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Original Article

Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation?☆

Franziska A. Stressmann [a], Geraint B. Rogers [a,*], Peter Marsh [b], Andrew K. Lilley [a], Thomas W.V. Daniels [c], Mary P. Carroll [c], Lucas R. Hoffman [d], Graeme Jones [b], Collette E. Allen [b], Nilesh Patel [e], Benjamin Forbes [e], Andrew Tuck [b], Kenneth D. Bruce [a]

[a] *Molecular Microbiology Research Laboratory, Institute of Pharmaceutical Sciences, 150 Stamford Street, Franklin-Wilkins Building, King's College London, London, SE1 9NH, UK*

[b] *Health Protection Agency South East, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK*

[c] *Cystic Fibrosis Unit, Southampton University Hospitals NHS Trust, Tremona Road, Southampton, SO16 6YD, UK*

[d] *Department of Pediatrics, University of Washington, Box 356320, HSB RR338, Seattle, WA 98105, USA*

[e] *King's College London, Institute of Pharmaceutical Sciences, 150 Stamford Street, Franklin-Wilkins Building, London, SE1 9NH, UK*

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Abstract

Background: Cystic Fibrosis (CF) lung disease is characterised by an inexorable decline in lung function, punctuated by periods of symptomatic worsening known as pulmonary exacerbations (referred to here as CFPE). Despite their clinical significance, the cause of CFPE remains undetermined. It has been suggested that an increase in bacterial density may be a trigger, although this has not been shown empirically.
Methods: Here, a previously validated quantitative PCR-based approach was used to assess numbers of *Pseudomonas aeruginosa* and of total bacteria in respiratory secretions from patients during the period leading up to CFPE. Sputum samples collected from 12 adult CF patients were selected retrospectively to fall approximately 21, 14, 7 and 0 days prior to CFPE diagnosis. In addition, the relationships between clinical parameters (FEV_1, temperature and patient reported outcome measures) and microbiological data were investigated.
Results: No significant changes either in total bacterial or *P. aeruginosa* numbers were identified prior to CFPE. Of all the correlations tested, only temperature showed a significant correlation with total bacterial numbers in the period leading to CFPE.
Conclusions: These findings strongly suggest that CFPE do not generally result from increased bacterial density within the airways. Instead, data presented here are consistent with alternative models of pulmonary exacerbation.

Keywords: Bacterial enumeration infection molecular respiratory

1. Introduction

Pulmonary disease in adult cystic fibrosis (CF) patients is typified by chronic polymicrobial lung infections [1] with intermittent periods of pulmonary exacerbation (referred to here as CFPE). CFPE presents clinically with changes in cough, sputum production, dyspnoea, decreased energy level and appetite, weight loss and decreases in spirometric parameters [2]. These periods of exacerbation affect quality of life of CF patients [3] and have been considered to be both the single most important cause of CF morbidity [4] and strong predictors of 5 year survival [5]. Exacerbations are risk factors for a more rapid lung function decline [6], and recent evidence indicates that loss of lung function that occurs during exacerbations can be permanent [7]. Despite their importance to CF patient health, the triggers for the onset of CFPE remain unknown.

Given the infective nature of CF lung disease, the initiation of CFPE has generally been considered to represent a shift in the

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* Corresponding author. Tel.: +44 207 848 4467; fax: +44 207 848 4500.
E-mail address: geraint.rogers@kcl.ac.uk (G.B. Rogers).

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complex relationship between host defence and airway microbiology. Changes in this balance impact on sputum production and airflow obstruction, and may be influenced by factors such as respiratory viral infections [8], fungal presence [9] and air pollution [10]. Antibacterial agents are routinely administered at times of CFPE to reduce symptoms and improve lung function [11,12]. Further, regular maintenance antibiotic treatment may be used in an attempt to decrease CFPE frequency [1]. However, some studies have failed to demonstrate a clear relationship between the impacts of antibiotics on bacterial density and clinical outcomes during treatment for CFPE [13]. Further, antibiotics that alter the behaviour, but not viability, of CF pathogenic bacteria can decrease risk of exacerbations [14]. Therefore, whether a simple increase in the number of bacteria present in respiratory secretions prior to the onset of CFPE acts as a direct trigger, or whether a change in the behaviour of colonising species, or other as yet unknown factors, are required, remains unclear.

To date, little quantitative analysis has been performed to investigate whether bacterial density increases prior to CFPE. This may be partly due to the lack of a universally accepted methodology for quantitative analysis [15]. In addition, whilst useful information can be gained from culture-based enumeration of bacterial cells in CF respiratory samples, the technique is associated with a level of inherent inaccuracy due to a number of factors, including the presence of slow-growing bacterial variants [16] and potentially viable but non-culturable bacterial cells [17,18], as well as the viscous nature of CF respiratory secretions, resulting in incomplete homogenisation and sampling errors [15].

The application of culture-independent quantitative (Q)-PCR potentially offers a more accurate means by which changes in bacterial numbers prior to the onset of CFPE can be characterised [19], regardless of whether species are refractory to in vitro culture [20]. Q-PCR technology is well-validated and used widely in the characterisation of bacteria in clinical samples [21], and has been shown to be a sensitive, accurate and reproducible method of bacterial enumeration in samples of CF airway secretions [22,23]. However, where clearance of material from the site of infection is poor, as in the lower CF airways [24], the presence of nonviable cells and extracellular DNA can have a substantial impact on the accuracy of quantification by molecular methods. To address this, new molecular protocols have been developed that exclude from analysis any DNA not derived from viable bacterial cells [25]. These protocols, involving propidium monoazide (PMA) photo-crosslinking chemistry to block extracellular DNA or DNA from non-viable cells from serving as a template for PCR reactions, have been adapted to, and validated in, the CF respiratory context [26]. Further, they have been shown to be capable of identifying short-term changes in bacterial levels in sputum not detected by standard Q-PCR techniques, such as a reduction in bacterial density in CF respiratory secretions following the intravenous (IV) administration of certain antibiotics [27].

The aim of this study was to determine whether a relationship exists between bacterial density in airway secretions and the onset of CFPE in adult CF patients with chronic lung infections. Q-PCR was used to determine levels of viable *P. aeruginosa* and total bacterial cells in PMA-treated sputum samples in the period prior to exacerbation. Further, *P. aeruginosa* and total bacterial density were compared with clinical signs (FEV_1, temperature) and patient reported outcome (PRO) scores (sputum production, cough severity, breathlessness, and general well-being).

