Author's response to reviews

Title: Aspergillus-PCR in bronchoalveolar lavage for detection of invasive pulmonary aspergillosis in immunocompromised patients

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

Thank you very much for the constructive comments of the reviewers and opportunity to revise our manuscript accordingly. Kindly find attached the point-by-point response to all reviewers’ comments.

We hope our manuscript is now suitable for publication in the Journal.

Kind regards,

Daiana Stolz

Reviewer's report
Title: Aspergillus-PCR in bronchoalveolar lavage for detection of invasive pulmonary aspergillosis in immunocompromised patients
Version: 1 Date: 15 April 2012
Reviewer: Mark Reinwald
Reviewer’s report:
Major Compulsory Revisions
I. Abstract:
In your analysis you counted the “possible” cases, which make up the majority of your “suspected aspergillosis population” as having IFD (invasive fungal disease). However, I find it questionable to include the possible cases as having IFD. The clinical reality reflects the fact that most cases where aspergillosis is suspected you fail to obtain a microbiological criterion (based on EORTC like GM, culture, BDG) and therefore these patients may have IFD based on radiological and clinical signs however the true cause cannot be identified. This is reflected by the relative number of cases (proven&probable vs. possible). I would at least comment on that because in most available diagnostic studies that adhere to the EORTC/MSG criteria the “positive” IA population are the patients who would be classified as probable& proven, the possibles are usually left out. As all surrogate parameters (GM, PCR) and BAL culture are subject to reduced sensitivity by underlying antifungal treatment this has a major impact on the low sensitivity you describe for BAL PCR. Therefore I would include the sentence about antifungal therapy into the results section.

We agree with the reviewer. As the reviewer also acknowledges, most cases where aspergillosis is suspected one fails to obtain a microbiological criterion and patients are treated based on radiological and clinical signs only. Thus, even patients classified as “no” IFD according to the EORTC are commonly treated for IFD. Accordingly, we present the performance of aspergillus PCR for all 5 clinical “gold-standards” - depicted in Table 2 – (only proven cases, proven&probable, proven&probable&possible, patients on antifungal therapy and with suspicion based on radiological studies). Please note that the sensitivity of the test remained low irrespectively of the situation taken, ranging from 0% in proven cases to 36% in proven&possible cases. To highlight the fact that prior anti-fungal therapy has an impact on the low sensitivity of the test, we have now added a sentence about antifungal therapy into
the results section of the abstract and of the manuscript, strengthening the message that most patients were on antifungal therapy at the time of bronchoscopy. Moreover, we now refer to “potential IFD” when discussing the combination of proven&probable&possible. (please see also discussion bellow).

II. Introduction:
1. In the paragraph “Typical features of IFD…… “ you cite several publications for serum GM performance (Pazos C, Moragues, Verweij ) in several sentences. I would recommend combining these single statements into something like “sensitivity for Serum GM ranges from XXX to XX % (Citations) as this makes the statement more concise. Furthermore as you reflect on Serum GM I would also recommend mentioning BAL GM as it is acknowledged as a microbiologic criterion in the EORTC/MSG criteria and its sensitivity is probably higher than Serum GM sensitivity (Luong et al, Chest 2010)

We thank the reviewer for the suggestions. In the new, revised manuscript we have rephrased the statements on serum GM in the introduction, Moreover, we now mention BAL GM and its higher sensitivity as compared to serum GM. The suggested reference (Luong et al, Diagn Microbiol Infect Dis 2010) has been included.

2. In the paragraph “Recently, molecular diagnostic methods for detection …..” I would strongly suggest leaving out the term “recently” as BAL PCR is used and reported on, although not externally validated since 1993 (Spreadbury et al, Tang et al). I would however advise mentioning that the major problem in PCR diagnostics is the missing external standardization and the wide plethora of different methods (real-time, nested, etc) and that harmonization efforts are ongoing in order to help integrating PCR into the next revision of the EORTC criteria (White et al, J Clin Microbiol 2010)

As suggested, we have now deleted the term “recently”. We also comment on the major problems in PCR diagnostics and have included the appropriate reference. The new paragraph in the introduction now reads: „Although major concerns related to the lack of external standardization, wide plethora of different methods (real-time, nested, etc) and need for larger studies have been widely acknowledged, the molecular diagnostic in the BAL
seems to be a promising approach for the diagnosis of IFD. Hence, harmonization efforts are ongoing in order to help integrating PCR into the next revision of the EORTC classification.”

