Author's response to reviews

Title: Flocked Nasal Swab versus Nasopharyngeal Aspirate for Detection of Respiratory Tract Viruses in Immunocompromised Adults: a Matched Comparative Study

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Author's response to reviews: see over
Dear Editor,

Thank you for considering the manuscript "Flocked Nasal Swab versus Nasopharyngeal Aspirate for Detection of Respiratory Tract Viruses in Immunocompromised Adults: a Matched Comparative Study" (MS: 4451761024480615) for publication in BMC Infectious Diseases. We have carefully read the reviewers’ valuable comments which we hereby aim to respond to point by point; partly in this letter and with amendments indicated in the revised resubmitted manuscript. The suggestions have, according to our opinion, improved the manuscript and we hope that the revised version now is acceptable for publication in your journal.

Yours sincerely, on behalf of all authors,

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Response to comments from referee no. 1-3. Added words and sentences are underlined.

REFEREE #1:
Several mistakes regarding dots and commas before references.
We have throughout the manuscript put the dots and commas AFTER the references.

Pag.3, line 6 – need space between children and citation [15]
Space is now inserted between children and citation [15]

Pag.3, line 14 – I would suggest “the sensitivity” instead of “the efficacy”. This is the parameter which the authors are dealing with.
We have changed “the efficacy” to “the sensitivity”.

Pag.3
- the authors should explain which type of hematological disorders have the patients recruited into study
We now clarify that by adding “…adults with any hematological disorder presenting…//…for febrile neutropenia…”

- how was assessed the temperature? Axillary, oraly, auricular?
This is now described: “(auricular temperature >38.0°C twice or ≥38.5°C at one occasion...)”

Pag.4 collection and storage of material
- did the patients receive antiviral therapy before collection of nasal swabs or nasopharyngeal aspirate?
Yes, in half of the patients (high risk patients with seropositivity for certain herpes viruses) aciclovir were used as antiviral prophylaxis against viruses from the herpes family, foremost HSV-1, HSV-2 but also VZV. The proportion of patients receiving this prophylaxis is now described: “In total, 49 (50%) of the patients were on antiviral prophylaxis with aciclovir.”

- Did the patients receive any antibiotics before collection of nasal swabs or nasopharyngeal aspirate?
Yes, all patients with febrile neutropenia are treated with broad-spectrum antibiotics which we now describe in the method section: “At admission, the patients received empirically administrated broad-spectrum antibiotics; ceftazidime or piperacillin-tazobactam.”

- The authors should explain how many collections were made in the first 24 hours, in the 24-48 hours, and 48-72 hours interval from onset of fever?
- I would suggest that authors explain the interval of time of collection of swabs/aspirates from admittance
This information is added to the result section: “The patients were sampled within one day from admittance (n=54, 55%), but due to practical obstacles, in 32 (33%) and 9 (9%) of the
cases, the samples were collected on the second and third day after admittance, respectively.”

This is further addressed in the Discussion: “This study has several limitations. The time interval between fever onset and sampling varied between the patients…”

Pag.5 – how was collected 98 paired samples from 89 patients? I suppose some patients had at least 2 collections. What was the reason for additional collections?
These patients follow programs with repeated treatments of anti-neoplastic chemotherapy. Thus, they have repeated episodes of neutropenia in which sometimes fever develop. The higher number of episodes than patients is due to repeated episodes of fever in the same patient. We now make that clearer in the method section: “The patients were allowed to participate more than once provided that an afebrile period of at least three weeks separated the episodes of febrile neutropenia.”

Pag.6 – I would suggest the authors could clarify or rewritten the first 6 lines. For readers which don’t have solid statistical background could be difficult to understand. We have now tried to describe the calculations clearer: “We plotted the difference in cell count between fNS and NPA against the difference in Ct values between the methods for the positive pairs. Although not statistically significant, the correlation indicate a possible association between a high cell yield and low Ct value (r=-0.46, p=.15).” Moreover, as a low Ct value actually means a high viral copy number, this is also clarified: “In pairs with virus detected in both methods, the Ct values were lower (thus viral load higher) for NPA than for fNS.”

Furthermore, regarding the correlation indicating that a lower Ct value is associated with a high number of cells yielded, this result is also disussed: “Even though not statistically significant, the Ct value negatively correlated to the number of cells collected, and thus, the finding of NPA being superior to the fNS in collecting cells invite us to speculate that the fNS sometimes may collect inadequate amount of specimen in order to reach the PCR method’s detection limit.”

REFEREE #2:
The major weakness of this report is that conclusions are based upon a very limited number of samples: the difference in sensitivity for rhinovirus is calculated on the basis of 9 vs 7 positive samples, while the difference in sensitivity for any virus is relevant to 18 vs 13 positive samples.

