

## **Cough-generated Aerosols of *Pseudomonas aeruginosa* and other Gram Negative Bacteria from Cystic Fibrosis Patients.**

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**Abstract** (Word Count 250)

**Background:** *Pseudomonas aeruginosa* is the most common bacterial pathogen in cystic fibrosis (CF) patients. Current infection control guidelines aim to prevent transmission via contact and respiratory droplet routes and do not consider the possibility of airborne transmission. We hypothesized that with coughing, CF subjects produce viable, respirable bacterial aerosols.

**Methods:** Cross-sectional study of 15 children and 13 adults with CF, 26 chronically infected with *P. aeruginosa*. A cough aerosol sampling system enabled fractioning of respiratory particles of different size, and culture of viable Gram negative non-fermentative bacteria. We collected cough aerosols during 5 minutes voluntary coughing and during a sputum induction procedure when tolerated. Standardized quantitative culture and genotyping techniques were used.

**Results:** *P. aeruginosa* was isolated in cough aerosols of 25 (89%) subjects of whom 22 produced sputum samples. *P. aeruginosa* from sputum and paired cough aerosols were indistinguishable by molecular typing. In 4 cases the same genotype was isolated from ambient room air. Approximately 70% of viable aerosols collected during voluntary coughing were of particles  $\leq 3.3$  microns aerodynamic diameter. *P. aeruginosa*, *Burkholderia cenocepacia*, *Stenotrophomonas maltophilia* and *Achromobacter xylooxidans* were cultivated from respiratory particles in this size range. Positive room air samples were associated with high total counts in cough aerosols ( $P=0.003$ ). The magnitude of cough aerosols were associated with higher FEV<sub>1</sub> ( $r=0.45$ ,  $P=0.02$ ) and higher quantitative sputum culture results ( $r=0.58$ ,  $P=0.008$ ).

**Conclusion:** During coughing, CF patients produce viable aerosols of *P. aeruginosa* and other Gram negative bacteria of respirable size range, suggesting the potential for airborne transmission.

**Key Words:** cystic fibrosis; cough aerosol; airborne transmission; *Pseudomonas aeruginosa*

## Introduction

*Pseudomonas aeruginosa* is the most common bacterial pathogen in patients with cystic fibrosis (CF).[1] The prevalence of chronic *P. aeruginosa* increases with age, and is a major predictor of mortality and morbidity.[2] It is unclear to what extent cross-infection of *P. aeruginosa* between patients with CF occurs.[3, 4] Whilst siblings with CF can harbor the same *P. aeruginosa* strain, it was thought until recently that most patients had their own individual strain acquired from the environment.[5] With the advent of molecular typing methods there is now convincing evidence of clonal *P. aeruginosa* infection in patients attending some pediatric and adult CF centers.[6, 7] *P. aeruginosa* has been cultured from soap holders held at up to 40 cm from the mouth of coughing patients with CF, supporting large respiratory droplet spread.[8] The exact mechanisms involved in the spread of bacteria in CF clinics remain unclear.[6, 7] Two studies have isolated clonal *P. aeruginosa* during environmental air sampling up to ten metres from CF patients infected with clonal strains whilst performing physiotherapy and lung function testing suggesting the potential of person-to-person spread via the airborne route.[9, 10]

Current guidelines for infection control for patients with CF recommend only contact and droplet precautions, i.e., focusing on hand hygiene and avoiding close contact between patients with CF, who are advised to maintain at least one meter distance from other patients.[11, 12] It is possible that airborne transmission of *P. aeruginosa*, *Burkholderia cepacia* complex and other bacteria may occur in addition to other modes of transmission.[13] The relative contribution of the airborne route may be opportunistic in nature and occur in certain circumstances such as in enclosed spaces with favorable ambient temperature and humidity as may occur in hospital, clinic and congregate settings.

Particle size distribution of aerosols is a key determinant for both deposition in the respiratory tract and for the ability of particles to remain airborne. To our knowledge, the particle size distribution of aerosols from patients with CF has never been reported. We hypothesized that during voluntary coughing and during sputum induction, subjects with CF produce viable bacterial aerosols that are respirable. To test this hypothesis, we modified a cough aerosol sampling system recently developed to measure cough-generated aerosols from patients with *Mycobacterium tuberculosis*. [14]

Our primary aim was to determine the concentration and particle size distribution of cough aerosols containing culturable *P. aeruginosa* and other Gram-negative bacteria from children and adults with cystic fibrosis. We also sought to determine whether concentrations of cough aerosols detected were related to clinical parameters and clonality of *P. aeruginosa* strains.

## Methods

**Subjects:** Subjects with CF were recruited from both the inpatient and outpatient services at the Royal Children's Hospital and The Prince Charles Hospital in Brisbane, Australia. Inclusion criteria were age older than 9 years, a confirmed diagnosis of CF and culture of *Pseudomonas aeruginosa* or *Burkholderia cepacia* complex (*Bcc*) from sputum on at least one occasion within the previous 12 months. Exclusion criteria included known pregnancy, pneumothorax within the previous six months, history of cough syncope, or vomiting associated with coughing. After the first subject experienced recurrence of mild hemoptysis during the cough study, we excluded those with hemoptysis in the previous 7 days. Subjects were excluded from hypertonic saline inhalation if there was a history of intolerance of hypertonic saline, presence of asthma

symptoms, or a forced expiratory volume in one second ( $FEV_1$ )  $\leq$  40% predicted and no previous trials of hypertonic saline. Subjects were asked to withhold all nebulized therapy for 12 hours prior to testing.

