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Original Article

Acute effects of viral respiratory tract infections on sputum bacterial density during CF pulmonary exacerbations

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Abstract

Background: Airway proliferation of *Pseudomonas aeruginosa* bacteria is thought to trigger CF exacerbations and may be affected by the presence of viral infections.
Methods: A 2-year prospective study was conducted on 35 adults with CF. *P. aeruginosa* sputum density was analyzed during stable, exacerbation and post exacerbation assessments. Upon exacerbation, samples were sent for PCR detection of respiratory viruses and the sputum density of *P. aeruginosa* in patients with a viral infection versus those without was compared.
Results: Twenty-two patients experienced 30 exacerbations during the study period; 50% were associated with a viral infection. There was no change in sputum density of *P. aeruginosa* from the stable to exacerbation state when measured by quantitative culture or by PCR. Virus-associated exacerbations did not result in significant increases in *P. aeruginosa* sputum density compared to non-viral exacerbations.
Conclusion: Sputum density of *P. aeruginosa* was not increased at the time of CF exacerbation and was not influenced by the presence of viral infection.

Keywords: Cystic fibrosis; *Pseudomonas aeruginosa*; Viral infection; CF exacerbation

1. Introduction

Lung disease in cystic fibrosis (CF) is characterized by chronic bacterial airway infection punctuated by acute deteriorations termed pulmonary exacerbations. Pulmonary exacerbations are defined clinically by a combination of signs and symptoms such as increased cough, sputum purulence, dyspnea, and weight loss [1]. CF exacerbations have been associated with declining lung function, have a negative impact on quality of life, and are associated with significant cost [2–4]. In addition, frequent exacerbations are associated with an increased risk of death and lung transplant [3].

The pathophysiology of exacerbations remains unclear. They have been linked with clonal expansion of existing bacteria rather than acquisition of new bacteria [5], and it has been hypothesized that exacerbations might be caused by increased airway bacteria proliferation and changes in bacterial phenotype [1,6]. Some studies have found that sputum bacterial density decreases following exacerbations as patients improve clinically

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with antibiotics [7–12]. These findings lead to the hypothesis that an increase in bacterial density immediately before, or at the time of exacerbation, might contribute to the pathophysiology of exacerbation. However, prospective studies investigating this hypothesis are relatively lacking.

Viral infections are thought to be triggers of CF exacerbations, though their prevalence in studies varies from 5 to 68% [13–16]. Exacerbations caused by viral infections have been associated with longer courses of antibiotics and worsening lung function [15,17]. In addition, patients with virus-associated exacerbations have been reported to have more severe clinical presentations and a shorter duration to the next exacerbation than those with non-virus associated exacerbations [15].

The evidence suggesting that viral infections are associated with changes in bacterial density in the lungs is relatively scanty. In a study of 17 adult patients with CF and chronic bacterial infection, viral infections were associated with a small increase in sputum bacterial density at the time of CF exacerbation [13]. An association between viral infection and bacterial burden has also been shown in other respiratory illness such as chronic pulmonary obstructive disease (COPD). Experimental rhinovirus infection in patients with COPD resulted in an increase in sputum bacterial density, which peaked 15 days post-rhinovirus infection [18,19]. These results suggest that viral infection may be instrumental in precipitating secondary bacterial infections.

The objective of this study was to determine if there was a change in the sputum density of *Pseudomonas aeruginosa* at the time of pulmonary exacerbation compared to the stable state. In addition, we wanted to determine whether changes in bacterial sputum density were affected by the presence of acute viral respiratory tract infections.

2. Methods

2.1. Study design and participants

This was a two-year prospective observational study of adult CF patients who were evaluated during stable, exacerbation, and post-exacerbation states. Subjects were recruited from the CF clinic at The Ottawa Hospital starting in June 2012 and were followed until April 2014. Adult patients (>18 year) with confirmed CF who were able to spontaneously produce sputum and had a history of chronic infection with *P. aeruginosa* (at least 2 positive sputum cultures for *P. aeruginosa* in the 12 month period before study entry) were recruited. Ethics approval for this study was obtained from the Ottawa Hospital Research Ethics Board in accordance with the amended Declaration of Helsinki and informed written consent was obtained from all subjects.

