Author's response to reviews

Title: Nasal swab samples and real-time polymerase chain reaction assays in community-based, longitudinal studies of respiratory viruses: the importance of sample integrity and quality control

Authors:

Asma N Alsaleh (a.alsaleh@uq.edu.au)
David M Whiley (d.whiley@uq.edu.au)
Seweryn Bialasiewicz (Seweryn@uq.edu.au)
Stephen B Lambert (sblambert@uq.edu.au)
Robert S Ware (r.ware@sph.uq.edu.au)
Michael D Nissen (michael_nissen@health.qld.gov.au)
Theo P Sloots (t.sloots@uq.edu.au)
Keith Grimwood (k.grimwood@uq.edu.au)

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Author's response to reviews: see over
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Professor Dale Barnard
Associate Editor, BMC Infectious Diseases.

Dear Professor Barnard

We would like to express our sincere thanks and appreciation to the editorial team at BMC Infectious Diseases and the reviewers for their constructive comments and thoughtful suggestions.

Set out in the following pages are our responses to each of the points raised. We believe the revisions we have made as a result of the feedback received have helped to strengthen our manuscript and we look forward to hearing from you in due course.

Please note that we have submitted 2 versions of our revised manuscript; a clean version as the main file and a tracked one as an additional file.

Kindest regards

Asma N. Alsaleh

(Corresponding author on behalf of all co-authors)
Editor’s comment: "The authors need to measure sample quality and RNA integrity using the collection method of choice. It should be an intrinsic part of any pre-study evaluation. That data must be made available for this study. Unless such data is provided the manuscript will be recommended for rejection."

AUTHOR RESPONSE: Thank you for highlighting the importance of the point raised by the second reviewer, C. Moore. You will observe that in our response to Dr Moore we now provide detailed information supporting the use of our protocol, including references to previous work published by ourselves and others using this method of specimen collection and transport as well as unpublished evaluations evaluating viral RNA stability we undertook prior to beginning the ORChID project.

Reviewer: M. Hargreaves

1. **Is it legal to send used specimens in containers by Australia Post? Was any special labelling required?**

AUTHOR RESPONSE: Sending biological specimens by Australia post is legal for domestic destinations under specific regulations and conditions. Special labelling and packaging are required. For this study, they were sent as exempt human specimens in accordance with Australia Post’s Dangerous and Prohibited Goods and Packaging requirements (Australia Post, 2009) [1].

We now note in the ‘Sample collection’ section of the Methods section at the top of page 8 that samples were sent by mail …“(in accordance with Australia Post regulations [23])”.

2. **Were the samples that were sent in late from outlying or rural participants, or was the lateness just due to oversight? I ask this because such areas might be expected to have a high airborne level of fungal spores emanating from disturbed soil and plant matter.**
AUTHOR RESPONSE: All participants lived within the greater Brisbane metropolitan area, none were from rural communities. This has now been clarified in the ‘Swab samples’ section of the results (page: 10).

3. The statistically significant correlation between mould and season is not unexpected, however relative humidity and temperature may explain this relationship. The increase in mould is noted in terms of number of swabs contaminated per season – was there also an increase in the amount of mould on each swab in Summer and Spring? The authors may find the work reported in: Rutherford, S., Owen, J. A. K. & Simpson, R. W. 1997. Survey of airspora in Brisbane, Queensland, Australia. - Grana 36: 114-121. ISSN 0017-3134, to be of some use in the interpretation of Seasonal variations in airborne fungal spores.

AUTHOR RESPONSE: Thank you for this suggestion. We have examined the association between season and level of mould for our samples, and found there was no significant association. For example in summer there were 261 samples with mould, of which 13.4% had high level mould; in autumn there were 167 samples with mould, of which 10.2% had high level mould; in winter there were 74 samples with mould, of which 18.9% had high level mould; and in spring there were 260 samples with mould, of which 12.7% had high level mould. In the ‘Mould’ section of the results we have noted the lack of association between season and level of mould (page: 12).

4. Whilst there is a clear correlation between amount of mould and the elapsed time before the swabs were received, I wonder if this indicates that the mould was, in fact, responsible for the lower ERV3 responses from swabs with visible mould.

