Author's response to reviews

Title: 2-aminoacetophenone as a potential breath biomarker for Pseudomonas aeruginosa in the cystic fibrosis lung

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Author's response to reviews: see over
Dear Dr Shipley

Thank you for forwarding the peer review comments regarding our manuscript to us. After careful consideration most suggested alterations have been made to the manuscript with some additional results added in the micro-organism *in vitro* testing. We would also like to address some of the general concerns raised in the reviewers’ reports, which can not be addressed in the manuscript itself.

**Reviewer: Douglas Curran-Everett**

**Minor Essential Revisions:**

1. The Welch t-test reference on page 10 has been modified to a *t*-test as requested.

2. 5-parameter logistic function: In the methods section a detailed description of the 5PL function has been inserted as follows: Degradation of 2-AA in the glass bulb was tested with the *t*-test adapted for unequal variances. A five parameter log logistic (5PL) model was fitted to the data and the EC$_{50}$ estimated using the R package drc [16]. The 5PL model may be parameterised by equation 1 where $c$ and $d$ are the minimum and maximum concentrations respectively, $d$ the hill slope, $e$ the EC$_{50}$ and $f$ the asymmetry parameter.

$$Concentration = c + \frac{d - c}{1 + \left(\frac{\text{time}}{e}\right)^b}$$  

The four parameter model has the same parameterisation as the 5PL with $f$ set to 1, forcing symmetry about the point of inflection. ANOVA shows that the 5PL had significantly better fit to the data than the 4PL model ($P < 0.0002$). The estimated minimum concentration for the 5PL model was -3.18 (SE 8.99) hence we fitted a 5PL model with the minimum concentration, $c$, constrained to 0 to aid interpretability. Table 1 shows the Akaike Information Criterion (AIC) for each model and the estimated EC$_p$ where the 95% confidence intervals were estimated by the delta method [16]. The AIC is a measure of fit which penalises additional parameters; lower values indicate better fit so the table shows that the 5PL model fits better than the 4PL while fixing the minimum concentration provides a marginal improvement.

3. In the manuscript the referencing on pages 12 & 14 regards to Figure 5 has been modified with the removal of the +/- symbols.

**Reviewer: Huw Williams**

In response to Reviewers Report:

The reviewer brought up some interesting points in his report which we would like to address.

1. Regarding the detection of 2AA in healthy breath and CF subjects not colonised with *Ps. aeruginosa* is an area of research that is ongoing in our laboratory. We agree with the reviewer, there must be significant discrimination between the levels of 2-AA detected in the healthy subjects compared to the CF patients. Determining the correct breath collection maneuver is extremely important as oral colonisation with *Ps. aeruginosa* may result in a positive signal. Also alveolar breath may produce an enhanced signal for 2-AA in comparison to the current mixed breath sampling we
perform. Other areas currently being investigated is whether consuming 2-AA rich foods immediately prior to giving a breath sample results in the increase of 2-AA on the breath. Cystic Fibrosis medication and over the counter supplements are also being tested for the release of 2-AA.

2. The point raised regarding culture conditions was a valid observation made by the reviewer. Culturing conditions of *Ps. aeruginosa* will indeed vary the level of 2-AA released, as will the culture media itself. To-date we have found that *Ps. aeruginosa* produces 2-AA reliably on any culture media; however it does release larger concentrations in complex media. Although this manuscript only reports results from aerobic batch culture, we are aware that oxygen tension and growth state of the microorganism may alter the level of 2-AA released. Our laboratory has since started studying the growth of *Ps. aeruginosa* in continuous cultures and biofilms with varying oxygen tensions, to determine the levels of 2-AA that could potentially be released by the micro-organism during growth in the CF lung. We hope to publish these findings shortly.

**Minor Revisions:**

1. The labelling of Figure 1A-D has been changed to Figure 1 – Figure 4 as suggested and all following figure numbers have been updated. Figure 1 has also had labels added to designate the parent and daughter ions’ and also to indicate the structure of 2-AA.

2. We appreciate the suggestion of moving the tabulated data into the supplementary information. We would like this information for be readily accessible to anyone that reads our paper however we are more than happy to move this to the supplementary information if the Editor so wishes.