II. Material & Methods:
1. Disease definitions: I don’t understand the sentence “Besides BAL, the choice of further sampling techniques used during bronchoscopy was at the pulmonologist discretion”. What does that mean? And why did you cite the Stolz Chest 2007 article in that regard? Please elaborate.

This sentence refers to the fact that further diagnostic broncoscopic procedures, such as protected brushing, endobronchial or transbronchial biopsies, transbronchial needle aspiration of the mediastinum or parenchyma could have been performed during bronchoscopy, in case the attending respiratory physician considered it to be required. Indeed, 15 (7.9%) transbronchial biopsies, 11 (5.8%) endobronchial biopsies and 3 (1.6%) TBNA have been performed in the study cohort. The citation provides information about the bronchoscopic technique itself (sedation, monitoring, etc) applied to the patients participating in the study. We have now rephrased the sentences and added the number of further bronchoscopic procedures performed to the results section of the manuscript. The reference has been relocated for better understandability.

2. In the paragraph “Severe neutropenia was defined as an absolute neutrophil count of ….” after this statement, which is important for immunocompromised individuals you write about nonspecific interstitial lung disease, organizing pneumonia, respiratory bronchiolitis, alveolar hemorrhage, etc. However I do not see the relevance to the actual topic of your manuscript, especially as these clinical conditions are not presented in the patient characteristics tables and not elaborated on later in the results or discussion section. I would recommend leaving that out as it doesn’t yield additional information.

Agree. As suggested, irrelevant disease definitions have been deleted.

3. In the paragraph “Patients were examined, treated, and followed up…” I would recommend providing the numbers of how often CT was performed in order for the reader to understand the population you are describing as the classic radiologic criteria for defining IA (nodules, halo-sign, air crescent sign, etc) are often not seen on conventional X-Ray.
Agree. Indeed, in only 48 patients (25.1%) attending physicians refrained from performing a chest-CT scan for evaluation of the respiratory symptoms leading to bronchoscopy. Thus, CT scans were obtained in 143 cases (74.9%). This information has been added to the paragraph.

III. Results:
III. 1. In the paragraph “A total of 191 immunocompromised....” You mention that the vast majority of patients required bronchoscopy due to fever and cough. However, only “Fifty-five percent of patients presented infiltrates in the Chest X-Ray. Do you perform BAL even when no infiltrate can be detected in your institution? or did the remaining 45 % have infiltrates in CT ?

Right. In the presence of respiratory symptoms, BAL was performed irrespective of the absence of an infiltrate. We have now improved the information provided in the methods section of the manuscript: “In patients with no or diffuse pulmonary infiltrates, BAL was performed either in the right middle lobe or the lingula. For patients with focal lung infiltrates, BAL was performed in the pulmonary segment corresponding to the radiologic abnormality. BAL fluid was recovered by suction.”

2. In the paragraph “ Bronchoalveolar lavage was performed in all cases”. I would leave that sentence out as this is a prerequisite for this study.

Agree. We have rephrased the sentence to accommodate the description of the further bronchoscopic procedures, as discussed above.

3. In the paragraph " Therefore, there were 53 Patients with IFD" I would disagree with that statement. You had at least 11 patients with IFD (proven&probable acc. To EORTC/MSG, see my comments on the abstract).?

Agree. We now refer to the group proven&probable&possible as patients with potential IFD. This reflects our wish to strength the fact that also patients with possible IFD would require specific therapy on clinical grounds. In addition, previous publications have also opted for incorporating the possible group for diagnostic calculations, for instance Bergeron, A., Chest 2010.
IV. Discussion:
1. In the paragraph “The low sensitivity of the assay in our study…” you correctly (at least from my point of view) identify the explanation why you have a low number of cases (11 proven/probable) because of the low pre-test-probability. However that by itself not the explanation for the low PCR sensitivity (0 % in proven, 36 % in proven& probable cases according to Table 2) but might be the explanation for the reduced sensitivity when taking the possible cases into account (as more cases are included which do not suffer from IA). I would elaborating on that.

Agree. There is a decrease of the sensitivity from 36% to 26%. The following sentence has been added to the discussion: “The low sensitivity of the assay in our study might have several reasons. Firstly, we have additionally computed the diagnostic performance of the assay by incorporating the group of “possible” IFD as potentially having IFD. As more cases not “formally” suffering from IFD are included as diseased, there is a decrease in the pre-test-probability, thus further reducing the sensitivity of the test from 36% to 26%, as depicted in Table 2.”

