Author's response to reviews

Title: Galactomannan testing of bronchoalveolar lavage fluid is useful for diagnosis of invasive pulmonary aspergillosis in hematology patients

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Version: 3 Date: 24 October 2009

Author's response to reviews: see over
The Editor  
BMC-series Journals  

24 October 2009

Dear Dr Koutsos,

Re:  **MS 1032021729503600 – Galactomannan testing**

Thank you very much for giving us the opportunity to submit a revised version of our manuscript to your journal.

We would like to thank all referees for their instructive and helpful comments. Our reply to each specific comment is given below, and I hope that we will have sufficiently answered all questions.

Yours sincerely,

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**Referee 1 (Alessandra Pasqualotto)**

1. Cases of probable/proven IA were defined following published EORTC/MSG criteria, with a single serum galactomannan OD result of ≥ 0.7 being considered positive (this last part added into the text). A new table (Table 2) with more clinical details of the cases as well as the test results has been included.

2. Because galactomannan is relatively specific for *Aspergillus* spp. and is not approved for diagnosis of other mould diseases, we feel that “invasive aspergillosis” rather than “invasive mould disease” would be more appropriate in this setting. For the 3 cases where invasive hyphae were observed, the BAL cultures were negative.

3. Actually, the BAL was performed only in those cases where patients already fulfilled “possible” invasive fungal disease (i.e. met host and clinical – including radiological – criteria but lacking microbiological evidence). Hence the patients are not exactly at an early stage of disease. The delay between reaching a diagnosis of possible IFD (i.e. suggestive CT changes in the right clinical setting) and time of bronchoscopy was actually up to 6 days in one case because galactomannan testing is only done twice weekly at our institute. This was mentioned as one of the limitations of the study.

   a. We excluded patients with positive serum GM from the study for ethical reasons (this was listed as a study limitation as well) – if a diagnosis of probable IPA was already made via positive serum
galactomannan, it was unnecessary to subject these fragile patients to the small but real risks of an additional bronchoscopy/BAL.

4. We performed only one bronchoscopy per subject, and therefore BAL GM was tested only once. For serum galactomannan, the highest value for each subject within 2 weeks of bronchoscopy/BAL was used – this is now reflected in Table 1. Serum galactomannan testing is performed twice weekly for at-risk patients at our institute, and positive results are not repeated.

5. Thank you for agreeing with our main conclusion. We have added a paragraph in the Discussion calling for standardization of BAL sampling, but acknowledge that this is difficult given different patient populations (for example the amount of fluid instilled in a pediatric patient would be different from an adult). Perhaps for adults, 40 or 60 mls should be the standard – these figures are used in some of the published studies on BAL GM testing. One issue is that in such cases, rarely there would be no BAL fluid retrieved (especially right lower lobe BAL), and the bronchoscopist would have to instill more saline – how this would impact on the BAL GM result is unknown.

a. As part of the Platelia testing protocol, samples are already centrifuged and vortex. What we did is described in the Methods section – 40 to 60 mls of saline instilled during bronchoscopy, with 5 mls sent off for GM testing (which was then performed according to the manufacturer’s instructions).

6. Thank you, we have changed to EORTC/MSG.

7. No, unfortunately, the use of IV pip/tazo was an exclusion criteria for our study.

8. The sera results are shown in Table 1 and 2. We had 4 cases (this is a change from 3 reported previously – an omission on our part) where serum GM was ≥ 0.7. It is true that we did not perform bronchoscopy in patients whose initial serum GM was positive, but for these ten cases, BAL GM appeared to be more sensitive than serum GM for the diagnosis of IPA.

9. We have included this (higher titres in BAL specimens and earlier time to positivity) in our discussion. Thank you for referring us to the paper by William Hope.

Referee 2 (Emilie Frelalle)

1. Our apologies for not communicating this properly – the institution has 900 beds, but the size of the Hematology unit is far smaller: approximately 30 beds, with 40-70 bone marrow transplants performed each year (approx. 60% autologous).

a. A new Figure (Figure 1) has been created to display the study schematic. In essence, 18 patients with possible IFD underwent bronchoscopy during this period, while 13 others with hematological diagnoses who did not meet EORTC/MSG criteria for “possible IFD” underwent bronchoscopy.

b. The inclusion and exclusion criteria are described in the Methodology section. There was no matching performed between cases and controls. Not all proven/probable IPA cases underwent
bronchoscopy – those with initial positive serum galactomannan or those who had concomitant disease elsewhere (viz. sinuses where tissue samples were more easily obtained) did not undergo bronchoscopy because of the risks involved.

2. We have addressed this question of mycological sampling in the text and Figure 1. In essence, BAL was only performed for possible IFD (by EORTC/MSG criteria) cases after an initial negative serum galactomannan. All patients with hematological diagnoses underwent BAL galactomannan testing, BAL fungal smears/cultures, BAL cytology. Three cases underwent transbronchial lung biopsy (the rest could not be biopsied in view of low platelet counts). The culture results (and number tested) are shown in Table 1.
   a. The “10 subjects” with BAL fungal cultures mentioned on Page 7 Line 13 relate to 14 control subjects with BAL GM ≥ 0.5 – we have changed the wording of the text slightly so that this is now clearer. The four (of 14) without fungal cultures were from Control Group 2 where fungal cultures were not routinely performed (as the majority of these had no risk factors for IFD and mainly underwent bronchoscopy to biopsy lung masses).
   b. We apologize for the confusion. Sampling frequency of serum GM has been included in the Methods. Not all subjects in control group 1 are considered at-risk for IPA at the time of bronchoscopy, hence serum GM were not performed for 6 of the 17 group 1 subjects. This has been expressed in the Results section.
   c. Thank you for the good suggestion: antifungal therapy duration prior to BAL is now included in both Table 2 and the main text (Results).
   d. For Figure 1A, we have changed the labels to indicate the 3 groups clearly as suggested.
   e. We have had the manuscript proofread by a native English speaker.