AUTHOR RESPONSE: Yes, we believe that mould was partially responsible for reduced ERV-3 loads in some samples. This is noted in the ‘Results’ in the second paragraph on page 13 where we found a significant association between mould growth and ERV3 findings. Of the 762 samples with visible mould, 529 (69.4%) were positive.
for ERV3, which was significantly lower than rates in samples without visible mould. Furthermore, high levels of visible mould were shown to be an independent risk factor for reduced respiratory virus detection in a multivariate model that included several potential confounding factors (Table-4).

5. The authors state (page 5) that mailing of swabs has limited or no effect on respiratory virus detection by PCR, but that further investigation of long term effects was required. Is a >7 day period considered long-term? Is there a possibility that the longer time period not only permitted mould growth, but also caused some damage to the viruses (as two separate events)?

AUTHOR RESPONSE: Yes, we consider 7 days long-term. While a longer time in reaching the Laboratory was associated with increased mould levels and high visible mould levels were associated with reduced respiratory virus detection, the time to reach the laboratory was not an independent risk factor for reduced respiratory virus detection (Table 4).

6. On this matter, I note that the authors state (p13) that respiratory virus detection rates “increased with . . . time taken to reach the laboratory”. Is there a satisfactory explanation for virus detection rates increasing with time taken to reach the laboratory? How common was this phenomenon? This seems to be an unexpected finding, which I would think deserves comment.

AUTHOR RESPONSE: This was indeed an unexpected, and possibly a chance, finding. Following further discussion, we believe a plausible, but only partial, explanation may be due to the viral nucleic acids being protected as result of being encapsulated within the viral capsid, combined with the use of the viral transport medium used in the swabs. This is now discussed at the end of the 3rd paragraph of the discussion.

7. Another matter on this same topic is the “ambient temperature” situation. On page 5, it is stated that the “mailing of swabs at ambient temperature has limited
or no impact”. Ambient temperature varies with geographical location and season. The impact may vary accordingly.

AUTHOR RESPONSE: We agree with the reviewer and now note that further studies are needed at higher ambient temperatures. The last sentence of the first paragraph on page 5 has therefore been modified accordingly.

8. There are some other actions that may be considered in response to the findings, in addition to reminding the participants to send the samples in promptly. These may include alcohol washing of hands prior to sampling, speed in collection, keeping the transport tubes enclosed (in a bag, or the mouth of the tube covered with a cap or similar) while the sample is being taken, collecting the sample in a clean, dry room (preferably one without carpets, and definitely NOT the bathroom) with the windows closed. Rainy weather will also increase the fungal spore load in the air.

AUTHOR RESPONSE: We thank the reviewer for these practical suggestions.

9. I suggest that the opening sentence of the last paragraph on page 14 be altered to: “Fungal contamination probably occurred during sample collection”. Certainly, this was not the “source of” fungal contamination. That could have been the resident fungal spores in the room, in carpets, curtains and so on, or coming in windows from the many and various outdoor sources. Do you have any evidence for this assumption? I imagine that the quality control of the swabs was in order, but I wonder if you sacrificed any swabs to expose them to air in various conditions, to better support the “sample collection” proposal? It may even be possible that the contamination occurred during the extended transport period, since the swab tubes’ seal was disturbed by that time. If this was the case, re-sealing of the transport tube would be an appropriate response.

AUTHOR RESPONSE: We thank the reviewer for their careful reading of the article and have amended the sentence on page 14 as suggested by the reviewer.
As for sacrificing any swabs to expose them to air etc.: We did not conduct these experiments because (a) at the beginning of the study we were not aware of any mould issues (b) no mould was evident on any swab samples prior to use ie. unopened, and (c) it really makes no difference whether the source of the mould was from the patient or otherwise contamination from the patient’s surrounds during collection given we were not studying mould as a clinical indicator. The key point is that these swabs could not prevent mould growth and that we will in future ensure samples are transported more promptly to the laboratory to reduce this problem.

Reviewer: C. Moore

1. The initial marker used in this study was the presence of mould on the nasal swab. As the samples were being tested using the same exogenous and endogenous controls, were any problems not previously noted in relation to time of collection and sample receipt? For any sample collection system that requires home collection and postal transporation, measuring sample quality and RNA integrity using the collection method of choice should be an intrinsic part of any pre-study evaluation. Is this data available for this study?