Patients were enrolled in the study when stable and were reassessed every 3 months. At each visit, patients provided a sputum sample for quantitative bacterial density assessment and RT-qPCR (reverse transcription real-time polymerase chain reaction) for virus detection. During episodes of worsening respiratory symptoms, patients were instructed to contact the CF staff. The patients were assessed to determine if they met criteria for a pulmonary exacerbation defined by criteria published by the 1994 Cystic Fibrosis Foundation Microbiology and Infectious Disease Consensus Conference [20].

After confirmation of a CF exacerbation, the patient was asked to provide a spontaneous sputum sample, and a nasopharyngeal (NP) swab was taken for respiratory virus assessment. Patients were then treated with antibiotics if necessary and asked to return a minimum of 30 days later for a post-exacerbation assessment.

2.2. Quantitative bacterial culture of sputum

Quantitative cultures were processed within 4 h of collection at the Special Microbiology Laboratory at the Children's Hospital of Eastern Ontario as per Burns et al. using serial dilution [21]. Diluted samples were inoculated onto MacConkey agar and *Pseudomonas* Cetrimide Agar. The number of colony forming units (CFU) per milliliter (ml) of sputum was determined by colony counts.

2.3. Nucleic acid extraction for PCR

Sputum samples were diluted 1:10 with sterile Dulbecco's Phosphate Buffered Saline (D-PBS, 1×), vortexed to mix, and then centrifuged at 11,000 rpm to bring down particulates. Nucleic acids (DNA and RNA) were then extracted from 400 μl of the sputum supernatant using an automated extraction device (iPrep, Life Technologies, Carlsbad, CA) in a final elution volume of 100 μl. The bacteriophage virus MS2 (Zeptometrix Corp., Buffalo, NY) was added to each sample prior to nucleic acid extraction as an amplification and extraction control.

2.4. Real-time PCR for P. aeruginosa

A previously published 5′ exonuclease probe assay targeting the *P. aeruginosa ecfX* (extra cytoplasmic function sigma factor) was used to detect *P. aeruginosa* in the sputum samples by real-time PCR [22].

PCR was performed in 10 μl volumes in 96-well PCR plates using TaqMan Fast Advanced master mix (Life Technologies). Thermocycling was performed using a ViiA7 thermocycler (Life Technologies) in a fast mode, with an initial 20 seconds (s) at 95 °C followed by 40 cycles of two-temperature cycling at 95 °C for 3 s, followed by 60 °C for 30 s. Cycle threshold (Ct) values were obtained using the ViiA7 software, and then exported to Excel for analysis.

2.5. PCR for detection of viruses

A real-time PCR and RT-qPCR panel was used to test NP swabs and sputum samples for all major viral respiratory pathogens including: rhinoviruses, enteroviruses, influenza A and B, respiratory syncytial virus (RSV) A and B, human metapneumovirus A and B, parainfluenza virus 1, 2, 3, and 4, coronavirus OC43, coronavirus NL63, coronavirus HKU1, coronavirus 229E, bocavirus, and adenoviruses.

RT-qPCR was performed using a panel of 5′ exonuclease probe assays. The performance of these assays has been validated using molecular proficiency testing specimens and clinical samples

(manuscript submitted for publication to World Journal of Pediatrics). Briefly, each assay was mixed with TaqMan Fast Virus 1-Step master mix (Life Technologies) and the nucleic acid sample was placed in a 10 μl reaction volume in 96-well plates (Life Technologies). Thermocycling was performed using the ViiA7 thermocycler. Initially, a temperature of 50 °C was held for 5 min for reverse transcription, followed by 40 cycles of two-temperature cycling at 95 °C for 3 s, followed by 60 °C for 30 s. Ct values were obtained and exported to Excel for analysis.