**Reviewer: Craig Winstanley**

**In response to Reviewers Report:**

1. Our manuscript reports results of an initial pilot study to determine the suitability of 2-AA as a breath biomarker for *Ps. aeruginosa* infection. The goal of the 2-AA breath test is to detect the initial colonisation of the lung with *Ps. aeruginosa* to ensure swift action can take place. Our results are promising however we are aware that further improvements are necessary. We are agree, this work is preliminary and we are currently working on improving this test by altering the sampling maneuver, removing oral 2-AA contamination and analysing foods & medications that may cause false positives. Overall, the work presented here shows potential for the 2-AA breath test and further work continues to enhance this *Ps. aeruginosa* detection method.

**Major Essential Revisions:**

1. With regards to the microbiology portion of our paper. The micro-organisms chosen for study represent an overview of those species found in the CF lung of New Zealanders.

2. The *Burkholderia cepacia* strain tested and reported on in the previous *in vitro* work was not typed. We therefore collected three typed *Burkholderia multivorans* strains from Canterbury Health Laboratories, Christchurch, New Zealand to test for
the production of 2-AA. These strains were identified by API 20 NE and 16S rRNA sequencing and these results have been incorporated into this manuscript. We also tried to obtain *Burkholderia cenocepacia* however this strain is not common in our local CF population and rarely comes through Canterbury Health Laboratories. With the time constraints imposed to respond to the referees we have not been able to obtain any of this strain. We do believe that if we obtain further promising results with the 2-AA breath test then we will need to purchase some *B. cenocepacia* strains to test.

3. All control subjects were healthy adults. The CF patients were a mixture of children and adults. In order to test breath from CF patients not colonised with *Ps. aeruginosa* we had to obtain samples from the Paediatrics population. This meant the mean age of CF subjects not colonised with *Ps. aeruginosa* was indeed lower than that of the control group. These two populations were unpaired groups and this does not affect statistical evaluation. However, in up coming studies we will be testing match paired subjects to ensure clarity of results.

4. There was confusion regarding whether multiple strains of *Staphylococcus aureus* were tested. Initially each individual strain of each micro-organism was cultivated in replicate on different media and the levels of 2-AA were monitored. However, since receiving reviewers’ comments we have tested an additional two strains of each species and also added in *Aspergillus fumigatus* results. These results have been inserted into the manuscript and discussed. It is clear to us that the release of high concentrations of 2-AA is specific to *Ps. aeruginosa*.

Both the healthy group and CF subjects not-colonised with *Ps. aeruginosa* produced breath samples positive for 2-AA (controls 29 % and CF subjects not colonised with *Ps. aeruginosa* 30.7 %). The reservoir of 2-AA for these positive results is unclear at present however food intake is a possible source of contamination and is currently being tested. Oral colonisation with *Ps. aeruginosa* may also cause positive results due to contamination of the breath as it exits the mouth. It is proposed that a 2 % H$_2$O$_2$ mouthwash could eliminate any oral signal and is also to be tested by our laboratory. We agree that the 2-AA breath test will only be useful if it can detect the initial colonisation of the lung with *Ps. aeruginosa* so the appropriate anti-pseudomonal drugs can be administered. A long term study will need to be designed to address this issue monitoring the levels of 2-AA in breath in relation to the number of *Ps. aeruginosa* cultured in sputum samples. This experiment will take some careful planning and initial testing of all medications that could be prescribed to the patient for the release of 2-AA.

**Minor Essential Revisions:**

1. The legend for Figures 1-4 is currently situated in the Introduction as Figure 1 is talked about in the first paragraph. The remaining Figures are discussed in the Materials & Methods Section. The position of this legend has remained unchanged however we are happy for it to appear wherever the Editor see’s fit.

2. On page 14 Figure 5 has now been put in brackets.

3. On page 15 *Aspergillus* has been italicised.
We hope that with the suggested alterations made to the manuscript, the additional experimental results and the discussion regarding any concerns the reviewers had, our manuscript can now be published in your journal.

Yours sincerely

Amy Scott-Thomas, Mona Syhre, Philip Pattemore, Michael Epton, Richard Laing, John Pearson and Stephen Chambers