Here, we report no significant link between the abundance of bacteria in respiratory secretions and the onset of CFPE, and discuss the relationship of these findings to our current understanding of respiratory health in CF patients.

2. Materials and methods

2.1. Clinical samples

Patients were recruited for this study under local ethical approval from Southampton and South West Hampshire Research Ethics Committee (06/Q1704/26). Twelve adult CF patients from Southampton General Hospital, UK were followed over a period of 1 year with sputum samples collected and archived 2–3 times per week. This work was performed within the context of a wider investigation of the dynamics of CF lung disease. Patients were selected on the basis of four criteria: consistent sputum production, anticipated frequent exacerbations (here, low relative FEV_1 scores were taken to indicate more severe respiratory disease, and a greater likelihood of exacerbation), chronic *P. aeruginosa* infection (defined as at least 3 positive sputum cultures in the previous 12 months, in accordance with the modified Leeds criteria [28]), and no change over the past 12 months in maintenance therapy (nine patients received a macrolide antibiotic, seven patients received nebulised colomycin, patient 10 received nebulised tobramycin, and patient 8 and 11 received nebulised colomycin/tobramycin in alternating months, Table 1). Over the study period all patients experienced at least two periods of CFPE, as diagnosed using the scheme described previously by Fuchs et al. [29] (Table 1).

Archived samples were selected retrospectively for analysis that fell approximately 21, 14, 7 and 0 days prior to the start of antibiotic treatment for CFPE. Periods of CFPE were selected where the patient had not received antibiotics less than 21 days prior to the first sampling point (42 days prior to start of CFPE). There were two exceptions to these criteria; CFPE 2, Patient 1, and CFPE 5, Patient 7, where, in each case, a course of antibiotics had finished 8 days prior to day −21. Of the 39 periods of CFPE experienced by the 12 patients in the study, samples were available at all four of the sampling time points in 28 cases, and samples were available at three of the sampling time points in 6 cases. For the remaining five CFPE, samples were available for fewer than three of the sampling points, and these CFPE were excluded from further analysis. The 6 CFPE with three available time points (CFPE 2, Patient 1, CFPE 2, 3 and 4, Patient 4, CFPE 3, Patient 9 and CFPE 2, Patient 10) were included for all analyses in this manuscript apart from the regression analysis and the repeated measures one way ANOVA. Sputum samples were selected for analysis that fell 3 weeks, 2 weeks and 1 week prior to the start of antibiotic treatment and a fourth sample at the diagnosis of exacerbation, and/or prior

Table 1
Patient information.

Patient	Age	Gender	Genotype I	Genotype II	FEV_1 (% predicted)	BMI	CFPE episodes over preceding 12 months	CFPE episodes analysed here	Diabetic	Long-term medications									
										Antimicrobial therapy					Corticosteroids		Mucolytic	
										Macrolide	Col neb	Tob neb	Col/tob neb (alt. months)	Itra	Oral cortico- steroids	Inhaled cortico- steroids	DNAse	HS
1	30	Male	phe508del	Unknown	54.9	29	3	3	No	No	No	No	No	No	No	No	Yes	Yes	No
2	45	Female	phe508del	unknown	40.2	18.5	3	2	Yes	Yes	Yes	No	No	No	No	No	Yes	No	No
3	30	Female	phe508del	711+3A7G	38.2	25	3	2	No	Yes	No	No	No	No	No	No	Yes	Yes	No
4	22	Female	phe508del	phe508del	36.2	19	5	5	No	Yes	Yes	No	No	Yes	No	No	Yes	Yes	No
5	55	Male	phe508del	G85E	52.2	24.5	2	2	No	Yes	Yes	No	No	No	No	No	Yes	Yes	No
6	21	Female	phe508del	phe508del	56.6	19	3	3	No	Yes	Yes	No	No	No	No	No	Yes	Yes	No
7	22	Male	phe508del	phe508del	16.5	17.9	5	5	Yes	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes
8	18	Female	phe508del	phe508del	84	22.5	2	2	No	Yes	No	No	Yes	No	No	No	Yes	Yes	No
9	24	Female	phe508del	G542X	72.5	23.4	3	3	No	Yes	Yes	No	No	No	No	No	Yes	Yes	No
10	20	Male	phe508del	phe508del	26.8	30.4	4	3	No	Yes	No	Yes	No	No	No	No	No	No	No
11	20	Male	phe508del	phe508del	54.4	21	3	2	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No
12	23	Male	phe508del	phe508del	53.6	20.7	3	2	Yes	No	Yes	No	No	No	No	No	Yes	No	No

Abbreviations: BMI — body mass index, col neb — colomycin nebulised, tob neb — tobramycin nebulised, col/tob neb (alt. months) — colomycin/tobramycin nebulised on alternating months, itra — itraconazole, HS — hypertonic saline.

to antibiotic treatment. Samples were collected within a range of days prior to diagnosis of CFPE: 18–24 days, 11–17 days, 4–10 days and 0 days at the start of CFPE. These are referred to as weeks 3, 2, 1 and 0, respectively or as the time points '−21', '−14', '−7' and '0' days. Antibiotic therapy for CFPE was initiated on day 0 for all patients, however "day 0" sputum samples were collected prior to their administration.

The majority of patients provided sputum samples as part of their normal morning treatment routine, however some patients chose to produce samples in the evening. In such cases, samples were stored at 4 °C and collected by a courier the following morning. All patients gave sputum samples consistently at their chosen time of day. All sputum samples were stored (immediately after expectoration) and shipped at 4 °C. Samples were left at 4 °C for a maximum of 36 h (in accordance with the sputum handling guidelines of the Health Protection Agency, BSOP57). Once material had been removed for culture-based analysis, sputum samples were stored −80 °C prior to processing for molecular analysis.

2.2. Clinical data

Clinical measures (FEV_1 in litres, temperature) were recorded at the time of collection for each sample. In addition, PRO scores ("breathlessness", "cough severity", "sputum production", and "general well-being") were recorded using visual analogue scores (VAS) by means of a symptom sheet. VAS were recorded in millimetres from 0 (no symptoms) to 100 (worst symptoms).