2. You state that one of the main reasons for negative PCR and therefore low sensitivity in the proven&probable patients is underlying antifungal therapy. This is very important, I would recommend citing other papers showing influence of underlying antifungal therapy on surrogate parameter performance (Marr et al, 2005; Lass-Floerl et al 2005).

Thank you very much for the references: We could not find the reference for Lass-Floerl, but have included a newer reference (Reinwald et al, J Antimicrob Chemother 2012) instead.

3. You state that “in the second case, the patient was on cyclosporine, had a suspicion of fungal infection and a positive aspergillus culture in BAL, thus fulfilling only the “possible” criteria. This is not true. Host Factor = CSA, Infiltrate = radiologic abnormality, BAL culture = microbiologic criterion # Patient would have been classified as “probable” without histology based on EORTC 2008 (see de PAuw et al CID 2008)

Agree. Our mistake has now been corrected.

4. Were autopsies performed? (basically the earlier studies recruited their higher number of “proven cases “ usually based on autopsies. I would add this information
Yes. Out of 37 deaths observed in the study, a total of 24 autopsies were performed. In 2 cases without autopsy, further tissue sections were previously obtained for examination (transbronchial biopsy, open lung biopsy, one case each). Thus, histology was available in 26 (70.3%) of the deceased patients.

Overall, histological confirmation was sought in 35 patients. In addition to bronchoalveolar lavage, 15 (7.9%) transbronchial biopsies, 11 (5.8%) endobronchial biopsies and 3 (1.6%) TBNA from the parenchyma have been performed during bronchoscopy. Eight (4.2%) patients were submitted to open lung biopsy. Additionally, autopsy results were available in additional 22 cases. Thus, histological confirmation was possible in 57 (29.8%) of the cases.

This information has been added to the result section.

5. In your final sentence you state that “your results” suggest a limited clinical applicability of Aspergillus PCR in the BAL for detection of IFD in a population of immunosuppressed patients”. However, I do not agree with that statement the way it stands now. Firstly I would recommend changing IFD to IA as your PCR detects only aspergillus species. Secondly (based on your table 2) sensitivity for probable cases would be 50 % (4/8 cases) which I find acceptable, taking the amount of antifungal therapy nowadays prior to BAL sampling into account.

We have now specified that IFD refers specifically to invasive aspergillosis in the discussion section. As described below, the 2 cases with positive aspergillus culture classified as “possible” did not reach the “probable” classification due to the lack of a clinical criterion. The first case had no infiltrate, body temperature, positive aspergillus BAL culture and symptoms of LRTI but no tracheobronchitis. The second case had no infiltrate, body temperature, prolonged (13 weeks) use of corticosteroids in previous 60 days, positive aspergillus BAL culture and symptoms of LRTI but no tracheobronchitis. Thus both were classified as possible cases.
1. In your table you list your possible patients, which, according to the EORTC Criteria (de Pauw et al CID 2008) fulfill the definition of Host criteria and lower respiratory tract disease (imaging, etc). However in your possible group you have several patients who have a positive aspergillus culture. According to table 2 of the de Pauw classification (CID 2008) of the EORTC these should be classified as “probable” increasing your total number of probable cases (“Recovery by culture of a mold (e.g., Aspergillus, Fusarium, Zygomycetes, or Scedosporium species”, Table 2, de Pauw 2008, CID)

The 2 cases with positive aspergillus culture classified as “possible” did not reach the “probable” classification due to the lack of a clinical criterion. The first case had no infiltrate, body temperature, positive aspergillus BAL culture and symptoms of LRTI but no tracheobronchitis. The second case had no infiltrate, body temperature, prolonged (13 weeks) use of corticosteroids in previous 60 days, positive aspergillus BAL culture and symptoms of LRTI but no tracheobronchitis). Thus, both were classified as possible cases.

Minor essential revisions:
. you mentioned you had Serum GM for 105 patients, do you also have data on BAL GM ? Or is that not performed in your institution.

Unfortunately, BAL GM is not routinely performed in our institution and was not determined within the study.

I would mention in your limitation paragraph, that the low-pretest probability of your study design lead to a limited number of proven/probable cases (11 total)

As suggested, we have included this limitation to the discussion section.

Reviewer 2

General
The diagnosis of invasive fungal infections is a persistent challenge and an issue of current debate in the care of patients with immunosuppression and infectious complications / respiratory symptoms.