**Ethics:** The study was approved by the Ethics Committees of both CF centers and the University of Queensland and the Institutional Review Board of UMDNJ. Informed consent was obtained from all subjects and in addition from the parents or guardians of all young people under 18 years of age.

**Cough Aerosol Sampling System (CASS):** The equipment used was a modification of that developed previously.[14] In brief, a subject coughs through a mouthpiece connected to afferent tubing into a chamber where upon a vacuum pump draws exhaled air and generated respiratory particles through one of two Anderson six stage impactors. Each stage has 400 holes of decreasing diameter through which appropriated sized aerosolized particles will penetrate and deposit on an agar plate. A 'settle plate' of the same agar was placed inside the chamber to capture larger droplets. Larger particles (droplets) would be expected to deposit in the afferent limb tubing, the settle plate and the walls of the chamber. Additional details are provided in the online data supplement.

**CASS Protocol:** The Andersen impactors were loaded with agar plates at room temperature. The tubing from the vacuum pump was attached to the port for the first 6-stage impactor in the CASS. After the first session of coughing, the tubing was moved to the second sampler. All unused ports were occluded with plastic tape.

Subjects were instructed to cough into the CASS as frequently and as strongly as was comfortable for 5 minutes. At the onset of coughing, the timer (set for 5 minutes) controlling the power to the vacuum pump was started. Cough strength was assessed as strong, moderate or weak and cough frequency was assessed quantitatively.

If hypertonic saline could be tolerated, the first sampling was done during voluntary coughing, and the second five minute sample was collected during inhalation of 5mL of 4.5% saline delivered by a handheld ultrasonic nebulizer (Microneb Allersearch distributed by Becton Dickinson Pty. Ltd. North Ryde, Australia). Subjects were pretreated with albuterol MDI (88 mcg per puff), 4 puffs via spacer (Volumatic, Allen & Hanburys Ltd, UK). If hypertonic saline was not considered safe, sampling was done with the subject using tidal breathing for five minutes. Sputum samples were collected if produced.

#### **Clinical Parameters:**

**a. Pulmonary Function Testing:** Forced expiratory volume in one second ( $FEV_1$ ) and forced vital capacity (FVC) were obtained according to standard guidelines prior to the cough study.[15] Respiratory muscle strength was assessed using maximum inspiratory ( $PI_{max}$ ) and expiratory ( $PE_{max}$ ) pressures (Morgan Pmax) at the pediatric center and using a Micro Medical Respiratory Pressure Meter (Micro Medical LTD 2003) at the adult center.

**b. Other:** Age, gender, presence of current exacerbation of disease, height, weight and body mass index were recorded.

**Room Air Sampling and Air Exchange:** Using a centrifugal air sampler, two samples were obtained prior to each cough aerosol study: one during the subjects' performance of spirometry and one during the cough aerosol study. The indoor air temperature and relative humidity were measured with a thermohygrometer (Rotronic HygroPalm 2, Rotronic Instrument Corp., Huntington, NY, USA) at the beginning of each study. Effective air exchange rates in the consultation rooms used for CASS testing and in the pulmonary function laboratory at the adult center were determined using carbon dioxide as a tracer gas. Further details are provided in the on line supplement.

### **Microbiology:**

**CASS aerosol samples and chamber settle plate:** Cultures were performed using chocolate bacitracin (300 µg/mL) agar in aerobic conditions at 35°C. After 48 and 72 hours incubation, a colony forming unit (CFU) count was performed on each plate including individual colonial *P. aeruginosa* morphotypes, and the combined total CFU count of *P. aeruginosa* and other Gram negative bacteria. Following presumptive screening (characteristic colonial appearance, presence of oxidase, and growth at 42°C) each *P. aeruginosa* isolate's identity was confirmed by species-specific *oprL* gene PCR.[16]. Other non-fermenting Gram negative bacteria detected throughout the study were identified using a combination of API 20NE (bioMerieux), amplified rDNA restriction analysis (ARDRA), and *recA*-based PCR analysis.[17, 18]

Each Andersen sampler stage contains 400 holes and each colony forming unit is regarded as the result of an infectious particle within a specific size range impacting on the agar. Colony counts exceeding 400 have been interpreted in two ways: an accepted "positive-hole" correction model taking into account the probability of multiple hits through each hole and a conservative model of a maximum count of 400 only.[19, 20] The total sum of *P. aeruginosa* or *Bcc* colonies counted (total count) in all the Andersen stages for 5 minutes of voluntary cough and for 5 minutes hypertonic saline study or tidal breathing was calculated, as was the sum of the colonies from stages 4, 5 and 6 (smaller than 3.3 microns termed 'small aerosol fraction').

**Sputa, afferent limb cultures and air samples:** Standard quantitative culture methods were used.[21] For air samples and afferent limb cultures only Gram negative non fermentative bacteria were assessed. Isolates were identified as above with molecular strain typing of *P. aeruginosa* isolates. Further details are provided in the on line data supplement.