Indeed, and we now address this further in the manuscript: “This study has several limitations...//... The foremost important limitation is the low number of positive samples on which the calculation of sensitivity is based. However, the upper limit in the 95% confidence interval for the overall sensitivity was 85% which is still a questionable value for replacing an established method.”
Another major problem is the storage (up to six h) of NPA and fNS in the absence of transport medium. Thus, differently to NPA samples (containing 2-3 ml of sodium chloride solution), fNS likely reached the lab completely dried out, impairing the subsequent recovery of cells.

The cells where indirectly measured by means of amplification of human beta actin gene (reference gene). Thus, the vitality of the cells did not affect the estimation of cell yield.

REFEREE #3:

The meaning of qPCR needs to be explicitly stated.

“...quantitative polymerase chain reaction.” is now used in the abstract (appears only once) and the abbreviation explained in the method section “quantitative polymerase chain reaction (qPCR)”.

1) There are some concerns about the samples collection. In particular, the time interval of 72 hours after the fever onset can be too long and there is the possibility that the viral load in nasopharyngeal regions goes under the detection limit.

The viral load indeed decline over time and one can expect fewer positive cases as time increases between fever onset and sampling. However, as the primary goal of the study was to compare two methods rather than determine the prevalence of viral respiratory infections, we still believe the study design is adequate. We have now addressed this issue as described above: “The patients were sampled within one day from admittance (n=54, 55%), but due to practical obstacles, in 32 (33%) and 9 (9%) of the cases, the samples were collected on the second and third day after admittance, respectively.”

This is also further discussed: “This study has several limitations. The time interval between fever onset and sampling varied between the patients...” and “…as both sampling methods were used at the same time point in the same patient, the comparison is still valid.”

A good approach to this problem is to reduce the time interval to collect the sample after the fever onset. It can be a good idea to analyze more samples and to collect them within 24 hours from fever onset. If data are available about this issue, they need to be incorporated in the text. We agree that more positive samples would have contributed. Unfortunately, we have no such data available to incorporate in the manuscript.

2) Some data are missing. In particular:

- the median number of absolute neutrophil count and its standard deviation
  The median number of ANC followed by range is now added. As the lowest value, “<100”, is not a value we could not use SD without making assumptions of the true value of “<100”.
- the median age of patients and its standard deviation
  This data is now added.
- the number of women and men and their median ages
  This data is now added.
• the prevalence rates of hematological disorders in patients
This data is now added.

3) The potential limitations of this work are not stated. Concerns about the limits of the study design and the intrinsic variability due to inclusion of people with different diseases and treatments, need to be explicitly mentioned in the Discussion.
We strongly agree and have now added a paragraph to the discussion section:

“This study has several limitations. The time interval between fever onset and sampling varied between the patients, and they did not necessarily have respiratory tract symptoms. Furthermore, the patients suffered from different underlying diseases. However, this reflects the clinical reality and as both sampling methods were used at the same time point in the same patient, the comparison is still valid. The foremost important limitation is the low number of positive samples on which the calculation of sensitivity is based. However, the upper limit in the 95% confidence interval for the overall sensitivity was 85% which is still a questionable value for replacing an established method.”

4) Page 6, line 6: P value =.15 is not generally considered as statistically significant. If the P value is wrong it must be changed otherwise this interpretation isn't correct.
We agree. The P value is correct, so the results are indeed over-interpreted. We hope that we now have interpreted the results with more caution:

Result section: “We plotted the difference in cell count between fNS and NPA against the difference in Ct values between the methods for the positive pairs. Although not statistically significant, the correlation indicate a possible association between a high cell yield and low Ct value (r=-0.46, p=.15).”

Discussion section: “Even though not statistically significant, the Ct value negatively correlated to the number of cells collected, and thus, the finding of NPA being superior to the fNS in collecting cells invite us to speculate that the fNS sometimes may collect inadequate amount of specimen in order to reach the PCR method’s detection limit.”

5) Paired t-test assumes that the variables differences are normally distributed. Have you checked this assumption? If this differences aren't normally distributed the test needs to be changed (e.g.: Wilcoxon signed-rank test).
We thank referee #3 for noticing this. No, the differences are not normally distributed and the Wilcoxon signed-rank test was used. The t-test was incorrectly stated in Materials and Methods as the statistical method for the comparison. This is now corrected.

6) The sum of PCR reagents seems to be wrong, 25 (20+5) seems to be the right
The total volume is 50 uL: 25 uL of Master-mix, 5 uL of template, and 20 uL of primers and probe. This is now clarified:
“The PCR assay was carried out in a total 50-µL reaction mixture containing 25 µL of TaqMan Universal PCR Master Mix (Applied Biosystems) and 5 µL of template, leaving 20 µL to the primers and probe.”

-End of letter-