2.3. PMA cross-linking and DNA extraction

Sputum samples were removed from −80 °C storage and homogenised by hand. 250 µl portions were processed for DNA cross-linking using PMA and nucleic acid extraction as described previously by Rogers et al. [26].

2.4. Quantitative PCR

All quantitative PCR analyses were performed in triplicate. Total bacterial density was determined using a Taqman assay, in which a 466 bp fragment of the 16S ribosomal RNA gene was amplified, as described previously [20]. *P. aeruginosa* density was determined using a Taqman assay which amplified a 65 bp fragment of the *reg*A gene, as described previously [30]. Details of the relevant primers and probes used are shown in Table 2. Total bacterial primers and probe were used at a concentration of 100 nM each, whereas *P. aeruginosa*-specific primers were used at a concentration of 1000 nM each, and the probe at a concentration of 250 nM. 1 µl (~800 ng) of mixed template DNA (human and microbial) was used in the *P. aeruginosa* assay. 1 µl of a 100 fold dilution (~8 ng) was used in the total bacterial assay. All PCR reactions were carried out in a total volume of 25 µl in Taqman® Universal PCR Mastermix (Applied Biosystems, Warrington, UK). Quantitative PCR assays were carried out using the Rotorgene 6000 (Qiagen, Crawley, UK) with a temperature profile of 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s.

Table 2
PCR primers and probes used in this study.

Primer	Primer type	Sequence (5′ to 3′)	Reference
EubF	Total bacterial forward	TCCTACGGGAGGCAGCAGT	[20]
EubR	Total bacterial reverse	GGACTACCAGGGTATCTAATCCTGTT	[20]
EubPR	Total bacterial Taqman probe	FAM–CGTATTACCGCGGCTGCTGGCAC–TAMRA	[20]
PaerF	*P. aeruginosa* forward	TGCTGGTGGCACAGGACAT	[30]
PaerR	*P. aeruginosa* reverse	TTGTTGGTGCAGTTCCTCATTG	[30]
PaerPR	*P. aeruginosa* Taqman probe	FAM–CAGATGCTTTGCCTCAA–TAMRA	[30]

For both total bacterial and *P. aeruginosa*-specific quantitative PCR assays, densities (cfu/ml) were determined by comparison with standard curves generated from bacterial isolates. Separate nutrient broth cultures of methicillin sensitive *Staphylococcus aureus* (MSSA: ATCC 29213), *P. aeruginosa* (NCTC 12934/ATCC 27853), *Streptococcus pyogenes* (ATCC 19615) and *Escherichia coli* (NCTC 12241/ATCC 25922) isolates were incubated at 37 °C for 16 h, with cfu ml^{-1} estimated by incubation of dilutions (n=4) on Columbia Blood Agar at 37° for 24 h, followed by colony counts. DNA was extracted from ten-fold dilutions of these broth cultures in the same way as for the sputum samples, and RT-PCR was carried out as above on the DNA extracts. The total bacterial assay was tested against the standard curves of all species (*P. aeruginosa*, MSSA, *S. pyogenes* and *E. coli*) to test for any variation in density estimation between different species with different 16S ribosomal RNA gene copy numbers. The standard curve generated using *P. aeruginosa* was used for both *P. aeruginosa* and total bacterial enumeration to allow direct comparisons to be made.

2.5. Statistical analysis

Statistical analyses were performed using SPSS Statistics 16.0 (SPSS Inc., Chicago, USA). The data were tested for normality and homogeneity of variance, and cell densities were log_{10} transformed. Data were analysed by GLM repeated measures one way ANOVA, using Mauchly's Sphericity Test with 'Week' (4 levels) as the within-subjects factor. Linear regression and correlations were calculated using SPSS. Means given are averages of Log_{10} values. Simple Interactive Statistical Analysis (http://www.quantitativeskills.com/sisa/) was used to calculate Bonferroni corrections for the correlations.

3. Results

3.1. Total bacterial and P. aeruginosa sputum densities prior to the start of IV antibiotic treatment

Viable total bacterial densities ranged from 3.5×10^{6} cfu ml^{-1} to 5.9×10^{12} cfu ml^{-1}, and viable *P. aeruginosa* densities ranged from 5.3×10^{3} cfu ml^{-1} to 1.8×10^{11} cfu ml^{-1}. Mean values for each time point for the sample set as a whole (34 periods of CFPE) are shown in Fig. 1, (with mean FEV_1 values for these time points shown in Figure 2). For days −21, −14, −7, and 0, mean total bacterial density values of 9.6×10^{9}, 1.9×10^{10}, 1.8×10^{10} and 1.0×10^{10} cfu ml^{-1} were obtained respectively. Corresponding mean *P. aeruginosa* densities for these sample points were 5.8×10^{7}, 6.9×10^{7}, 9.0×10^{7} and 8.5×10^{7} cfu ml^{-1}, respectively.

Repeated measures one way ANOVA was used to test for significant differences between mean density values at each of the four time points (CFPE 2, Patient 1, CFPE 2, 3 and 4, Patient 4, CFPE 3, Patient 9 and CFPE 2, Patient 10 were excluded from these analyses due to there being only 3 data points). Mean log bacterial densities did not differ significantly between the four sampling points either for mean total bacterial density ($F_{3,81}=1.608$, $p=0.208$) or mean *P. aeruginosa* density ($F_{3,81}=0.348$, $p=0.791$).

3.2. Identification of trends in total bacterial and P. aeruginosa density prior to antibiotic treatment in individual patients

To test for significant rises or falls in bacterial densities (total and *P. aeruginosa*) prior to each exacerbation linear regressions

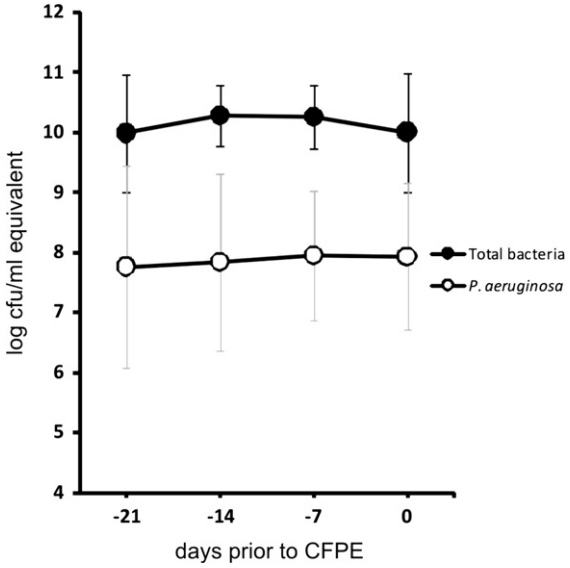

Fig. 1. Variation in total bacterial density and *P. aeruginosa* density three weeks prior to antibiotic therapy for increased severity of respiratory symptoms. Trend in mean log cfu/ml equivalent for total bacterial and *P. aeruginosa* density prior to CFPE onset for all 34 exacerbations. Bacterial density is expressed as log cfu ml^{-1} of sputum equivalent (y axis) over the days preceding exacerbation (x axis). Time points are plotted as '−21', '−14', '−7' and '0', relating to days prior to CFPE onset, however, some sample points did not fall on these exact days prior to CFPE. For the observed *P. aeruginosa* means, rises and falls from 0.7 to 0.75 (log values) were the minimal changes that would have resulted in a statistically significant (P<0.05) effect. For the observed total bacterial means, rises and falls of 0.2 to 0.6 (log values) were the minimal changes that would have resulted in a statistically significant (P<0.05) effect.

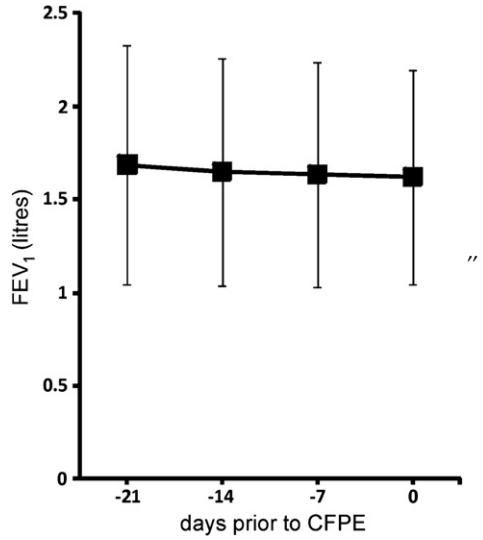

Fig. 2. Variation in FEV_1 3 weeks prior to antibiotic therapy for increased severity of respiratory symptoms. Trend in mean FEV_1 prior to CFPE onset for all 34 CFPE. FEV_1 is expressed in litres (y axis) over the days preceding exacerbation (x axis). In the case of Patient 7, it was not possible to collect spirometric data due to the poor respiratory health of the patient. Time points are plotted as '−21', '−14', '−7' and '0', relating to days prior to CFPE onset, however, some sample points did not fall at these exact times. FEV_1 values for the individual patients are shown in Supplementary Fig. 2.

were fitted to the data (excluding the CFPE with three time points). In each case, the rate of change, the slope, of the regression line over the four time points, and the statistical significance of the change, is presented as Log_{10}-fold/day change (Table 3). Of the 28 CFPE, four showed a significant trend in total bacterial density. Specifically, in Patient 3, CFPE 1, total bacterial density increased significantly at an average of 0.03 per day ($p=0.03$), and in Patient 6, CFPE 3, Patient 8, CFPE 1, and Patient 9, CFPE 2, the total bacterial density decreased significantly at an average of −0.07 ($p=0.05$), −0.09 ($p=0.05$) and −0.02 ($p=0.05$) per day respectively (Table 3).

For *P. aeruginosa*, only three of 28 exacerbations showed a significant change in density over the four time points. Specifically, in Patient 4, CFPE 5, *P. aeruginosa* density decreased significantly at an average of −0.04 per day ($p=0.017$), In Patient 5, CFPE 1, *P. aeruginosa* density decreased significantly at an average of −0.04 per day ($p=0.009$), and in Patient 7, CFPE 1, *P. aeruginosa* density increased significantly at an average of 0.12 per day ($p=0.05$). Examples of trends in total bacterial and *P. aeruginosa* densities for individual CFPE are shown for three patients in Supplementary Fig. 1.

3.3. Correlations between bacterial density, "time prior to CFPE", and clinical data

Correlation analysis was performed on the entire dataset and was used to test, in a pair-wise manner, for significant relationships between any of the nine variables relating to the samples. These variables included two clinical measures — FEV_1 and temperature (with ranges of 0.7–3.1 L, and 35.4–37.9 °C, respectively), four PRO scores — sputum production, cough severity, breathlessness, and general well-being (ranges 2–81 mm, 2–78 mm, 1–80 mm, and 1–76 mm, respectively), time before CFPE, and total bacterial and *P. aeruginosa* densities. Twelve significant relationships were identified, shown in Table 4.

The only variables that correlated significantly with total bacterial density prior to CFPE were *P. aeruginosa* density and temperature ($p=0.0001$, $n=132$ and 127 respectively) (Table 4). No other significant correlations were identified with *P. aeruginosa* density. Two significant correlations were identified for time before CFPE (sputum production, $p=0.031$, $n=128$, and cough severity, $p=0.019$, $n=128$).

Eight significant correlations were identified amongst clinical signs and PRO scores. Correlations between sputum production, general well-being, cough and breathlessness were all highly significant ($p<0.0001$, $N=128$ for all). Temperature significantly correlated with sputum ($p=0.001$, $n=127$) and cough ($p=0.011$, $n=127$).

4. Discussion

Despite the impact that episodes of pulmonary exacerbation have on the respiratory health and quality of life of CF patients, the reasons for their occurrence remain undetermined. This poor understanding of the underlying causes for the onset of CFPE hampers both the design of strategies to reduce the incidence of CFPE, and the ability to design and deliver effective, timely clinical therapy.

Antibiotic therapy represents a central component in the treatment of CFPE [1]. The use of antibiotics in this context is associated with an alleviation of respiratory symptoms, and improved patient outcome measures [11,31]. Despite the benefits associated with treatment strategies that might be expected to reduce bacterial numbers, there is no evidence that changes in the density of bacteria in the airways influence either the onset of CFPE or FEV_1.

It has been noted in at least one antibiotic trial that average improvement in lung function did not correlate well with changes in density of *P. aeruginosa* [31,32], and subjects in that trial in whom lung function improved with antibiotic treatment did not necessarily experience a drop in *P. aeruginosa* density (personal communication, B. Ramsey). Other studies have noted a poor correlation between bacterial density and measures of inflammation [33], and between these measures and either systemic symptoms or FEV_1 [12]. Nevertheless, a potential link between changes in bacterial numbers and the onset of CFPE remained plausible, influencing the manner in which antibiotics are usually administered, with high dose, combination therapy designed to maximise the reduction of viable bacterial cells in the airways. Whilst only one of many suggested triggers for the onset of CFPE, it is the possible link with an increase in the levels of the key CF pathogen *P. aeruginosa*, or airway bacteria generally, that is the focus of the study presented here.

Here, the start of an exacerbation was defined clinically as the initiation of antibiotic therapy administered in response to a worsening of respiratory symptoms, as used previously [29]. Whilst no universally accepted definition or measure exists to define the actual onset of an episode of CFPE [1,34], the start

Table 3
Summary of linear regressions fitted to trends in total bacterial and *P. aeruginosa* density prior to and at onset of CFPE, and total bacterial and *P. aeruginosa* density 21 and 0 days prior to onset of CFPE.

Patient	CFPE	Total bacteria						*P. aeruginosa*					
		Rate of change (Log_{10}-fold/day)	Significance (p)	Density (cfu/ml) on day −21	SD	Density (cfu/ml) on day 0	SD	Rate of change (Log_{10}-fold/day)	Significance (p)	Density (cfu/ml) on day −21	SD	Density (cfu/ml) on day 0	SD
1	1	−0.068	0.094	3.5×10^{10}	2.1×10^{9}	1.9×10^{9}	3.3×10^{7}	0.082	0.524	5.3×10^{3}	1.2×10^{3}	1.5×10^{5}	8.7×10^{4}
1	3	0.022	0.836	4.1×10^{7}	6.1×10^{6}	3.9×10^{8}	9.9×10^{6}	0	0.978	3.5×10^{6}	6.6×10^{5}	6.0×10^{6}	7.2×10^{5}
2	1	0	0.955	2.9×10^{9}	7.0×10^{7}	2.2×10^{9}	2.3×10^{8}	−0.004	0.889	6.1×10^{6}	3.6×10^{6}	3.2×10^{6}	1.1×10^{6}
2	2	−0.065	0.404	7.8×10^{10}	2.5×10^{9}	1.4×10^{9}	4.9×10^{8}	−0.011	0.596	8.7×10^{9}	2.0×10^{8}	3.6×10^{9}	5.3×10^{7}
3	1	0.034	0.026 *	1.1×10^{10}	1.4×10^{9}	5.0×10^{10}	2.5×10^{9}	−0.01	0.762	1.6×10^{5}	2.4×10^{4}	1.5×10^{5}	1.9×10^{4}
3	2	0.023	0.571	1.1×10^{9}	1.6×10^{7}	2.9×10^{9}	4.3×10^{7}	−0.028	0.125	2.0×10^{7}	2.0×10^{6}	5.2×10^{6}	1.6×10^{6}
4	1	0.087	0.289	5.1×10^{10}	2.2×10^{9}	5.9×10^{12}	1.9×10^{10}	−0.013	0.804	4.1×10^{8}	2.3×10^{7}	2.1×10^{8}	2.9×10^{6}
4	5	−0.002	0.918	2.3×10^{10}	3.4×10^{9}	1.7×10^{10}	2.9×10^{9}	−0.043	0.017 *	5.0×10^{9}	3.0×10^{8}	8.6×10^{8}	2.9×10^{7}
5	1	−0.018	0.101	7.3×10^{10}	6.9×10^{8}	3.7×10^{10}	1.8×10^{8}	−0.037	0.009 *	4.0×10^{8}	2.1×10^{8}	8.0×10^{7}	2.6×10^{7}
5	2	−0.035	0.194	2.8×10^{10}	1.8×10^{8}	5.6×10^{9}	2.6×10^{7}	−0.022	0.323	1.7×10^{9}	4.4×10^{8}	6.9×10^{8}	9.7×10^{7}
6	1	0.089	0.29	2.2×10^{8}	1.8×10^{7}	4.1×10^{10}	6.9×10^{9}	0.075	0.393	9.6×10^{5}	3.6×10^{5}	8.3×10^{7}	2.3×10^{6}
6	2	0.001	0.961	1.1×10^{10}	7.0×10^{9}	1.0×10^{10}	3.1×10^{9}	−0.011	0.637	3.2×10^{7}	2.0×10^{7}	2.0×10^{7}	2.6×10^{6}
6	3	−0.074	0.045 *	4.9×10^{10}	1.7×10^{9}	1.2×10^{9}	2.6×10^{6}	−0.013	0.663	6.0×10^{7}	4.4×10^{7}	3.8×10^{7}	9.7×10^{6}
7	1	0.038	0.275	3.9×10^{9}	5.4×10^{8}	2.8×10^{10}	1.5×10^{9}	0.117	0.047 *	7.3×10^{6}	4.2×10^{5}	2.1×10^{9}	1.1×10^{8}
7	2	−0.031	0.348	4.7×10^{10}	3.8×10^{9}	5.4×10^{9}	2.6×10^{8}	−0.016	0.707	7.6×10^{7}	8.3×10^{6}	1.8×10^{7}	5.2×10^{6}
7	3	0.025	0.572	4.5×10^{10}	6.2×10^{9}	3.8×10^{11}	8.0×10^{9}	0.036	0.329	8.1×10^{7}	6.1×10^{6}	5.3×10^{8}	9.8×10^{6}
7	4	0.001	0.959	1.5×10^{11}	3.7×10^{10}	1.7×10^{11}	4.5×10^{10}	−0.022	0.575	1.1×10^{9}	4.6×10^{7}	4.0×10^{8}	2.4×10^{7}
7	6	0.002	0.865	7.8×10^{10}	4.9×10^{9}	1.0×10^{11}	2.8×10^{10}	0	0.994	1.8×10^{9}	5.4×10^{7}	2.8×10^{9}	5.3×10^{7}
8	1	−0.086	0.046 *	6.7×10^{10}	9.9×10^{8}	5.1×10^{8}	2.1×10^{8}	−0.078	0.052	1.0×10^{9}	5.5×10^{7}	1.8×10^{7}	1.0×10^{7}
8	3	0.078	0.12	2.0×10^{8}	1.4×10^{8}	1.1×10^{10}	2.4×10^{9}	−0.011	0.459	6.7×10^{8}	1.6×10^{7}	5.6×10^{8}	8.9×10^{7}
9	1	0.069	0.245	3.1×10^{8}	1.8×10^{8}	3.0×10^{10}	1.1×10^{10}	0.12	0.256	9.0×10^{4}	4.4×10^{4}	2.6×10^{8}	1.2×10^{8}
9	2	−0.031	0.043 *	1.3×10^{10}	1.1×10^{10}	3.2×10^{9}	2.1×10^{9}	−0.039	0.065	6.3×10^{7}	2.3×10^{7}	1.1×10^{7}	4.7×10^{6}
10	1	0.036	0.371	2.7×10^{9}	9.9×10^{8}	2.6×10^{10}	2.0×10^{9}	0.061	0.639	2.2×10^{7}	2.7×10^{6}	4.0×10^{7}	2.7×10^{7}
10	3	−0.062	0.159	2.1×10^{10}	1.4×10^{10}	7.4×10^{8}	4.0×10^{8}	−0.059	0.105	1.2×10^{9}	1.0×10^{8}	5.6×10^{7}	4.5×10^{7}
11	1	−0.018	0.718	9.3×10^{9}	2.9×10^{9}	1.3×10^{8}	9.1×10^{7}	−0.009	0.799	2.6×10^{9}	2.9×10^{8}	7.6×10^{9}	4.7×10^{8}
11	2	−0.084	0.327	1.1×10^{11}	1.4×10^{10}	2.0×10^{10}	4.9×10^{9}	0.018	0.471	4.3×10^{8}	2.1×10^{8}	1.9×10^{8}	1.1×10^{8}
12	1	0.1	0.085	3.3×10^{8}	1.5×10^{8}	2.0×10^{10}	2.4×10^{8}	0.173	0.13	3.1×10^{4}	1.1×10^{4}	2.5×10^{7}	5.6×10^{5}
12	3	−0.03	0.386	3.3×10^{10}	9.2×10^{9}	6.2×10^{9}	5.6×10^{8}	−0.029	0.687	7.6×10^{9}	1.9×10^{7}	4.1×10^{9}	1.1×10^{6}