2.6. Statistical analysis

Colony counts measured by quantitative culture were analyzed using a $\log_{10}$ scale and data were tested for normality. The colony counts and PCR Ct at stable, exacerbation, and post exacerbation assessments, were compared using paired t-tests. The colony counts at exacerbation were compared to the stable assessment immediately preceding it. Generalized estimating equation analysis was conducted to compare viral vs. non-viral exacerbations to account for patients who experienced both types of exacerbations. Data are presented as means with 95% confidence intervals. Statistical tests were two sided, and significance of 0.05 was applied.

3. Results

Thirty-five patients with chronic *P. aeruginosa* airway infection were enrolled and were followed for an average of 533 ± 102 days. Twenty-two of these patients had a total of 30 exacerbations during the study period; with a maximum of 2 exacerbations per patient. The mean time from the most recent stable visit until exacerbation was 82 ± 65 days, and from exacerbation to post-exacerbation assessment was 115 ± 70 days. Patient demographic information can be found in Table 1.

Table 1
Baseline characteristics of study population.

Characteristic	All enrolled patients N = 35	Patients with exacerbations N = 22
Age (mean ± SD)	32.5 ± 12.6	32 ± 14.1
Sex, n (%)		
• Female	15 (43)	12 (55)
• Male	20 (57)	10 (45)
FEV1 (mean % predicted ± SD)	56.3 ± 22.4	54.7 ± 21.5
• Mild (≥80%) n (%)	6 (17)	4 (18)
• Moderate (50–79%) n (%)	15 (43)	7 (32)
• Severe (30–49) n (%)	10 (29)	9 (41)
• Very severe (<30) n (%)	4 (11)	2 (9)
BMI (mean ± SD)	22.2 ± 3.1	21.4 ± 5.4
Medications, n (%)		
• Inhaled tobramycin [a]	26 (74)	18 (82)
• Dornase alpha	10 (29)	6 (27)
• Inhaled colistin	3 (9)	3 (14)
• Azithromycin	23 (66)	16 (73)
Comorbidities, n (%)		
• CF-related diabetes	7 (20)	5 (23)
• Pancreatic insufficiency	34 (97)	21 (95)
• Liver disease	5 (14)	3 (14)
Colonized bacteria, n (%)		
• *P. aeruginosa*	35 (100)	22 (100)
• *S. aureus*	10 (29)	2 (9)
• *B. cepacia*	0	0
• *H. influenzae*	0	0
• *S. maltophilia*	0	1 (5)
• *Aspergillus* species	1 (3)	1 (5)
• Other	3 (9)	2 (9)
Mean bacterial density of *P. aeruginosa* during stable period (CFU/ml)	5.75×10^6	6.1×10^6

[a] Inhaled tobramycin may represent inhaled tobramycin, TOBI inhalational solution or TOBI podhaler.

Signs and symptoms present during the exacerbations, and differences between virus and non-virus associated exacerbations are shown in Fig. 1. All patients fulfilled criteria for a CF exacerbation.

At the time of exacerbation three (10%) patients required hospitalization and were treated with intravenous (IV) antibiotics. An additional 5 (17%) patients were treated with IV antibiotics at home, 19 (63%) were treated with oral antibiotics and 3 (10%) did not require treatment.

Three exacerbations were excluded from the quantitative bacterial sputum density calculations; two patients did not grow *P. aeruginosa* at the time of exacerbation, and one sample had insufficient volume. Fig. 2 illustrates the change in *P. aeruginosa* bacterial density in sputum collected during stable, pulmonary exacerbation, and post-exacerbation assessments via quantitative culture. There was no significant change in colony counts from stable state to exacerbation; the mean within-patient difference was 0.014 $\log_{10}$ CFU/ml (95% CI −0.51 to 0.53, p = 0.95). There was similarly no significant change in *P. aeruginosa* sputum density from steady state to exacerbation as measured by PCR. The mean change in Ct was −0.31 cycles (95% CI −1.35 to 0.73, p = 0.56) where a negative value indicates a higher bacterial density in the exacerbation sample than in the stable sample.

