Author’s response to reviews

Title: Antibiotic perturbation of mixed-strain Pseudomonas aeruginosa infection in patients with cystic fibrosis

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Author’s response to reviews:

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Professor Jean-Philippe Bouchara
Editor
BMC Pulmonary Medicine

Dear Professor Bouchara,

We submit this revised manuscript for the consideration of publication in the BMC Pulmonary Medicine journal. Revisions to the manuscript have been made in accordance with the feedback from the two reviewers outlined as follows:
REVIEWER 1 (Andrew M. Jones):

The paper details an impressively comprehensive analysis of P. aeruginosa isolates from 4 patients with CF at the start, during, end, and follow up visit, after IV treatment of an acute exacerbation. The investigators should be congratulated on delivering this study.

Author response: Thank you for the positive comments about our work.

1. The authors state that the patients with mixed strains show consistently more severe/advanced disease. I am unclear how this is defined? They have increased treatment requirements but similar %pred FEV1 - can the authors further clarify this.

Author response: We defined people with mixed strain infection as having signs of more advanced disease based on their treatment requirements before (hospitalisations and outpatient visits) and after (relapse, days to next exacerbation) the exacerbation studied. However, as highlighted by the Reviewer, patients with mixed strain infection had no significant difference in FEV1 % compared with single strain group. We have modified the text accordingly as follows:

(1) Abstract Section, Page 3, Lines 19-22: “Patients with mixed-strain infection had more long-term treatment requirement than those with single strains”.

(2) Results Section, Page 11, Lines 2-5: “Despite similar baseline FEV1% values and other between-group variables not being significantly different, patients harbouring mixed strains had more treatment requirements than those with single strain infection (Table 1).”

(3) Conclusion Section, Page 12, Lines 19-22: “In the year before recruitment and the months following their exacerbation episodes, those with mixed-strain infections had more treatment requirements than those with predominant single-strain infection, although this difference did not reach statistical significance.”
2. The study addresses P. aeruginosa, but do the authors have any data of co-pathogens in any of the samples?

Author response: We thank the Reviewer for this comment and agree that this information is important to include. We were able to retrieve historical culture results for each patient that were recorded as part of routine clinical care at the time the study.

We have now included the following text: (Results Section, Page 8, Lines 11-14): “A limited numbers of bacterial co-pathogens were isolated from sputum samples patients at baseline and within prior 3 months (4 patients with methicillin-susceptible Staph. aureus).”

3. Total viable P. aeruginosa count - the numbers are small - there does seem to be a trend that total count does seem to fall during iv treatment in comparison with end of treatment, although this with the numbers of participants involved will not be significant, - if so this would be intriguing, does this warrant discussion

Author response: We thank the Reviewer for this comment and agree that this is an intriguing observation. A recent culture-independent based study by Smith et al [1] showed that the relative abundance of P. aeruginosa decreased within the first few days of treatment of an acute exacerbation in CF; however, this effect was not maintained beyond the first week of treatment. Although our sampling time-points are not identical to those of Smith et al, we found a similar pattern: within one week of commencing IV antibiotics, the geometric mean total viable count decreased from 6.3 x10^7 CFU/mL at day 1 to 1.8 x10^7 CFU/mL but by the end-of-treatment, the total viable count had increased to 1.4 x10^8 CFU/mL. Therefore, we have added the following text to the Discussion Section (Page 13, Lines 16-19): “Although not statistically significant, we observed a transient decrease in the total P. aeruginosa load during the first 6-9 days of intravenous antibiotic treatment, which was reversed by the end of treatment. Similar transient effects have been previously described [1].”

4. Methods - the inpatients shared room facilities - does this means they shared bathrooms? did they have individual bedrooms or did they have beds in shared bays?
Author response: We thank the Reviewer for this comment. We have now modified the text for clarification as follows (Methods Section, Page 5, Lines 10-12): “While in hospital, CF patients with P. aeruginosa were not managed exclusively in single room accommodation and some were admitted to shared rooms [18].

5. Methods - Did the patients continue to take their usual long term antibiotics during the inpatient episode?

Author response: We can confirm that all patients continued taking azithromycin during the exacerbation episode studied but all inhaled antibiotics were ceased.

We have now added this information to the Results section (Page 8, Lines 7-10): “All patients had advanced lung disease, had been chronically infected with P. aeruginosa for several years, and with one exception were taking maintenance oral azithromycin with inhaled antipseudomonal antibiotics prior to the exacerbation episode.” and Page 8, Lines 14-16: “During the period of exacerbation treatment, all patients received combination intravenous antipseudomonal antibiotics and all patients continued taking azithromycin. Inhaled antibiotics were ceased.”

6. Methods - What was the mean/median and range of time difference for sampling for the follow up sputa taken after admission?

Author response: We have added this information in the Results section (Page 8; Lines 16-17): “The median (range) period from completion of intravenous antibiotics and follow-up visit was 42 (13 – 119) days.”
7. Results - Results - clinical response to antibiotic treatment - the authors compare a mean change for patients with single strain infection in comparison with a median change for mixed strain infection - these are very different and should not be used to compare the 2 groups.