### **Analysis**

Counts for individual components and totals across Andersen stages 1-6 (total) and for Andersen stages 4-6 (small fraction) were logarithmically transformed prior to analysis to correct for skewness. Means and 95 percent confidence limits were back-transformed from log to linear scales for presentation. The paired differences between counts during voluntary cough and each of hypertonic and tidal breathing studies were analysed by paired t-tests and mean differences were also back-transformed from log to linear scales to calculate the ratios of counts during voluntary cough and each of hypertonic and tidal breathing studies. Correlation coefficients were estimated between logarithmically transformed total counts and clinical and demographic factors where available for all subjects. Fisher's exact test was used for the association between positive air samples and high total counts. All reported p values are two-sided. Linear regression was used to estimate the slope of the relationship between FEV<sub>1</sub> and total count. All analyses were performed with SPSS software (version 15).

## Results

**CASS Studies.** 28 subjects (15 pediatric, 13 adult) were consecutively recruited and completed the five minutes of voluntary cough. Twenty subjects were administered nebulized hypertonic saline and seven subjects had measurements during tidal breathing. One subject performed voluntary cough only. Thirteen subjects were studied during a pulmonary exacerbation (Table 1).

**Sputum Microbiology.** In the 12 months prior to study, 27 subjects had sputum that cultured positive for *P. aeruginosa* and one subject had cultured *B. cenocepacia* (Table 2). Of the 27 patients with *P. aeruginosa* infection, all adults (n=12) and 14 children had chronic infection based on the Leeds criteria.[22] One child had recently cleared a new infection with *P. aeruginosa* following an eradication course of antibiotic therapy and cultured normal respiratory flora from a sputum sample collected on the day of testing. The patient with *B. cenocepacia* had chronic infection based on the Leeds criteria (Table 2).[22] On the study day, 23 subjects provided expectorated sputum samples. Of these, one subject grew *B. cenocepacia* as expected and *P. aeruginosa* was cultured in 21. Six subjects had *Staphylococcus aureus* cultured and two had Methicillin resistant *S. aureus* cultured. Other organisms cultured from sputum included alpha haemolytic Streptococci, Aspergillus species and yeasts. Molecular strain typing demonstrated a common clone, corresponding to the previously described Australian Epidemic Strain-2 (AES-2) in 16 subjects (6 adults, 10 children) and 5 had unique strains (4 adults, 1 child)(Table 2).[23]

**CASS Microbiology.** Of the 28 subjects, 25 had cough aerosols that grew *P. aeruginosa*. One subject cultured *P. aeruginosa* from cough aerosols only with the hypertonic saline study and not from voluntary cough. One subject cultured *B. cenocepacia* from cough aerosols. Two subjects had no Gram negative bacteria cultured from cough aerosols. In five subjects with cough aerosols with *P. aeruginosa*, additional Gram negative bacteria were co-cultured including *Stenotrophomonas maltophilia* in four and *Achromobacter xylosoxidans* in one (Table 2). Two of the subjects who co-cultured *S. maltophilia* did not produce sputum and sputum culture was negative for *S. maltophilia* for the other two. Three of the four subjects cultured *S. maltophilia* intermittently from sputum at other times. The subject with *A. xylosoxidans* in the cough aerosol culture did not culture the organism in the sputum sample on this occasion although the subject was known to be chronically infected with this organism which had been cultured repeatedly from previous sputum samples.

Corrected total count of colony forming units obtained from generated aerosols varied widely among subjects and was log-normally distributed (voluntary cough range 0 - 13,485 CFU) (figure 1). All subjects, but one, who cultured *P. aeruginosa* in sputum also cultured *P. aeruginosa* of identical genotype in the CASS cough aerosols. The total count from sputum correlated with total corrected count for voluntary cough from the aerosols ( $r=0.58$   $P=0.008$ ). Three out of seven subjects who had tidal breathing studies had positive CASS aerosol cultures, with *P. aeruginosa* cultured in low numbers (Total aerosol counts from tidal breathing 1, 5, 137 CFU).

**Settle Plate and Air Sampling Microbiology.** The chamber settle plate and afferent limb equipment was not changed between the two components of the study for individual subjects with quantitative culture reflecting large droplet deposition for both components of the study combined. The mean total count for the settle plate was 6 CFU (95% confidence interval 3, 14).

The mean total count for the afferent limb was 56 CFU/mL wash fluid (95% confidence interval 10, 303 CFU/mL).

Air exchange rates ranged between 9.77 +/- 0.06 and 19.40 +/- 0.70 exchanges per hour (+/- calculated error) in the testing rooms. 101 air samples were collected before and during testing. Sixteen samples cultured unique strains of *P. aeruginosa* during testing of 14 patients. The unique strains isolated did not match any sputum or CASS isolates. Five air samples cultured AES2 strain during testing of four subjects with AES2 strain of *P. aeruginosa*. For these four subjects, sputum, cough aerosol and air samples all cultivated the same strain. Three of the AES2 positive air samples were collected during pulmonary function testing, and two during background testing in the CASS study rooms. Positive air samples were associated with high concentration in cough aerosols. If only subjects with AES2 were considered, 4 out of 5 subjects with total cough aerosol CFU counts above 1000 CFU had positive air samples and no subjects out of the 10 with lower total CFU counts had positive air samples ( $P=0.003$ ). Temperature and humidity did not vary significantly between study sites or study days at each site (data not shown).