There was no significant change in bacterial density from exacerbation to post-exacerbation state. The mean within patient change in sputum density from exacerbation to the post-exacerbation visit was 0.20 $\log_{10}$ CFU/ml (95% CI −0.47 to 0.86) (p = 0.57). Seven patients did not return for post-exacerbation assessments and were excluded from this analysis.

Statistical analysis comparing changes in sputum bacterial density by quantitative culture against changes in nucleic acid detection by quantitative PCR showed a moderate correlation (Pearson Correlation Coefficient 0.38, p = 0.048).

Thirty exacerbations had paired sputum taken at baseline and at the time of exacerbation for analysis of viral infection. At baseline, we could only detect viral DNA/RNA by RT-qPCR in the sputum from one stable state sputum sample (coronavirus HKU1). In contrast at the time of exacerbation, respiratory viruses were detected in the sputum or NP swab from 15 of the 30 exacerbation samples. The viruses detected were rhinovirus (67%), RSV type A (13%), PIV type 3 (7%), corona HKU1 (7%) and corona NL63 (7%). Three viruses were detected in an NP swab only, 6 in the sputum only and 6 were detected in both the NP swab and sputum. The patient who was found to have

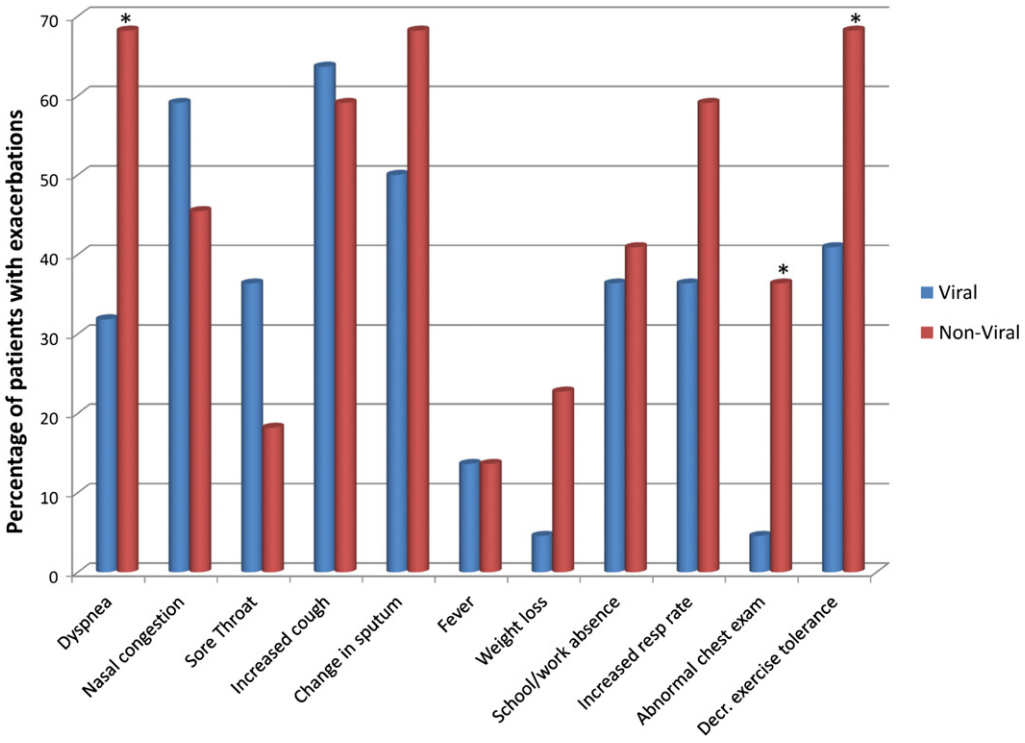

Fig. 1. Comparison of signs & symptoms of CF pulmonary exacerbations in patients with viral and non-viral exacerbations. Blue columns indicate patients with viral exacerbations, and red columns indicate patients with non-viral exacerbations. * indicates exacerbation criteria where the difference in the two groups was statistically significant. Those patients with virus-triggered exacerbations experienced significantly less dyspnea ($p = 0.009$), and were less likely to have a decreased exercise tolerance ($p = 0.0195$) or new findings on respiratory exam ($p = 0.0147$).

coronavirus HKU1 at stable state did not have coronavirus or any other virus detected at the time of his exacerbation.