Referee 3 (Prasanna Khot)

1. Laboratory investigators performing the BAL galactomannan testing were blinded to the clinical characteristics of the subjects. We have now reflected this in the Methods.

2. Thank you for the comment – we have included confidence intervals for diagnostic sensitivity and specificity values for BAL GM OD cut-offs of 1.1 and 0.5.

3. We have included predictive values (positive and negative) for both BAL GM cutoffs as well.

4. Thank you – unfortunately we were not able to test serum samples for the entire cohort. We have amended the conclusion to state that BAL galactomannan testing has potentially higher sensitivity (rather than just “higher sensitivity”) in at-risk hematological patients.

5. Discussion points:
   a. We have included the paper by William Hope (JID 2007), where in-vitro and rabbit models showed discordance in time-to-positivity and GM indices between serum and BAL samples. It is postulated
that this discordance occurs because of the longer time taken for advancing hyphae to breach the alveolar-capillary barrier (and hence longer times to GM positivity and lower OD indices in the serum compared to BAL samples).

b. Unfortunately we (and no one to date who has published on BAL GM testing) were not able to distinguish between infection and colonization. Part of the issue is that diagnosis of IPA is already difficult to establish short of postmortem examination, and that no criteria have been defined for diagnosing Aspergillus spp. colonization. Hence we are not able to discuss this matter in the manuscript.

c. With due respect, this issue of pros and cons of different molecular testing has been discussed in a large number of original and review papers, including the new EORTC/MSG guidelines (giving reasons for why PCR results were not included as part of the guidelines). As we did not perform either beta-D-glucan testing or PCR testing, we feel that it is not so important to revisit this issue.

6. Limitations of the study:

   a. The question of analytical specificity for broad-range fungal detection and false-positives due to other fungi has been included in the Discussion previously when the case of mucormycosis (with low BAL GM) was discussed.

   b. The inclusion of subjects with non-hematological diagnoses was to determine whether the BAL GM test performed differently between patients with/without hematological diagnoses (logically, there should not be any difference, given the test characteristics and that the main issue was whether patients had IPA). There was no difference between these control groups (in fact, there was a slight trend towards higher BAL GM OD indices in non-hematological patients) and hence the results of the control groups were combined for ROC testing. This has been clarified in Methods.

      i. If we had removed the results of non-hematological patients from the analysis, the specificity of using a cut-off of 1.1 would have been even higher (100%), as one patient had a BAL GM OD index of 1.1. Hence we feel that there is no bias in using non-hematological patients as controls in this particular instance.

7. We have included a new Table (Table 2) specifically listing these results for each case.

8. Thank you for the suggestion, we have included these cut-offs on the ROC curve.

9. This data is now given in Table 2 (list of cases).

10. Minor revisions:

    a. Changed as suggested.

    b. Changed as suggested

    c. Thank you for picking this up – changed as recommended.

    d. This is given in the line below – 10 mls of the BAL aspirate aliquot was used for GM testing. As for serum galactomannan testing, we followed the manufacturer’s requirements strictly.
e. Changed as suggested.

Referee 4 (Maria Yasuda)

1. Methods:
   a. Histoplasmosis and other endemic fungi are extremely rare in Singapore. We did not detect any of these fungi in our study (nor previously among our hematological patients).
   b. The diagnoses of the control groups are listed in Table 1. We believe that the data are sound.
   c. We have now included Table 2 listing all these results in our 10 cases (patients with proven/probable IPA).
   d. We apologize for the confusion – the “none” referred to the 4 subjects in control group 1 with possible invasive fungal disease by EORTC/MSG criteria, but where alternate pulmonary diagnoses were found for these. We have now changed this to “None of the four...” to minimize the potential confusion.
   e. The median GM OD values for each group were listed in Table 1. We have now also indicated these values on the Figure (now Figure 2A) in accordance with the recommendation.

2. We have now listed this data down in Methods and Results. There were 4 patients who met EORTC/MSG criteria for possible IFD but no pulmonary diagnosis could be confirmed. These were taken out of the study. Unfortunately, it is impossible to discuss about these patients with any degree of certainty. In Singapore, necropsies are rarely if ever performed, no autopsies were performed for any of the patients who died. Of these four patients, 1 had BAL GM OD index = 1.1 while all the others were below 1.1. Three were treated with anti-Aspergillus therapy and two became better clinically and radiologically (one patient progressed and died). The last patient was not treated and also recovered. But we reiterate that it is difficult to make any sense of these results without any definitive diagnosis reached.

3. The difference between our studies and others was briefly discussed – because of low number of proven/probable IPA cases, it is possible that we had a higher cut-off. As explained above, we did not include the group of “possible IPA” where a final diagnosis was not reached because this group is very ill-defined.

4. We have amended the title as suggested.