AUTHOR RESPONSE:

Regarding the appropriateness of the methods used in this study:

- The sample collection protocols used in this study [2] were based upon our own published (and unpublished pilot) studies as well as several additional studies conducted by others highlighting the suitability of these methods. [3–6]

- For example, previous studies of rhinovirus showed similar prevalence rates when using this protocol (swab collection + mailing samples) compared with more invasive sampling protocols combined with rapid transportation (ie. nasal aspirates + immediate transportation to the Laboratory) [7, 8].

- Other studies have shown that the mean Ct values for RNA viruses was lower (higher load) in the mailed than in the frozen specimens, suggesting that the
mailed samples may in fact be more suitable. This is despite RNA viruses being less robust than DNA viruses [3, 9].

- Of note was that the above studies (as well as several others) have used respiratory virus detection rate as the key performance indicator to show the suitability of their methods [7, 10, 11]. Similarly, in this study we did not observe any decrease in virus detection associated with prolonged transport times (see our comments to reviewer 1 above). This is now discussed at the end of the third paragraph of the Discussion at the top of page 15.

- As a part of our preliminary work for ORCHiD, we conducted a pilot study to examine the impact of time (transport duration) upon RNA stability. Briefly, we used 5 different influenza-A isolates to inoculate 45 swabs that were then managed differently; five control swabs were immediately stored at -80°C, 20 swabs were left at ambient temperature in the laboratory for 1, 3, 5 and 7 days prior to freezing and testing (5 swabs each), whereas the remaining 20 swabs were sent through our postal system at 1, 3, 5 and 7 days prior to freezing and testing. The differences in Ct values was typically less than 3 cycles (i.e. within 1 log) in the influenza-A PCR irrespective of whether swabs were stored immediately at -80°C, remained on the bench for up to 7 days or were sent by post over the same time period. This showed that there was little impact upon the influenza-A RNA as a result of the sample collection, transport method or time to storage in a -80°C freezer. (see Appendix for further details).

- Please note that the initial marker used in this study was the presence or absence of ERV3, which was used as a marker for the epithelial cell component of the sample and not for human DNA quality (discussed further below).

- The presence of mould on nasal swabs was unexpected for many reasons: (i) Participants were asked to send samples as soon as possible and previous studies by our group showed high levels of sample returns [12]. (ii) The swabs were bathed in both antifungal and antibacterial agents.
• As for the other unexpected findings of this study (absence of ERV3, and decreased virus detection as a result of poor sampling etc.): While there have now been several studies looking at community based collection and testing of respiratory samples [3, 7, 12, 13], none are of this sample size and sampling intensity and consequently they have lacked sufficient power to identify the problems observed here. It is for this reason that we believe this article will be of considerable benefit and interest to readers contemplating such studies.

We are a little unsure if the reviewer was also suggesting the testing of human RNA to investigate “RNA integrity”. If this is the case then we respectfully disagree with this for several reasons:

• We consider human RNA as unsuitable for use as a broader measure of “sample RNA quality.”

• Firstly, while human RNA is protected by a single barrier (cell wall), the RNA in the viruses is protected by a double barrier: the cell wall (being obligate intracellular parasites) and their own protein coats. Therefore, human RNA could be more sensitive to degradation.

• In addition, viral transport medium was also used, which helps to preserve viral nucleic acids by maintaining intact viruses during transport.

We also wish to emphasise that assaying ERV3 by PCR was not employed to examine DNA integrity, but as a check of appropriate sample collection (ie. the presence of epithelial cells).

2. It seems apparent that the major problem in posting samples and the growth of fungal or indeed bacterial contaminants despite the inclusion of antimicrobials in the transport medium is the presence of moisture. have the authors considered removing the moisture factor and having samples returned to the laboratory dry? This is a method employed for dry blood spot testing and has been successfully applied to the collection of respiratory samples from the community.

AUTHOR RESPONSE: The sampling protocol, including the type of swabs used, was established and selected according to previous published work [3, 4, 10]. Although
other studies suggested no significant impact of using dry swabs upon the prevalence of respiratory virus detection comparing with nasopharyngeal aspirates [12], we believe the presence of virus transport medium helped to achieve higher detection rates by maintaining viruses particles as long as possible.

3. Do the authors recommend the use of both an endogenous and exogenous control, or do they think that by using ERV3 alone that you fulfil both the sample integrity and process control elements?