Subgroup analysis showed that there was no significant difference in the change in *P. aeruginosa* sputum density between viral and non-viral exacerbations when measured by quantitative culture and PCR (Figs. 3 and 4). By quantitative culture, the difference in the average change in colony counts of *P. aeruginosa* was 0.59 $\log_{10}$ CFU/ml (95% CI −0.51 to 1.68;

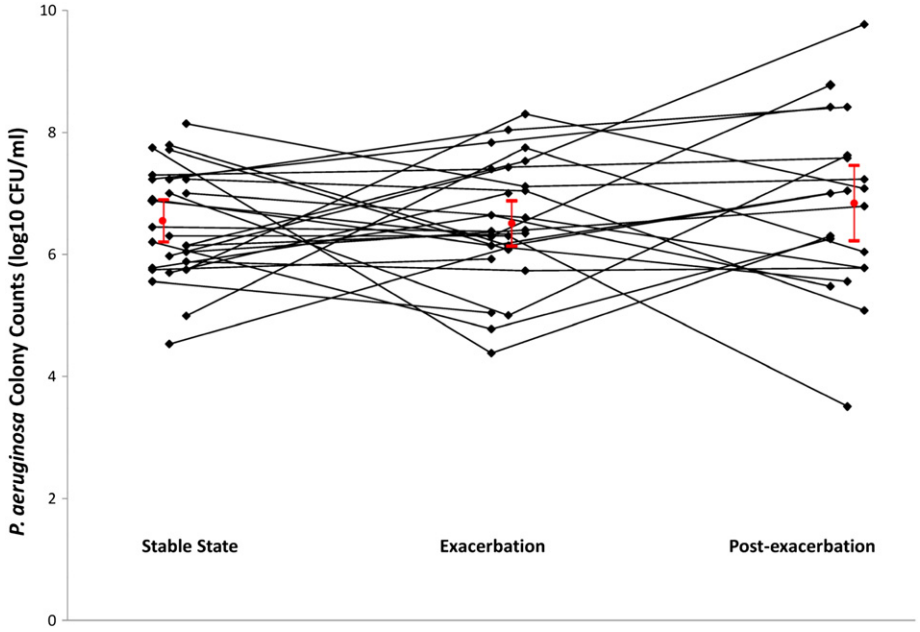

Fig. 2. Comparison of *P. aeruginosa* colony counts in sputum during stable, exacerbation, and post-exacerbation assessments. The black circles represent individual sputum samples at exacerbation while mean colony counts at each time point are shown in red circles.

p = 0.27) between the viral and non-viral groups. By PCR Ct value comparison, the mean between-group difference was 1.15 cycles (95% CI −1.03 to 3.38; p = 0.33).

4. Discussion

The results of our study suggest that bacterial density of *P. aeruginosa* is not increased when patients present with a CF exacerbation compared to when they are in a stable state. While respiratory viruses were commonly detected during CF exacerbations, those exacerbations associated with viral infections were not characterized by greater changes in bacterial density of *P. aeruginosa* compared to non-viral associated exacerbations.

To date, few studies have investigated whether sputum bacterial density increases at the time of CF exacerbation. To our knowledge, three studies have been published recently that also addressed this question. The first, by Stressman et al., measured the bacterial density of *P. aeruginosa* at the time of exacerbation and weekly for three weeks prior. They found no change in sputum bacterial density [23]. The second publication by Carmody et al. found no change in total or *P. aeruginosa* bacterial density in the sputum between steady state and exacerbation where the mean time between these states was 84 days [24]. Lastly, Fothergill et al. measured the sputum bacterial density of *P. aeruginosa* by qPCR in 10 patients over a two-year period. At the time of exacerbation, the sputum bacterial density of *P. aeruginosa* did not consistently change [25].

Post-exacerbation bacterial density of *P. aeruginosa* in sputum has been found to be decreased in multiple studies [7–12]. In Ordoñez et al., the density of *P. aeruginosa* in sputum decreased by 2.4 ± 3.1 $\log_{10}$ CFU/g after 14 days of antibiotics [7]. These results are consistent with a study published by Tunney et al. which found that sputum bacterial density of *P. aeruginosa* decreased by 89% immediately following antibiotic therapy, and returned to baseline levels 137 days later [8]. Finally, in a study published by Regelmann et al., parenteral antibiotic therapy resulted in a significant decrease in the sputum density of *P. aeruginosa*. However, 2 weeks after therapy was discontinued, the sputum density of *P. aeruginosa* was the same as at the time of acute exacerbation [9]. The combination of these studies, and ours, which measured bacterial density an average of 115 days post-exacerbation and showed no change compared to the time of exacerbation, provides evidence that the decrease in bacterial density in the sputum of CF patients associated with antibiotic therapy for exacerbation is a transient phenomenon.

The second question we sought to answer with this study was whether the presence of a viral infection at the time of CF exacerbation influences the bacterial density in sputum. Viruses are well recognized as triggers of exacerbations of airway diseases including COPD, asthma, and CF [13–19,26]. The frequency of virus associated exacerbations in our study was 50%. While somewhat higher than previously reported, this is