This well written manuscript is focussed on an interesting and up to date topic, especially for pneumologists / bronchoscopists as well as specialists for infectious diseases /microbiology, haematological malignancies and transplant medicine etc.

Therefore the paper provides useful clinical information with impact on the diagnostic strategy in these patients. Furthermore it presents real life data as many of these patients are already on antifungal therapy.

Thank you!
Major comments
The authors mention technical problems with the PCR procedure. This is a major issue that might jeopardize the whole data collection. Therefore these technical problems should be further addressed and potentially affected samples removed from the analysis.

We apologize for the imprecision. Technical problems with the PCR procedure are a possible, although not probable, explanation for the low sensitivity of the assay in this study. We have no concrete suspicion on a technical problem with the assay or particular patient probes. A discussion about this theoretical limitation is present in the discussion section of the manuscript.

The risk of invasive fungal infection is depending on the type and severity of immunosuppression. The authors might provide information about the type of immunosuppression, e.g. steroids, solid organ transplantation, stem cell transplantation, neutropenia) and the incidence of IFD and results of aspergillus PCR resp.

We agree with the reviewer. In our study, BAL Aspergillus PCR was positive in 36/129 patients with hematologic disorders, 8/26 cases of solid organ transplants, 2/12 with AIDS and 9/24 with autoimmune disorders. Thus, the underlying cause of immunosuppression did not significantly influence PCR results (p=0.605) There was no difference in PCR positivity among alogen (13/48), autolog (5/12) and no (37/131) stem cell recipients either (p=0.589). This information has been added to the revised version of the manuscript.

There is a high rate of false positive and false negative aspergillus PCR. What were the final diagnoses in the cases with an incorrect PCR result? Was there any association between PCR results and non aspergillus lung disease?

This is an interesting question. Unfortunately, we could not find any association between PCR results and a particular lung disease. Specifically, there was no association between a positive PCR result and a positive Aspergillus cultures in the
BAL, which could suggest colonization without IFD. Further diagnosis encountered in these patients included a plethora of disorders such as bacterial and viral (VZV, CMV) pneumonia, PCP pneumonia, BO, lymphocytic pneumonia, sarcoidosis, Wegener granulomatosis, NSCLC, etc. Due to the lack of a specific association with either false positive or negative results, we refrained from listing the further diagnoses in the manuscript.

Did you perform a follow up on patients with a positive Aspergillus PCR result without signs of invasive Aspergillus infection? How many of them developed Aspergillus infection in the follow up?

Patients were followed up until the specific episode of respiratory symptoms leading to study inclusion has been conclusively evaluated, e.g. a final diagnosis has been made. Thus, if a patient has been diagnosed with IFD as the cause of the respiratory symptoms within the give episode, he has been considered to have the disease. Due to the chronic nature of the underlying immunosuppressive disorder and associated recurrent symptomatology, we believe that the correlation between aspergillus PCR results at the present episode and the development of future IFD cases could be misleading, as some of these patients would expectably develop (new) IFD in the course of their chronic disease.

The authors discuss the vascular spread of invasive aspergillosis. Did you use Aspergillus PCR also in blood samples?

No. Aspergillus PCR was performed solely in the BAL samples.

How many of the patients received a histological confirmation of the diagnosis e.g. by transbronchial biopsy or open lung biopsy / resection and what was the clinical criteria to perform histological examinations?

Histological confirmation was seeked in 35 patients. In addition to bronchoalveolar lavage, 15 (7.9%) transbronchial biopsies, 11 (5.8%) endobronchial biopsies and 3
TBNA from the parenchyma have been performed during bronchoscopy. Eight (4.2%) patients were submitted to open lung biopsy. Additionally, autopsy results were available in additional 22 cases. Thus, histological confirmation was possible in 57 (29.8%) of the cases. The decision to obtain tissue for histological examination was left at the discretion of the attending physician. This information has been added to the manuscript.

Minor comments
Parts of the introduction belong to discussion.
After several modifications, the introduction section of the revised manuscript became more concise.

It should be noted that the study was performed at one center = monocentric Study
This information has been added to the material and methods section of the manuscript.

Why the authors did mentioned the diagnostic criteria for BOS, DAH, but also NSIP
Thank you. This superfluous information has been now deleted.
Please cite the literature on the consensus of IFD (page 9).
We apologize. The reference of the consensus on IFD has been now included in the revised version of the manuscript.

Several typing errors are noted.
We hope to have now corrected all typing errors.