The table shows the slope and significance of the linear regressions. The slope, or rate of change, is expressed as the Log_{10}-fold change per day (the change in the Log_{10} density per day), with the sign of the fold change indicating an increasing or decreasing trend. Total bacterial and *P. aeruginosa* densities (and respective standard deviations (SD)) are expressed in cfu ml^{-1} of sputum on day −21 and 0 prior to the onset of CFPE, however, some sample points did not fall on these exact days prior to CFPE. The values for day −14 and day −7 are shown in Supplementary Table 1.
* $p<0.05$.

Table 4
Significant correlations between clinical variables, PRO scores and total bacterial density and *P. aeruginosa* density.

Correlation of clinical signs or PRO scores with	Correlate 1	Correlate 2	Pearson coefficient *R*	Significance (*p*)	n
Total bacterial numbers	Log Ps	Log Tot	−0.374	0.0001	132
	Temperature	Log Tot	−0.31	0.0001	127
Time	Sputum	Time	– 0.19	0.031*	128
	Cough	Time	– 0.207	0.019*	128
Self	Sputum	GWB	0.898	0.0001	128
	Sputum	Temperature	0.28	0.001	127
	Cough	Sputum	0.96	0.0001	128
	Cough	GWB	0.882	0.0001	128
	Cough	Temperature	0.224	0.011*	127
	Breath	Cough	0.827	0.0001	128
	Breath	Sputum	0.776	0.0001	128
	Breath	GWB	0.802	0.0001	128

Nine correlates (FEV_1, temperature, sputum production, cough severity, breathlessness, general well-being (GWB), time before CFPE, total bacterial (Log Tot) and *P. aeruginosa* (Log Ps) density) were tested for the pooled data for the 34 exacerbations analysed. Only significant correlations are shown ($p<0.05$). Bonferroni corrections were performed on the comparisons. Values marked * denote correlations where *p* would be greater than 0.05 after Bonferroni correction.

date of antibiotics for respiratory worsening is used often because it provides a clinically replicable, defined time point. Because changes in bacterial density could precede the symptomatic onset of an exacerbation, samples were selected to encompass a three-week period prior to antibiotic therapy, an interval that seemed adequate for capturing any related changes in bacterial density should they occur.

Culture-independent analysis of respiratory samples was performed using quantitative (Q)-PCR, which offers many potential advantages over cultivation-based approaches to bacterial enumeration, and has been employed extensively in a wide range of contexts [21]. Whilst a number of key issues must be considered when using these culture-independent techniques, such as the potential for incomplete cell lysis, nucleic acid degradation, and PCR bias or inhibition [35], Q-PCR has been shown to be accurate and reproducible in the analysis of CF airway samples [22,23]. Despite this, molecular quantification of viable bacteria in CF respiratory secretions is challenging due to the persistence of bacterial DNA from dead or lysed cells in airway mucus [24,36]. This DNA can contribute to PCR amplification, and thus result in an overestimation of the true bacterial density present. In order to remove this potentially confounding factor, clinical samples were pre-treated with PMA, a method demonstrated previously to restrict amplification to DNA templates derived only from viable bacterial cells [26,27]. Previous, culture-based, studies of CF respiratory secretions have reported between 10^6 and 10^9 colony forming unit equivalents per ml of sample [37,38]. The bacterial densities we observed using a Q-PCR-based approach agree well with those previous studies, with mean densities of $\sim 10^7$ and $\sim 10^{10}$ colony forming unit equivalents per ml for *P. aeruginosa* and total bacteria, respectively. Further, the difference between total bacterial and *P. aeruginosa* densities was not unexpected, given the many non-*Pseudomonas* species that are now known to be present in CF airway secretions, often outnumbering *P. aeruginosa* [39]. This study set out to determine whether a clonal expansion of bacteria colonising the CF airways was a trigger for the onset of CFPE. We identified no significant differences in total bacterial or *P. aeruginosa* density amongst any of the sampling points during the 3 weeks prior to antibiotics being administered, or between any of those points and mean values for the entire study. Whilst no evidence was found for a general trend in bacterial density over the period leading up to treatment, we did observe significant changes during this period in individual patients. Nevertheless, the direction of these changes varied, and they did not occur consistently for given patients, indicating that they were unlikely to be causally related to exacerbations. However, in patients where no trend was observed, it is possible that relatively high bacterial densities masked variations, that whilst substantial, represented only a small proportion of the total bacteria present.