Author response: We thank the Reviewer for the comment and have modified the text in Results Section (Page 11, Lines 5-13) accordingly: “During treatment of the exacerbation, there was a greater improvement in FEV1% predicted in patients with single-strain infection [median change (range): 7.2% (3.1 to 15.8%); P=0.02; Wilcoxon signed-rank test; Table 2a] compared to those with mixed-strain infection [median change (range): 2.4% (0 to 14.5%); P=0.07; Wilcoxon signed-rank test; Table 2a], despite the median FEV1% predicted being similar between both groups at start-of-treatment [single-strain infection: 31.2% (range: 20.2% to 84.0%); mixed-strain infection: 41.7% (Range: 30.3% to 63.1%); P=0.5, Mann-Whitney U test].” Similarly we have modified the Results Table 2a (Page 25) to reflect the change in data presentation from mean to median.

7. Discussion para 2 -the authors could additionally state that this present data would suggest that IV antibiotic courses do not seem to be the driver for evolution of single strains dominating infection in CF patients.

Author response: We thank the Reviewer for this comment and have addressed this comment in the Discussion section (Page 12; Lines 24-25; Page 13; Line 1): “The data also potentially suggest that short-term intravenous antibiotic courses might not be a driver of the evolution of single strains dominating infection in CF patients.”

REVIEWER 2: Craig Winstanley: Although others have looked at sets of multiple isolates from CF patient sputum samples in order to study populations of P. aeruginosa, this study is interesting because some of the findings are different. For example, whereas with the Liverpool Epidemic Strain (LES), no mixed strain infections are evident in chronically infected patients, even when large numbers of isolates are examined, here AUST-02, another transmissible strain, co-exists with other strains. In addition, whereas infection with the LES is associated with greater morbidity but is a single "strain" infection, here there is some evidence for more severe
disease in multi-strain infections. Although the evidence is fairly weak because of the small numbers of patients in each group, the findings are worth reporting because they will help in the design of better studies to specifically address that question.

Author response: Thank you for your comments.

1. Page 4 - the authors could also refer to a recent paper by Hilliam et al. in ERJ (currently online) where genome sequence data was used to show that there are often multi-strain P. aeruginosa lung infections in bronchiectasis patients.

Author response: We thank the Reviewer for this comment. We have included this reference in the Background Section (Page 4, Lines 17-22): “Once P. aeruginosa becomes established within the airways of patient with chronic lung disease, it is usually by a single strain that evolves through micro-adaptation into multiple sub-lineages of the original ancestral clone [11, 12]. There are however, reports of co-infection with two or more distinct P. aeruginosa genotypes in both CF [13-15] and, non-CF bronchiectasis [12].”

2. Page 9 - it is notable that from two of the patients included in the study and identified as chronically infected with the AUST-02, no AUST-02 isolates were obtained. The patient were initially positive using PCR of sweeps. (1) Is this the method used routinely in the clinic to define these patients, or are single colonies tested? (2) Are the authors saying that PCR assays of sweeps were negative for subsequent samples (or just that AUST-02 was not identified amongst the colonies selected)? Please clarify.

Author response: We thank the Reviewer for this comment. (1) The PCR sweep methodology used here is not routinely undertaken in the clinical setting; rather, individual colonies are selected from the primary culture plates which then undergo genotyping in the research laboratory. (2) We have edited the Discussion Section (Page 15, Lines 22-25; Page 16, Lines 1-4) to clarify this point: “Furthermore, despite confirming AUST-02 by culture sweep at study entry, using a random sampling culture approach, in two patients who had previously had chronic AUST-02 infection, AUST-02 was not subsequently identified in the genotyping of 48
randomly selected colonies, and only AUST-06 and AUST-01 were detected. It is possible AUST-02 might have constituted a minority of the *P. aeruginosa* population in these cases (at the time of study recruitment), and therefore, were not selected because of limitations in sampling.”

3. It is interesting that overall resistance was greater amongst AUST-02 colonies yet AUST-06 seems to prosper during the exacerbation periods. The authors do discuss the limitations of in vitro antimicrobial susceptibility. There could also be other factors involved favouring AUST-06 during these periods, such as other species of microorganisms or host responses.

Author response: In accordance with the Reviewer’s comment, we have modified the text in the Discussion Section (Page 15; Lines 1-4): “In addition, other as yet unrecognized AUST-06 virulence determinants may impact on the host inflammatory response and co-infection with other CF pathogens [46-48]. Such factors could potentially favor the selection of AUST-06 during intravenous antibiotic treatment.”

4. The Discussion perhaps focuses a little too much on the issue of more severe disease in the mixed infection patients. It would be interesting to see some discussion about the obvious contrast between this transmissible strain and the LES.

Author response: We thank the Reviewer for the comment. We have added further discussion on the contrast between AUST-02 and LES in the Discussion Section (Page 11, Lines 23-25; Page 12, Lines 1-5; Lines 16-19).

5. Page 13, sentence beginning "These findings contrast…” I wasn't convinced that they do contrast. These other studies were not addressing changes in the relative abundance of lineages (or sub-lineages) within *P. aeruginosa* populations over short periods of time.
Author response: We thank the Reviewer for this comment and upon reflection we now agree. We have modified the text in the Discussion Section (Page 14, Lines 7-13): “Whilst previous studies have shown within-host microevolution leads to co-existing sublineages of single P. aeruginosa strains emerging over months-to-years [28, 35-57], this study demonstrates rapid multi-strain turnover in mixed-strain infections within-host during antibiotic treatment for pulmonary exacerbations.”

We are grateful for your consideration regarding this article, which we believe will make a valuable contribution to available literature in this field. I also hereby confirm that this manuscript has not been and will not be submitted elsewhere for publication.

Yours sincerely,

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Reference: