | 83.05 | 0.402 | 110.14 | **0.004**** | 90.11 | 0.265 |
| Swimming | mm² | 70.40 | 72.12 | 0.836 | 66.33 | 0.658 | 74.20 | 0.705 |
| Twitching | mm² | 67.82 | 53.53 | 0.167 | 63.76 | 0.723 | 66.69 | 0.928 |
| Colony Size | mm | 2.5 | 2.83 | 0.077 | 2.49 | 0.941 | 2.74 | 0.288 |

**Part 2: Age at isolation of a strain**

| Virulence factor | Units | <12mths | 12-23mths | pvalue[a] | 24-35mths | pvalue[a] | 36+mths | pvalue[a] |
|---|---|---|---|---|---|---|---|---|
| Pyocyanin | % | 28.57 | 33.33 | 0.752 | 17.39 | 0.372 | 14.75 | 0.238 |
| Pyoverdine | % | 54.76 | 36.67 | 0.245 | 18.12 | **0.008**** | 34.43 | 0.152 |
| Swarming | % | 14.3 | 13.3 | 0.932 | 8.0 | 0.477 | 15.3 | 0.924 |
| Elastase | mm² | 94.33 | 75.31 | 0.147 | 80.82 | 0.275 | 85.28 | 0.451 |
| Rhamnolipid | mm² | 63.86 | 58.86 | 0.574 | 55.33 | 0.309 | 64.84 | 0.904 |
| PLC[b] | mm² | 101.29 | 85.21 | 0.200 | 85.01 | 0.169[b] | 94.43 | 0.550 |
| Haemolysin | mm² | 115.72 | 87.01 | 0.137 | 56.49 | **0.001**** | 98.03 | 0.317 |
| Total Protease | mm² | 100.41 | 81.45 | **0.047*** | 81.51 | **0.036*** | 91.07 | 0.285 |
| Biofilm Mass | %[c] | 85.28 | 83.14 | 0.894 | 88.46 | 0.832 | 92.29 | 0.634 |
| Swimming | mm² | 74.37 | 72.51 | 0.871 | 70.12 | 0.696 | 66.72 | 0.469 |
| Twitching | mm² | 81.43 | 64.42 | 0.221 | 60.70 | 0.114 | 53.09 | **0.031*** |
| Colony Size | mm | 3.07 | 2.95 | 0.626 | 2.54 | **0.025*** | 2.44 | **0.006**** |

**Part 3: Time since isolation of initial strain**

| Virulence factor | Units | 0mths | 1-11mths | pvalue[a] | 12-23mths | pvalue[a] | 24-35mths | pvalue[a] | +36mths | pvalue[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| Pyocyanin | % | 18.03 | 20.00 | 0.822 | 19.05 | 0.918 | 0.00 | 0.061 | 33.33 | 0.202 |
| Pyoverdine | % | 40.98 | 26.67 | 0.180 | 25.40 | 0.171 | 18.18 | 0.166 | 26.67 | 0.309 |
| Swarming | % | 13.7 | 11.1 | 0.725 | 4.8 | 0.292 | 18.2 | 0.695 | 8.9 | 0.552 |
| Elastase | $mm^2$ | 89.88 | 79.36 | 0.203 | 89.20 | 0.942 | 100.84 | 0.367 | 49.75 | **0.000*** |
| Rhamnolipid | $mm^2$ | 62.07 | 59.60 | 0.690 | 60.23 | 0.794 | 66.77 | 0.607 | 66.93 | 0.545 |
| PLC[b] | $mm^2$ | 97.72 | 88.68 | 0.287 | 95.62 | 0.827 | 96.20 | 0.903 | 65.56 | **0.003** |
| Haemolysin | $mm^2$ | 96.88 | 76.96 | 0.147 | 83.34 | 0.385 | 90.92 | 0.767 | 59.88 | **0.037*** |
| Total Protease | $mm^2$ | 91.50 | 84.34 | 0.249 | 88.99 | 0.722 | 94.35 | 0.755 | 73.82 | **0.028*** |
| Biofilm Mass | $\%^c$ | 95.07 | 70.61 | **0.033*** | 92.71 | 0.850 | 100.82 | 0.721 | 85.02 | 0.480 |
| Swimming | $mm^2$ | 78.01 | 61.71 | **0.034*** | 68.68 | 0.284 | 75.34 | 0.812 | 58.90 | *0.054†* |
| Twitching | $mm^2$ | 70.96 | 62.13 | 0.354 | 45.34 | **0.018*** | 55.89 | 0.282 | 42.67 | *0.054†* |
| Colony Size | mm | 2.86 | 2.83 | 0.874 | 2.43 | **0.027*** | 2.64 | 0.375 | 1.93 | **0.000*** |

1   [a] Wald statistic; p values obtained from unadjusted regression analysis [b] Phospholipase C [c] % of *P. aeruginosa* PAO1 biofilm grown concurrently

2   * $p<0.05$; ** $p<0.01$; ***$p<0.001$; †borderline $p<0.05$