The findings presented here also indicate little correlation between bacterial density and either PRO measures or other clinical variables. The only significant correlation found was that between temperature and total bacterial density. Whilst a decrease in FEV_1 is often used clinically as an indication of short-term decline in respiratory health, the absence of such a decline seen here (regardless of airway disease severity), was consistent with previous studies that demonstrated poor correlation with exacerbation onset [40].

To the best of our knowledge, this is the first culture-independent investigation of bacterial density in serially collected sputum in the period preceding CFPE. The patient group studied here was of limited size. Further, patients were selected who had moderate to severe lung disease (as indicated by low FEV_1) in anticipation of a high likelihood of exacerbation, and as such, do not represent a cross-section of CF patients. However, three separate statistical analyses each failed to detect significant trends, differences or correlations in the relationship between bacterial density and onset of CFPE.

The data presented here suggest that the airway densities of *P. aeruginosa* and/or total bacterial cells do not rise consistently in the period prior to the onset of CFPE. However, if the volume of sputum produced during this period increased, this might have the effect of masking an increase in bacterial numbers due to a dilution effect. The patient reported levels of sputum production indicate a rise during this time. Nevertheless, the impact of any such increase on bacterial density data is unknown, and would have to consistently mirror changes in bacterial numbers in order to prevent detection of bacterial trends. Previous work suggests that expectorated sputum rates change less than 10 fold between CFPE and clinical stability [41]. However, the total volumes of sputum produced by CF patients in the period prior to CFPE onset warrants further investigation. It is possible that, whilst no trends in total bacterial abundance were detected here, changes in the abundance of individual non-pseudomonal species that represent only a small proportion of the total bacterial community, could play a role in precipitating CFPE. This possibility could be

investigated through the application of a wider range of species-specific assays. Furthermore, investigation of changes in levels of inflammatory or microbial biomarkers in the period prior to exacerbation might provide additional insight into the mechanisms of CFPE onset, and the role played by bacterial density in this [42].

An increase in bacterial density in CF airway secretions is only one proposed trigger for CFPE [11,43]. Changes in the bacterial mode of growth [44], the occurrence of respiratory viral infections [4,8,45,46], or changes in the expression of virulence [47], might also be triggers of CFPE. For example, certain *P. aeruginosa* virulence factors have been shown to be elevated during periods of CFPE [47]. Factors such as these could still underlie the observed impact of antibiotic treatment during CFPE [43], but go undetected by the analytical approaches employed here. Analysis of bacterial gene expression, as well as the investigation of acute viral infections, are therefore likely to be important to include in future studies.

The data presented here challenge the model that periods of CFPE are generally precipitated by a change in bacterial density in the CF lower airway secretions. Given the focus of both short and long-term antibiotic therapy in CF care on reducing bacterial load, these findings necessitate a reappraisal of the way in which antibiotic strategies are designed. Further, identification of factors that do trigger the onset of CFPE would provide both novel opportunities for therapeutic intervention and the biomarkers that are predictive of such episodes. As such, this represents an important area of continuing investigation.

Conflict of interest

There are no existing conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jcf.2011.05.002.

References

[1] Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med 2003;168:918–51.
[2] Goss CH, Burns JL. Exacerbations in cystic fibrosis. 1: epidemiology and pathogenesis. Thorax 2007;62:360–7.
[3] Britto MT, Kotagal UR, Hornung RW, Atherton HD, Tsevat J, Wilmott RW. Impact of recent pulmonary exacerbations on quality of life in patients with cystic fibrosis. Chest 2002;121:64–72.
[4] Balfour-Lynn IA, Elborn JS. Respiratory disease: infection. In: Hodson M, Geddes D, Bush A, editors. Cystic fibrosis. 3rd Edn. London: Hodder Arnold; 2007. p. 137–58.
[5] Liou TG, Adler FR, Fitzsimmons SC, Cahill BC, Hibbs JR, Marshall BC. Predictive 5-year survivorship model of cystic fibrosis. Am J Epidemiol 2001;153:345–52.
[6] Konstan MW, Morgan WJ, Butler SM, et al. Risk factors for rate of decline in forced expiratory volume in one second in children and adolescents with cystic fibrosis. J Pediatr 2007;151:134–9 139.e1.
[7] Sanders DB, Hoffman LR, Emerson J, et al. Return of FEV1 after pulmonary exacerbation in children with cystic fibrosis. Pediatr Pulmonol 2010;45:127–34.
[8] van Ewijk BE, van der Zalm MM, Wolfs TF, van der Ent CK. Viral respiratory infections in cystic fibrosis. J Cyst Fibros 2005;4(Suppl 2):31–6.
[9] Jubin V, Ranque S, Stremler Le Bel N, Sarles J, Dubus JC. Risk factors for Aspergillus colonization and allergic bronchopulmonary aspergillosis in children with cystic fibrosis. Pediatr Pulmonol 2010;45:764–71.
[10] Goss CH, Newsom SA, Schildcrout JS, Sheppard L, Kaufman JD. Effect of ambient air pollution on pulmonary exacerbations and lung function in cystic fibrosis. Am J Respir Crit Care Med 2004;169:816–21.
[11] Regelmann WE, Elliott GR, Warwick WJ, Clawson CC. Reduction of sputum *Pseudomonas aeruginosa* density by antibiotics improves lung function in cystic fibrosis more than do bronchodilators and chest physiotherapy alone. Am Rev Respir Dis 1990;141:914–21.
[12] Smith AL, Redding G, Doershuk C, et al. Sputum changes associated with therapy for endobronchial exacerbation in cystic fibrosis. J Pediatr 1988;112:547–54.
[13] Gold R, Overmeyer A, Knie B, Fleming PC, Levison H. Controlled trial of ceftazidime vs. ticarcillin and tobramycin in the treatment of acute respiratory exacerbations in patients with cystic fibrosis. Pediatr Infect Dis 1985;4:172–7.
[14] Saiman L, Marshall BC, Mayer-Hamblett N, et al. Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: a randomized controlled trial. JAMA 2003;290:1749–56.
[15] Pye A, Stockley RA, Hill SL. Simple method for quantifying viable bacterial numbers in sputum. J Clin Pathol 1995;48:719–24.
[16] Schneider M, Muhlemann K, Droz S, Couzinet S, Casaulta C, Zimmerli S. Clinical characteristics associated with isolation of small-colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. J Clin Microbiol 2008;46:1832–4.
[17] Colwell RR. Viable but nonculturable bacteria: a survival strategy. J Infect Chemother 2000;6:121–5.
[18] Rogers GB, Daniels TT, Tuck A, et al. Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. BMC Pulm Med 2009;9:14.
[19] Fenollar F, Raoult D. Molecular diagnosis of bloodstream infections caused by non-cultivable bacteria. Int J Antimicrob Agents 2007;30(Suppl 1):S7–S15.
[20] Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 2002;148:257–66.
[21] Espy MJ, Uhl JR, Sloan LM, et al. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 2006;19:165–256.
[22] Deschaght P, De Baere T, Van Simaey L, et al. Comparison of the sensitivity of culture, PCR and quantitative real-time PCR for the detection of *Pseudomonas aeruginosa* in sputum of cystic fibrosis patients. BMC Microbiol 2009;29:244.
[23] Zemanick ET, Wagner BD, Sagel SD, Stevens MJ, Accurso FJ, Harris JK. Reliability of quantitative real-time PCR for bacterial detection in cystic fibrosis airway specimens. PLoS One 2010;5:e15101.
[24] Robinson M, Bye PT. Mucociliary clearance in cystic fibrosis. Pediatr Pulmonol 2002;33:293–306.
[25] Nocker A, Sossa KE, Camper AK. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J Microbiol Methods 2007;70:252–60.
[26] Rogers GB, Stressmann FA, Koller G, Daniels T, Carroll MP, Bruce KD. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. Diagn Microbiol Infect Dis 2008;62:133–41.
[27] Rogers GB, Marsh P, Stressmann AF, et al. The exclusion of dead bacterial cells is essential for accurate molecular analysis of clinical samples. Clin Microbiol Infect 2010, doi:10.1111/j.1469-0691.2010.03189.x.
[28] Proesmans M, Balinska-Miskiewicz W, Dupont L, et al. Evaluating the "Leeds criteria" for *Pseudomonas aeruginosa* infection in a cystic fibrosis centre. Eur Respir J 2006;27:937–43.
[29] Fuchs HJ, Borowitz DS, Christiansen DH, et al. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and