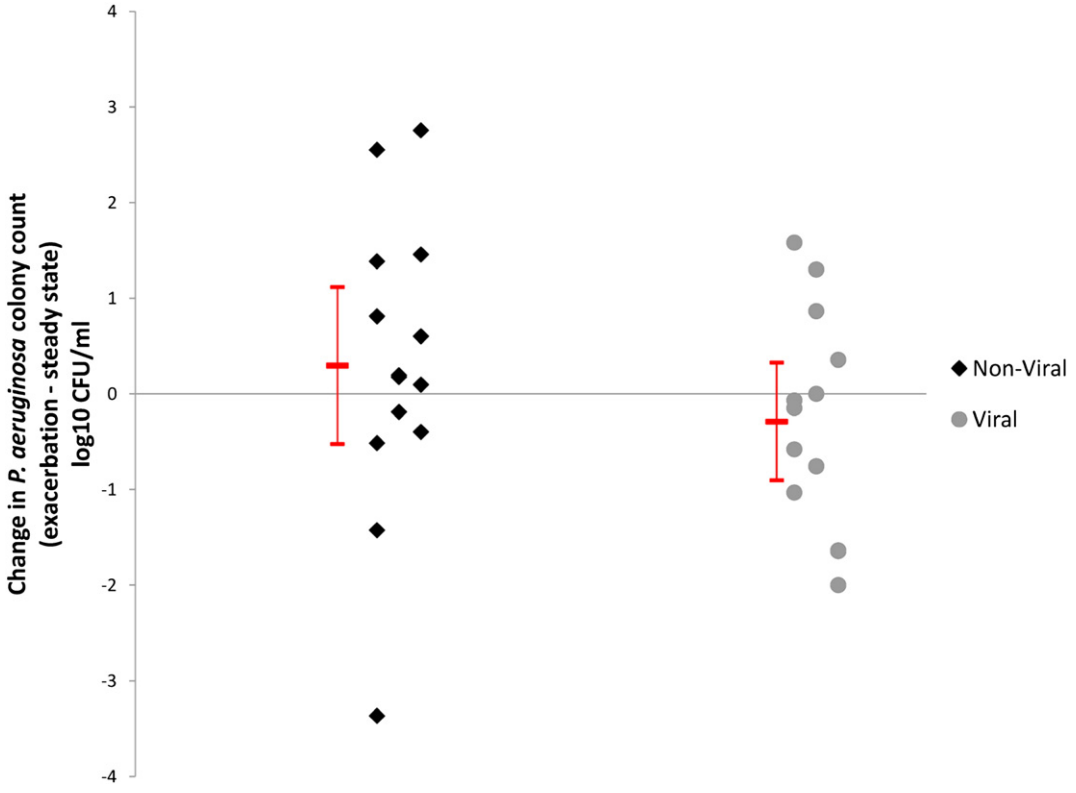

Fig. 3. Comparison of the change in *P. aeruginosa* colony counts from stable to exacerbation states, in viral and non-viral exacerbations (reported as $\log_{10}$ CFU/ml). Mean change (red bar) for non-viral exacerbations was 0.30 $\log_{10}$ CFU/ml (95% CI −0.52 to 1.11); mean change for viral exacerbations was −0.29 $\log_{10}$ CFU/ml (95% CI −0.90 to 0.33).

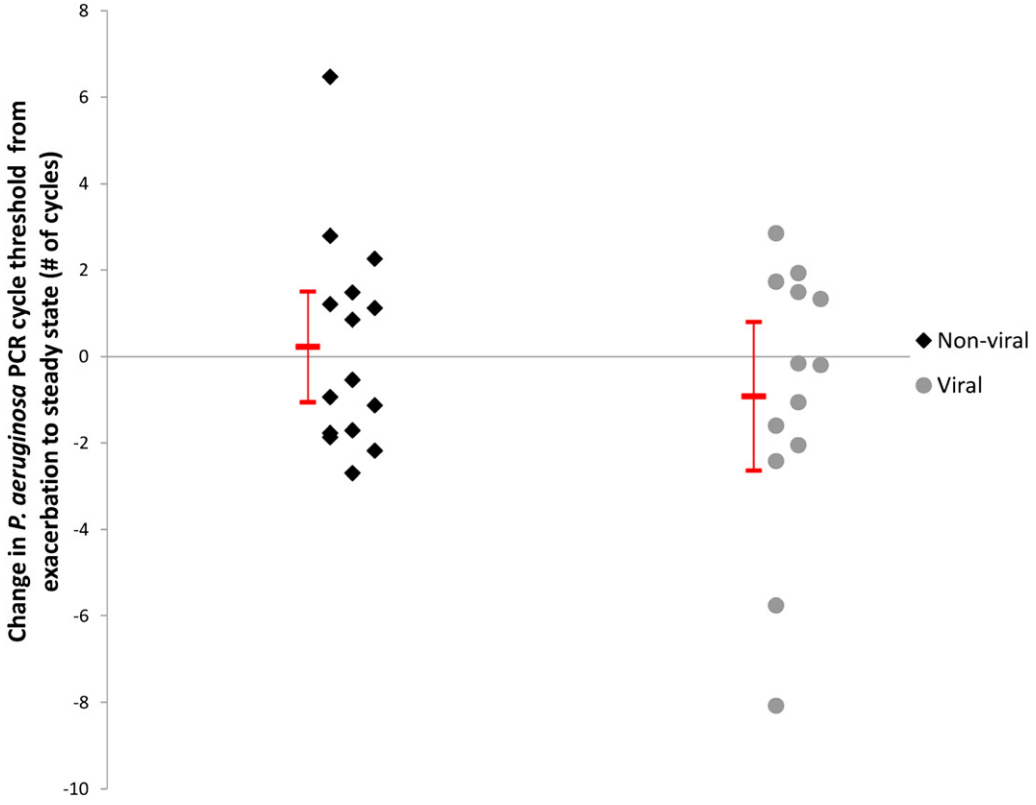

Fig. 4. Comparison of the change in *P. aeruginosa* PCR cycle threshold from stable to exacerbation states, in viral and non-viral exacerbations. Mean change (red bar) for non-viral exacerbations was 0.22 cycles (95% CI −1.06 to 1.50); mean change for viral exacerbations was −0.92 cycles (95% CI −2.64 to 0.80).

consistent with other studies that used sensitive viral PCR detection techniques [27].