AUTHOR RESPONSE: We believe it is important to use both endogenous and exogenous controls when employing nasal swabs for sampling. Nasal swabs collected by parents have been used successfully in other settings (mentioned above). Moreover, as nasal swabs are less invasive and more convenient than nasopharyngeal aspirates/swabs collected by health professionals, they are now recommended as the specimen of choice for community-based studies and following the 2009 influenza pandemic they are also being used more often in clinical practice [3, 14, 15]. However, this method is still prone to suboptimal sample collection. The ERV3 control is ideal as a means of investigating the quality control aspects of sampling. In fact, we believe that this study could be used to help establish a criterion for the quality of nasal swab collection process.

The ERV3 control does however have some limitations that warrant the use of the exogenous control (in our case we used equine herpes virus). These are mainly associated with potential problems in the laboratory and may include such issues as a suboptimal extraction run. For example, a sample containing very high loads of ERV3 DNA may still provide a moderate ERV3 load result upon ERV3 testing despite suboptimal extraction. It is this type of the problem that will be detected by utilising the exogenous control at a standard concentration.

We have refrained from discussing this in the article as the above is well recognised and is in fact now used in many commercial NAAT systems.


The impact of ambient temperature upon influenza-A real-time PCR screening.
(unpublished data)

For the study, we used 5 influenza-A positive NPAs to inoculate a total of 45 Virocult swabs; each strain was inoculated onto 9 swabs which were then treated in nine different ways (as described in the text and table below):

- five swabs (one of each strain) was immediately frozen at -80 (‘Swab day 0’)
- five swabs each were left on the laboratory bench for 24 hours prior to freezing and testing (‘Bench day 1’ swabs)
- five swabs were sent by post & received back within 24 hours prior to freezing and testing (‘Mail Day 1’ swabs)
- five swabs each were left on the laboratory bench for 3 days prior to freezing and testing (‘Bench day 3’ swabs)
- five swabs were sent by post & received back within 3 days prior to freezing and testing (‘Mail Day 3’ swabs)
- five swabs each were left on the laboratory bench for 5 days prior to freezing and testing (‘Bench day 5’ swabs)
- five swabs were sent by post & received back within 5 days prior to freezing and testing (‘Mail Day 5’ swabs)
- five swabs each were left on the laboratory bench for 7 days prior to freezing and testing (‘Bench day 7’ swabs)
- five swabs were sent by post & received back within 7 days prior to freezing and testing (‘Mail Day 7’ swabs)
The results:

<table>
<thead>
<tr>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
<th>Strain 5</th>
<th>Mean Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Swab day 0’</td>
<td>29.26</td>
<td>30.97</td>
<td>28.29</td>
<td>33.06</td>
<td>32.53</td>
</tr>
<tr>
<td>‘Bench day 1’</td>
<td>33.42</td>
<td>31.91</td>
<td>33.74</td>
<td>35.51</td>
<td>33.73</td>
</tr>
<tr>
<td>‘Mail day 1’</td>
<td>32.48</td>
<td>34.58</td>
<td>33.03</td>
<td>34.61</td>
<td>33.27</td>
</tr>
<tr>
<td>Bench day 3</td>
<td>33.26</td>
<td>28.97</td>
<td>33.06</td>
<td>34.81</td>
<td>33.66</td>
</tr>
<tr>
<td>Mail day 3</td>
<td>28.6</td>
<td>30.76</td>
<td>31.27</td>
<td>32.95</td>
<td>33.27</td>
</tr>
<tr>
<td>Bench day 5</td>
<td>30.55</td>
<td>31.57</td>
<td>34.17</td>
<td>34.93</td>
<td>33.87</td>
</tr>
<tr>
<td>Mail day 5</td>
<td>30.69</td>
<td>33.08</td>
<td>32.64</td>
<td>34.52</td>
<td>33.87</td>
</tr>
<tr>
<td>Bench day 7</td>
<td>35.05</td>
<td>27.97</td>
<td>31.96</td>
<td>33.68</td>
<td>34.15</td>
</tr>
<tr>
<td>Mail day 7</td>
<td>33.08</td>
<td>33.91</td>
<td>33.06</td>
<td>34.66</td>
<td>33.65</td>
</tr>
</tbody>
</table>

- Compared to the Day 0 swabs, there was no substantial change to the Ct values of the influenza-A detection, irrespective if they sat on the bench or mailed, or over time; all average Ct values were within 3 cycles showing that any decrease in viral load was within 1 log.