**CASS Microbiology: Voluntary cough, hypertonic cough and tidal breathing.** The infective particle size distribution of cough aerosols of *P. aeruginosa* or *B. cenocepacia* during voluntary cough is shown in Figure 2. Using the corrected total counts 71.8% of particles (95% confidence interval 66.8, 76.8 %) containing culturable aerosol isolated from voluntary coughing were on Andersen stages 4, 5 and 6 of the Andersen samplers (small aerosol fraction  $\leq 3.3$  microns). The conservative model gave similar results with 69.9% (95% confidence interval 64.6, 75.4%) in the small aerosol fraction. Mean total corrected counts were much lower during tidal breathing (2, 95% confidence interval -0.5, 15) compared with voluntary cough (85, 95% confidence interval 28, 238,  $P<0.001$ ) or hypertonic saline (68, 95% confidence interval 21, 215). There was no significant difference in total corrected counts between voluntary cough and hypertonic saline ( $P=0.12$ ). The pattern of differences was unaffected by utilizing the conservative model (data not shown).

**CASS Microbiology: clinical correlates.** FEV<sub>1</sub> correlated with the total corrected count from the voluntary cough aerosol ( $r=0.45$ ,  $P=0.019$ ) (figure 3) and also with the corrected small aerosol fraction ( $r=0.45$ ,  $P=0.018$ ). Similarly, FEV<sub>1</sub> correlated with the conservative total count ( $r=0.39$ ,  $P=0.044$ ) and conservative small aerosol fraction ( $r = 0.39$ ,  $P= 0.047$ ) during voluntary cough. There was no significant association between cough aerosol colony forming unit counts and any other clinical factor including gender, age, testing at pediatric or adult center, current exacerbation status, FVC, MIPs, MEPs, percentage predicted FEV<sub>1</sub>, presence of clonal *P. aeruginosa*, quality or actual number of coughs counted (data not shown). There was a trend for association of peak expiratory flow ( $r=0.36$ ,  $P=0.079$ ) and of body mass index ( $r=0.37$ ,  $P=0.058$ ) with total corrected count for voluntary cough.

## Discussion

This is the first study to report the magnitude, variability, and particle size distribution of culturable aerosols of Gram negative bacteria produced by coughing in patients with CF. Although there is evidence of culturable Gram negative bacteria in the large droplets within the afferent tubing and settle plates in the cough chamber, a large proportion of culturable particles were found to be in a size range that is likely to deposit in the lower respiratory tract. Genetically

indistinguishable bacteria were identified in expectorated sputum and in the cough-generated aerosols, and in four experiments the same organisms were also isolated from the ambient room air. This supports the assertion that the sources of the bacteria are the patients rather than the hospital or nearby environment.

Aerosolisation of respiratory tract particles during cough and sneeze and even during tidal breathing, is a well recognized phenomenon associated with the spread of many infections including measles, influenza, and tuberculosis.[24-26] The majority of respiratory pathogens have been thought to be spread by large droplets that settle within an approximate one meter range of an individual, providing a low risk of airborne infection. Infection control practices for most cystic fibrosis centers reflect recently published infection control guidelines suggesting that patients should maintain a distance of at least one meter to reduce the risk of cross infection.[12]. The risk of acquisition of infection from respiratory aerosols is complex and likely relates to the pathogen type, concentration of organism in aerosol, the susceptibility of exposed individuals and environment (air movement, relative humidity, temperature etc). Limited studies have examined particle size distribution of respiratory aerosols and most have reported large droplet formation, predominantly particles having diameter greater than 8 microns.[27, 28] More recently Papineni reported 85% of particles were under one micron and that coughing produced more aerosol particles than did breathing or talking.[29] The first published study to use a CASS examined patients with tuberculosis and like our study, found the majority of respiratory particles to be less than 3.3 microns.[14] Our study demonstrates that CF patients produce culturable aerosols in a wide range of particle sizes, including both respiratory droplets and infectious droplet nuclei. We have shown this predominantly for clonal *P. aeruginosa* and, for a small number of patients, for other non-fermentative Gram negative organisms, including *B. cenocepacia*. We do not know the ideal site of deposition in the respiratory tract for *P. aeruginosa* to establish infection in people with CF and either large or small droplets or both may be important in the pathogenesis.

Until relatively recently, cross infection with *P. aeruginosa* was believed to be uncommon and limited to siblings with CF and cohorts attending the same residential CF camps.[30] The identification of genetically related *P. aeruginosa* strains in many CF centers in the UK, Europe and Australia has suggested cross-infection between patients.[23, 31] Clonal strains of *P. aeruginosa* contaminating the air close to patients with the same infection during physiotherapy or lung function testing have been reported.[9] Our results provide further evidence that cross-infection may result from direct inhalation of aerosolized bacteria.

This study demonstrates widely varying bacterial counts in cough aerosols with a log normal distribution. Such a distribution is consistent with descriptions of highly infectious patients as 'disseminators', e.g. in tuberculosis or 'super-spreaders', e.g. in Severe Acute Respiratory Syndrome.[32, 33] Factors influencing the extent of isolation of Gram negative bacteria in cough aerosols are likely to be complex, including both host factors and bacterial factors such as enhanced survival in air. Our data demonstrate that the concentration of bacteria in the sputum and the forced expiratory flow rates were related to cough aerosol concentration, with a trend for association with higher peak flow and higher body mass index. These data suggest that patients with milder lung disease, perhaps as a result of stronger cough, may have an increased risk of producing infectious aerosols. This warrants further investigation as the improvement in clinical outcomes in patients with CF may potentially increase the risk of spread of clonal strains of *P. aeruginosa* and other Gram negative bacteria.

The only air samples that cultured *P. aeruginosa* which matched clinical samples from sputum or CASS samples were clonal AES2 strains. Given that patients who had positive air sample associated also had high total aerosol counts, we were unable to determine if the density of infection on its own, or whether in addition the nature of the specific infection, contributed to positive air samples. The source of the *P. aeruginosa* air isolates that did not match any clinical samples is unknown and environmental sampling of surfaces was not undertaken. It is possible that environmental sources such as sinks may have been involved as hand washing occurred during testing. The measured air exchange rates in the study rooms provide an important perspective as air sampling was performed for 12 minutes on each occasion and during this period 2-4 complete air exchanges occurred. Higher rates and density of positive air samples may be anticipated in less well ventilated rooms.

Nebulized hypertonic saline is now a recognized therapy which improves mucociliary clearance.[34] We sought to determine if hypertonic saline induced cough further enhanced the production of bacterial aerosols, however we noted similar results to those seen with voluntary coughing and much greater than seen during tidal breathing. Therapies including physiotherapy, mucolytic agents and even nebulized antibiotics, which can induce coughing, are likely to result in similar cough induced aerosols as seen with voluntary cough. Although only seen in three of seven patients tested during tidal breathing, the presence of *P. aeruginosa* in the cough aerosols and in the small aerosol fraction from two patients warrants further study as any reassessment of infection control recommendations to incorporate the role of airborne transmission may not only apply to coughing patients.

The significance of aerosol positive, sputum negative results for isolation in low numbers of *S. maltophilia* (n=2) and *A. xyloxidans* (n=1) is uncertain but it is possible that separating respiratory particles by size negates the obscuring of individual colony morphotypes by other flora which may occur with direct sputum culture.

There are several limitations to this study. First, the study was not powered to examine the effects of many of the clinical variables, such as exacerbations or strain of *P. aeruginosa* on the production of cough aerosols. In particular there were few patients with unique strains of *P. aeruginosa* and none who produced high concentrations of cough aerosols. Therefore the association between specific strain and obtaining a positive air sample could not be determined. Second, the media in the Andersen plates was selective for Gram negative organisms, and thus it is not possible to generalize these results to patients with CF infected with Gram positive bacteria, mycobacteria, or fungi. Third, we did not perform reproducibility or efficiency studies of the CASS. Fourth, the studies of tidal breathing were a select group of patients who did not undertake hypertonic saline-induced cough studies and further work is required to evaluate the extent tidal breathing is associated with the generation of potentially infective particles. Finally, whilst this study provides evidence that patients with CF and Gram negative infection can produce potentially infectious cough aerosols, we cannot draw conclusions about transmission to susceptible individuals. A recent study examined the survival of *P. aeruginosa* in vitro and found bacterial survival, at least for a limited time period of less than 90 seconds, to be favoured by lower temperature and mucoid phenotype.[35]. While providing further evidence that airborne transmission is plausible, transmission by this route is yet to be proven beyond doubt.

In conclusion, this study demonstrates that patients with CF infected with *P. aeruginosa* can produce respirable infectious cough aerosols in a wide range of concentrations of a log normal distribution. We also detected other non-fermenting Gram negative bacteria, including *B. cenocepacia* in the small aerosol fraction, suggesting airborne transmission of such organisms is biologically plausible. Further studies of potential airborne transmission of bacterial pathogens among CF patients are warranted to provide a scientific basis for infection control recommendations to prevent the spread of multidrug-resistant or clonal strains of *P. aeruginosa* and other Gram negative bacteria in this patient population.

Table 1. Subject Demographics and Baseline Clinical Factors

|   | <b>Paediatric Center<br/>n=15</b> | <b>Adult Center<br/>n=13</b> | <b>All<br/>n=28</b> |
|---|-----------------------------------|------------------------------|---------------------|
| <b>Age in years median (range)</b>      | 13.5 (9.9-16.6)                   | 25.8 (18.8-48.8)             | 16.4 (9.9-48.8)     |
| <b>Gender (male/female)</b>             | 8/7                               | 9/4                          | 17/11               |
| <b>Current exacerbation</b>             | 7 (47%)                           | 6 (46%)                      | 13 (46%)            |
| <b>Body mass index mean (SD)</b>        | 17.9 (2.5)                        | 22.6 (3.7)                   | 20.1 (3.9)          |
| <b>Z score for weight mean (SD)</b>     | -0.6 (0.9)                        |                              |                     |
| <b>Z score for height mean (SD)</b>     | -0.5 (1.2)                        |                              |                     |
| <b>FEV1 % predicted mean (SD)</b>       | 67.0% (22.5 )                     | 52.4% (19.6)                 | 60.2% (22.1)        |
| <b>FVC % predicted Mean (SD)</b>        | 77.0% (19.6)                      | 70.7% (15.4)                 | 74.1% (17.7)        |
| <b>Peak flow L/sec mean (SD)</b>        | 4.6 (2.2)                         | 6.1 (1.9)                    | 5.4 (2.2)           |
| <b>MIPs cm H<sub>2</sub>O mean (SD)</b> | 82.8 (32.8)                       | 101.0 (30.1)                 | 91.1 (32.3)         |
| <b>MEPs cmH<sub>2</sub>O mean (SD)</b>  | 113.1 (42.9)                      | 103.6 (29.5)                 | 109.1 (37.4)        |

Table 2. Microbiology of Sputum and CASS samples

| CASS study<br>n=28                           | Infection status prior<br>to study                              | Expectorated<br>Sputum<br>n= 23 | CASS study<br>Voluntary Cough<br>n=28 /hypertonic<br>saline n=20 | Tidal study<br>n=7 |
|--|---|---------------------------------|--|--------------------|
| <b><i>P. aeruginosa</i><br/>not isolated</b> | Cleared <i>P.aeruginosa</i><br>n=1                              | 1                               | 3  | 4                  |
| <b><i>P. aeruginosa</i><br/>isolated</b>     | Chronic <i>P.aeruginosa</i><br>(n=12 adult,<br>n=14 paediatric) | 21                              | 25*  | 3                  |
|  | <b><i>P. aeruginosa</i> (unique)</b>                            | 5                               | 7  | 1                  |
|  | <b><i>P. aeruginosa</i> (AES 2)</b>                             | 16                              | 18   | 2                  |
| <b><i>B. cenocepacia</i><br/>isolated</b>    | Chronic <i>B. cenocepacia</i><br>n=1                            | 1                               | 1  | 0                  |

\* 5 subjects also cultured additional gram negative bacteria in cough aerosols ( 4 *Stenotrophomonas maltophilia* and 1 *Achromobacter xylosoxidans*)

**Figure 1.**

Distribution of total corrected voluntary cough aerosols.

**B** Subject with *Burkholderia cenocepacia*

+ Positive ambient air samples isolated

**Figure 2.**

Particle size distribution of logarithmic corrected total cough aerosol counts in colony forming units with 95% confidence intervals during voluntary cough according to Anderson stage.

**Figure 3.**

Correlation of baseline forced expiratory volume in one second with logarithmic total corrected count from cough aerosols during voluntary cough. .

+ Positive ambient air samples isolated

Supported by Royal Children's Hospital Foundation, Brisbane, Australian Cystic Fibrosis Research Trust and a University of Queensland Travel Grant Award.

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## Online Data Supplement

### **Cough-generated Aerosols of *Pseudomonas aeruginosa* and other Gram Negative Bacteria from Cystic Fibrosis Patients.**

Claire E Wainwright , Megan W France , Peter O'Rourke , Snehal Anuj Timothy J Kidd , Michael Nissen , Theo Sloots , Chris Coulter , Zoran Ristovski , Megan Hargreaves , Barbara R Rose , Colin Harbour , Scott C Bell , Kevin P Fennelly

#### ***Cough aerosol sampling equipment (CASS):***

Two Andersen 6-stage cascade impactors for viable air sampling (Thermo Andersen, Smyrna, GA, USA) were positioned in the horizontal position in a stainless steel chamber cylinder 39cm in length and 31.3 cm in diameter. The impactors were connected to a vacuum pump (Gast Manufacturing, Inc. Benton Harbor, MI, USA). The flow through the vacuum pump was maintained at 28.3 LPM after calibration against a primary flowmeter (Dry Cal® DC Lite, BIOS International, Butler, NJ, USA) and was monitored using an in-line flow meter (Field Rotameter, SKC, Inc., Eighty Four, PA, USA). The vacuum pump was run for five minutes on an electronic timer for each cough session. The mouthpiece into which subjects coughed was attached to 45.7 cm of flexible non-compressible tubing that was connected to the chamber. A smaller mouthpiece was used for paediatric subjects. A high-efficiency particulate air filter (Scott HEPA respirator filters #642-P100, Scott Health and Safety, Monroe, NC, USA) allowed for equalization of pressures and provided make-up air flow if needed. A single stage impactor (SKC, Inc., Eighty Four, PA, USA) was used to sample the ambient air in the study room with the same agar. All equipment was cleaned and sterilized by standard methods between subjects. A minimum time period of 120 minutes occurred between patient studies to allow at least 18 room air exchanges.

#### **Air sampling:**

Sample volumes of 41L per minute for 12 minutes were collected onto non-selective agar strips (Kelly Company, Australia) for total count determination, using a Biotest HYCON RCS Centrifugal air sampler (Gelman Sciences, Australia). The centrifugal sampler was placed at head height two meters from the subject.

Air exchange rates were calculated using carbon dioxide as a tracer gas. The decay of carbon dioxide was measured using an indoor air quality monitor (Q-Trak, TSI, Inc., Shoreview, MN, USA). The decay in the carbon dioxide concentration was measured over time until the carbon dioxide levels reached background concentration. The measured decay in carbon dioxide concentration versus time was then fitted to a theoretical exponential curve. From the fitting procedure the value of the exchange rate was determined and expressed as number of exchanges per hour +/- the calculated error obtained from the fitting procedure.

#### ***Culture of sputum, afferent limb tubing and air samples:***

Sputum was homogenized in an equal volume of Sputasol (0.1% dithiothreitol Oxoid, UK) and 100 µL of the treated or diluted sample (1/10 to 1/10<sup>5</sup>) was cultured onto horse blood agar,