In a study performed by Wark et al. of 17 adult patients with CF (13 of whom were chronically infected with *P. aeruginosa*), sputum bacterial density increased slightly but significantly, by a one-fold log change, during exacerbations triggered by viruses when compared to baseline, whereas no changes in sputum bacterial density were seen in non-viral associated exacerbations [13]. Our study did not show the same result. In our study, there was a statistically insignificant 0.58-log difference in the change in sputum density of *P. aeruginosa* when comparing virus associated exacerbations to those not associated with viruses, and in contrast to the Wark study, non-viral exacerbations exhibited a trend to greater increases in sputum density comparatively. In both studies rhinovirus was the most frequent virus implicated. The reasons for the different results seen in these two studies might be due to differences in bacteria; our study was limited to patients chronically infected with *P. aeruginosa*, whereas the Wark study was not. Differing results may also be explained by the differences in severity of exacerbations studied or imprecision related to relatively small sample sizes.

Our study has a few limitations. The first is a relatively small sample size which may have impeded our ability to detect a significant change in bacterial density during pulmonary exacerbations, as well as a difference in those exacerbations that were associated with viruses. The second is that the effects of viral infection on bacterial proliferation might be delayed and occur days after exacerbations present clinically. In patients with COPD infected with rhinovirus, peak bacterial density was on day 15 following infection. These rhinovirus-associated COPD exacerbations were mild in severity, and these patients were not treated with corticosteroids or antibiotics [19]. In CF exacerbations the possibility of delayed bacterial proliferation following a virus-associated exacerbation would be difficult to investigate since antibiotics are a standard therapy at presentation and are rarely withheld. Finally, while viruses do not appear to affect the sputum density of *P. aeruginosa*, this does not preclude other pathogen–pathogen interactions such as effects of viral infections on bacterial phenotype and expression of bacterial virulence factors. For example, in a study where human CF bronchial cells were co-infected with RSV and *P aeruginosa*, direct binding of RSV to *P. aeruginosa* was observed, and *P aeruginosa* was found to exhibit increased adherence to human CF cells in vitro in the presence of RSV [28]. Such interactions were not investigated in our study, and further research on viral–bacterial interactions in CF lung disease is needed.

A final potential limitation is that some of the patients included in our study experienced mild pulmonary exacerbations. Interestingly, in a study by Reid et al., while the majority of patients improved clinically with antibiotics, those patients with a decrease in their sputum density of *P. aeruginosa* upon completion of treatment were found to have the greatest

improvement in their lung function [12]. One hypothesis generated from this finding might be that the changes to bacterial sputum density are greater with more severe exacerbations and might explain why we did not observe a change with the inclusion of mild exacerbations.

5. Conclusion

In conclusion, our study did not show evidence of significant changes in sputum density of *P. aeruginosa* at the time of CF exacerbation compared to the stable state. Although 50% of exacerbations were associated with a viral respiratory tract infection, viral infections had no acute effect on changes in *P. aeruginosa* sputum bacterial density. Given the significance of CF exacerbations, further work is needed to determine if viral infections elicit an immune response that leads to exacerbation that may be independent of *P. aeruginosa* bacterial load in CF patients [29]. As well, further investigation is required to understand the etiology of exacerbations in patients who do not have viral infections, since increased *P. aeruginosa* bacterial density in sputum does not appear to be responsible.

Competing interests

None of the authors have any competing interests.

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Author contributions

S.D.A., R.S., and T.F.M. contributed to the conception and design of this study. S.D.A., K.L.V., R.S., E.G., L.H., F.C., and W.F. contributed to the acquisition of data. S.D.A., R.S., M.C., M.D.Z. and R.M. contributed to the analysis and interpretation of data. M.C., M.D.Z. and S.D.A. drafted the manuscript, and all authors critically revised it for important intellectual content and approved the final version to be submitted.

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