on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. N Engl J Med 1994;331:637–42.
[30] Shannon KE, Lee DY, Trevors JT, Beaudette LA. Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Sci Total Environ 2007;382:121–9.
[31] McLaughlin FJ, Matthews Jr WJ, Strieder DJ, et al. Clinical and bacteriological responses to three antibiotic regimens for acute exacerbations of cystic fibrosis: ticarcillin–tobramycin, azlocillin–tobramycin, and azlocillin–placebo. J Infect Dis 1983;147:559–67.
[32] Ramsey BW, Pepe MS, Quan JM, et al. Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. Cystic fibrosis inhaled tobramycin study group. N Engl J Med 1999;340:23–30.
[33] Gibson RL, Emerson J, McNamara S, et al. Significant microbiological effect of inhaled tobramycin in young children with cystic fibrosis. Am J Respir Crit Care Med 2003;167:841–9.
[34] Dakin C, Henry RL, Field P, Morton J. Defining an exacerbation of pulmonary disease in cystic fibrosis. Pediatr Pulmonol 2001;31:436–42.
[35] Roudiere L, Lorto S, Tallagrand E, Marchandin H, Jeannot JL. Jumas-Bilak. Molecular fingerprint of bacterial communities and 16S rDNA intra-species heterogeneity: a pitfall that should be considered. Pathol Biol 2007;55:434–40.
[36] Potter J, Matthews LW, Lemm J, Spector S. Composition of pulmonary secretions from patients with and without cystic fibrosis. Am J Dis Child 1960;100:493–5.
[37] Tummler B, Bosshammer J, Breitenstein S, et al. Infections with *Pseudomonas aeruginosa* in patients with cystic fibrosis. Behring Inst Mitt 1997;98:249–55.
[38] Aitken ML, Greene KE, Tonelli MR, et al. Analysis of sequential aliquots of hypertonic saline solution-induced sputum from clinically stable patients with cystic fibrosis. Chest 2003;123:792–9.
[39] Tunney MM, Field TR, Moriarty TF, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med 2008;177:995–1001.
[40] Rosenfeld M, Emerson J, Williams-Warren J, et al. Defining a pulmonary exacerbation in cystic fibrosis. J Pediatr 2001;139:359–65.
[41] Smith JA, Owen EC, Jones AM, Dodd ME, Webb AK, Woodcock A. Objective measurement of cough during pulmonary exacerbations in adults with cystic fibrosis. Thorax 2006;61(5):425–9.
[42] Rogers GB, Hoffman LR, Johnson MW, et al. Using bacterial biomarkers to identify early indicators of cystic fibrosis pulmonary exacerbation onset. Expert Rev Mol Diagn 2011;11(2):197–206.
[43] Moskowitz SM, Foster JM, Emerson J, et al. Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. J Clin Microbiol 2004;42(suppl 2):1915–22.
[44] VanDevanter DR, Van Dalfsen JM. How much do Pseudomonas biofilms contribute to symptoms of pulmonary exacerbation in cystic fibrosis? Pediatr Pulmonol 2005;39(suppl 6):504–6.
[45] Hiatt PW, Grace SC, Kozinetz CA, et al. Effects of viral lower respiratory tract infection on lung function in infants with cystic fibrosis. Pediatrics 1999;103(suppl 3):619–26.
[46] Chattoraj SS, Ganesan S, Jones AM. Rhinovirus infection liberates planktonic bacteria from biofilm and increases chemokine responses in cystic fibrosis airway epithelial cells. Thorax 2011;66:333–9.
[47] Jaffar-Bandjee MC, Lazdunski A, Bally M, et al. Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by *Pseudomonas aeruginosa*. J Clin Microbiol 1995;33(suppl 4):924–9.
[48] Stressmann FA, Rogers GB, Daniels T, et al. Are pulmonary exacerbations triggered by an increase in bacterial load? Pediatr Pulmon 2009;Suppl. 32: 331-331.