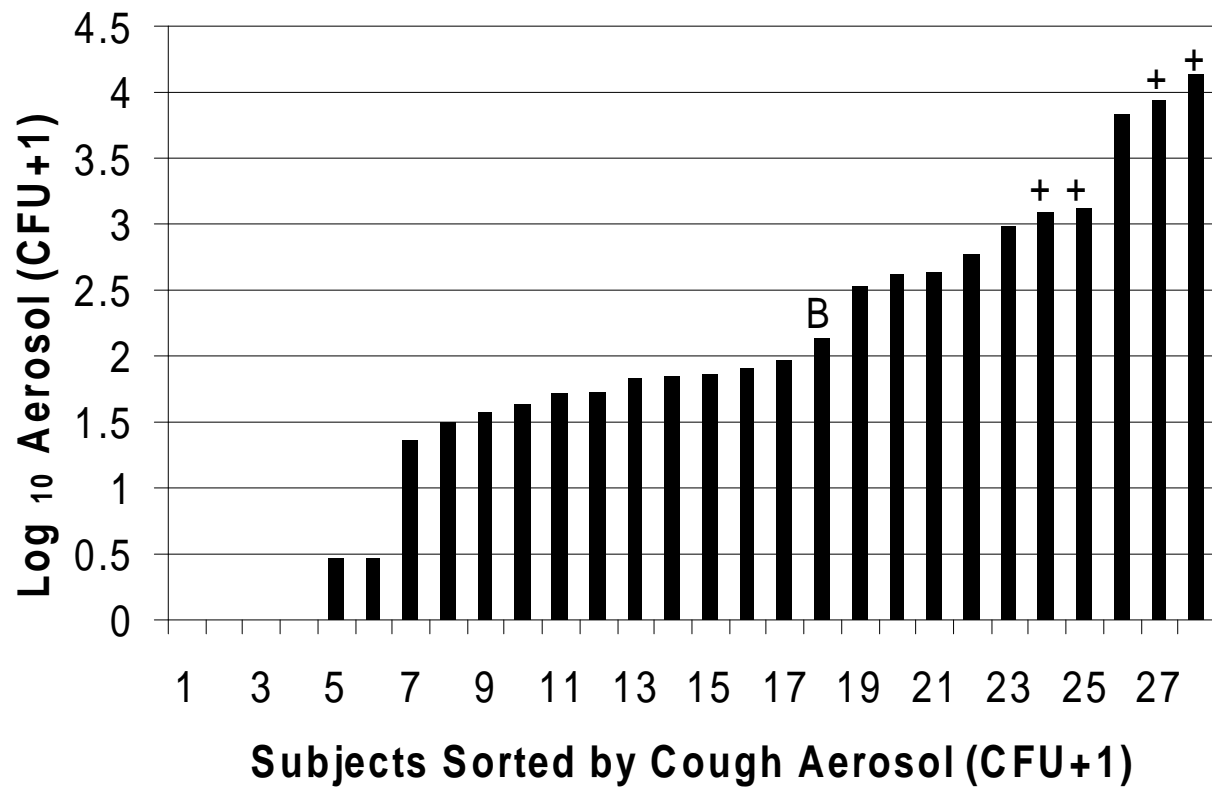
MacConkey agar, mannitol salt agar, chocolate bacitracin agar and *B. cepacia* selective agar. The horse blood, MacConkey, and chocolate bacitracin agar plates were incubated in 5% CO<sub>2</sub> at 37°C and the mannitol salt agar and *B. cepacia* selective agar plates were incubated aerobically at 37°C. Media was made 'in-house' from commercial base reagents (Oxoid, United Kingdom) and tested for quality by standard methods. Examination for bacterial growth was performed at 24, 48 and 72 hours. Final colony counts were recorded after 72 hours incubation.

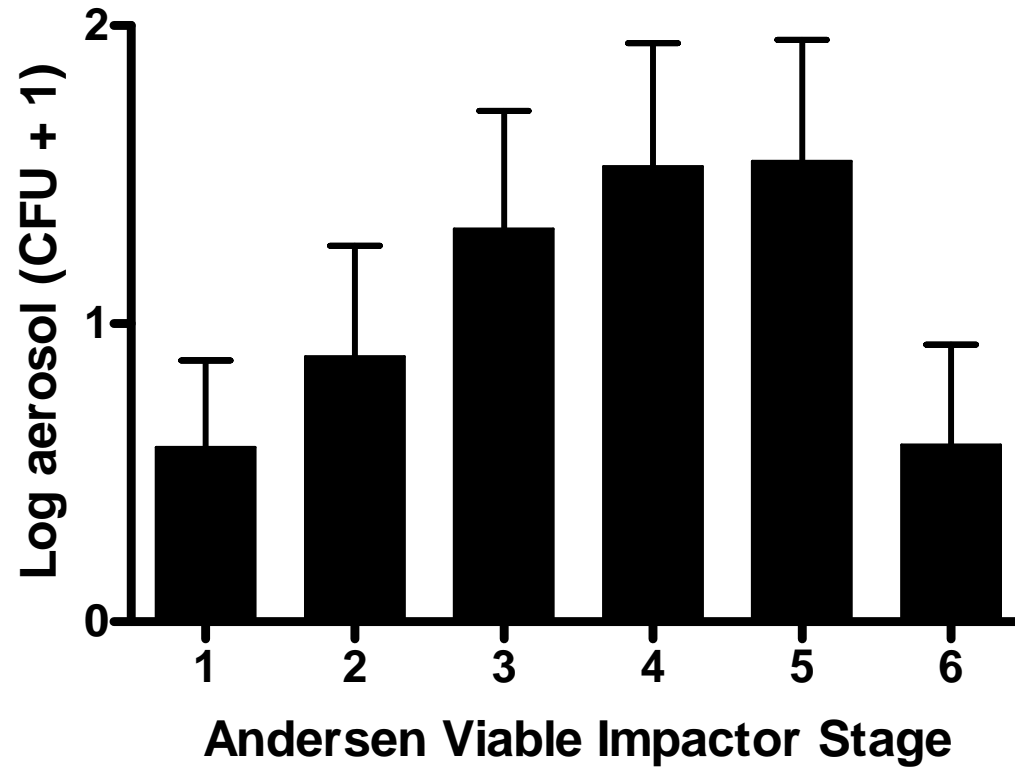
The 'afferent limb' tubing was instilled with 20 mL of wash fluid (0.1% peptone, 0.85% NaCl) and inverted 20 times. Thereafter, 100 µL of the wash fluid was inoculated onto chocolate bacitracin agar, incubated at 37°C and examined at 48 and 72 hours. Culture results from sputum and afferent tubing were quantified and expressed as cfu/ml. The air sampling agar strips were incubated at 37°C for 24 to 48 hours. Presumptive *P. aeruginosa* colonies were identified as described in the methods.

**Molecular studies:** DNA extraction was carried out on each isolate using the MagNA Pure LC DNA Isolation Kit III (Roche Applied Science, Mannheim, Germany) as per the manufacturer's instructions. Molecular strain analysis was performed using repetitive element based PCR as described previously with minor modifications.[1] Repetitive PCR genotypes which resembled the local clonal strain (AES 2) were confirmed by pulsed field gel electrophoresis.[1] Each repetitive element PCR and pulsed field gel electrophoresis fingerprint was examined by visual analysis.

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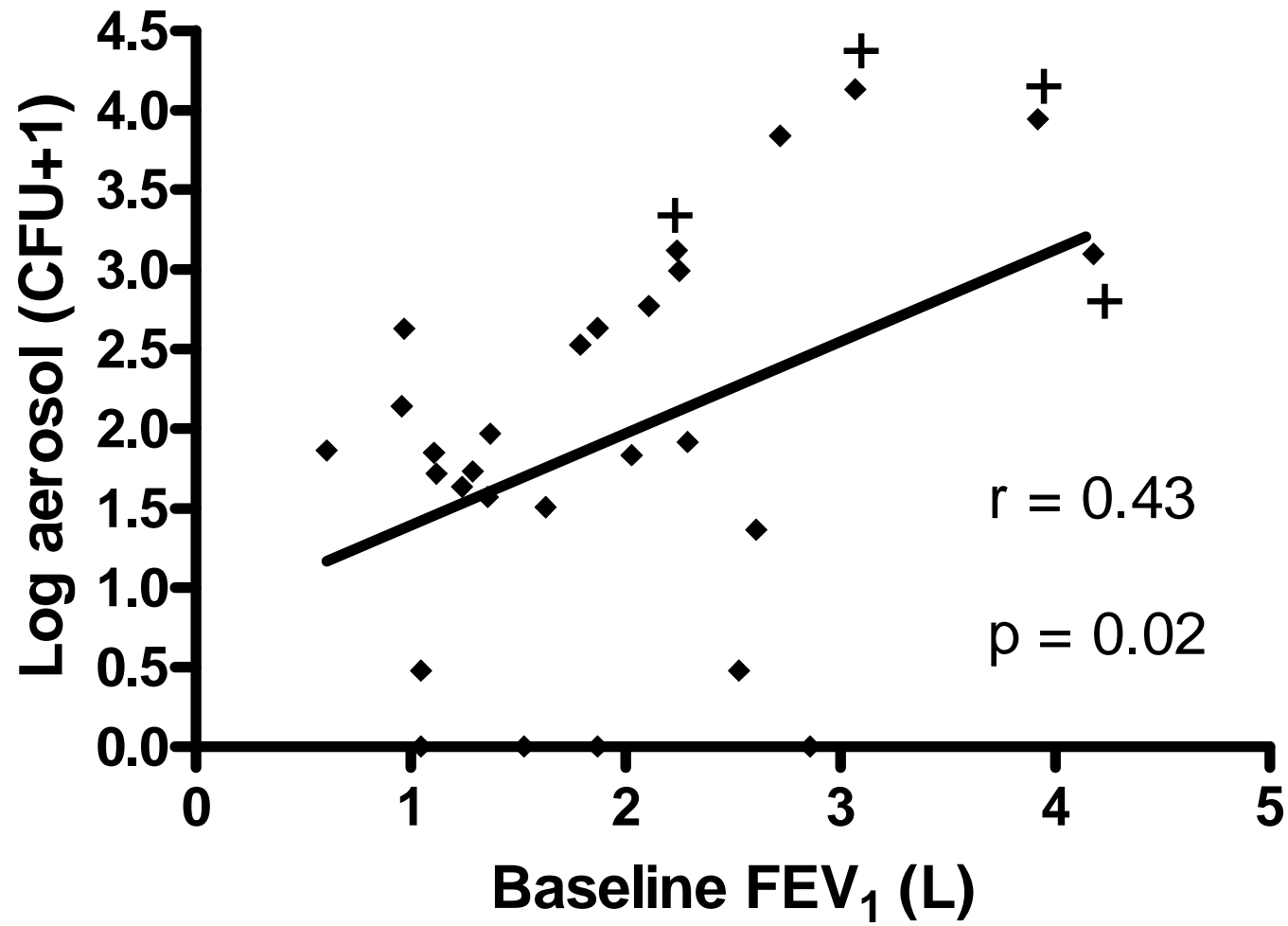


**Lower limit of size range (microns)**

**7.0    4.7    3.3    2.1    1.1    0.65**

**Anatomical deposition site**

**Upper airway - - - bronchi - - - alveoli**





## Cough-generated Aerosols of *Pseudomonas aeruginosa* and other Bacteria from Cystic Fibrosis Patients.

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*Thorax* published online July 1, 2009

doi: 10.1136/thx.2008.112466

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