Author's response to reviews

Title: A multiplex nested PCR for the detection and identification of Candida species in blood samples of critically ill paediatric patients

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Author's response to reviews: see over
To Nathaniel Nazareno  
Journal Editorial Office  
BioMed Central  

Dear Editor,  

We are re-submitting the manuscript MS: 7095512071244286 “A multiplex nested PCR for the detection and identification of Candida species in blood samples of critically ill paediatric patients”. We would like to thank the Editor for the opportunity of re-submitting the manuscript, as well as the reviewers, who helped us to improve it with their input. The manuscript was revised according to their suggestions: all the modifications that were incorporated to the text are highlighted in yellow and discussed in the response to reviewers. We feel that the revised manuscript has been much improved. Below are our point-by-point answers to the comments.  

With regard to referee 1 comments (3 and 4) about missing and faint bands, we would like to say that the figure has lost its resolution during uploading and processing to the PDF file. The original figure with better resolution (and clearly perceptible bands) is shown in the response to referee 1. We would like to know what we can do to overcome this problem.  

Sincerely yours,  

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Referee 1

1. Lines 174-177: Limit of detection was performed on C. albicans DNA only. It is well known that PCR efficiency differs between targets, particularly in multiplex reactions. The authors should provide limit of detection values for each of the seven targets within each respective multiplex assay.

Answer: We agree with the reviewer that this information was missing from the original manuscript. Detection limit assays were performed for all seven Candida species. Actually, in both the Results and Discussion sections, the detection value of 150 fentograms/mL of blood referred to all seven Candida species. The detection limit assays were performed 10 times in different days (10 replicates), following recommendations of the CLSI EP12-A guideline (2002) - [User Protocol for Evaluation of Qualitative Test Performance]. Results are shown below.

<table>
<thead>
<tr>
<th>Species (DNA)</th>
<th>1.5 ng/mL</th>
<th>150 pg/mL</th>
<th>15 pg/mL</th>
<th>1.5 pg/mL</th>
<th>150 fg/mL</th>
<th>15 fg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>10*</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>05</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>05</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>04</td>
</tr>
<tr>
<td>C. krusei</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>04</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>02</td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>04</td>
</tr>
<tr>
<td>C. pelliculosa</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>03</td>
</tr>
</tbody>
</table>

* = number of replicate tests with positive results

Once the 15 fg/mL value was positive in less than 50% of tests for most Candida species, the concentration of 150 fg/mL of blood sample was defined as limit detection of the assay. In the revised version of the manuscript (Methods section) C. albicans DNA sample was replaced by the seven Candida species DNA samples and we added that the tests were performed for each Candida species separately (lines 187-189).

2. Specificities of the assays were measured against bacteria and non-Candida DNA. It is unclear whether specificity testing was also performed among different Candida species. It is well known that Candida dubliniensis is genetically closely related to Candida albicans – is there potential for cross reaction? Were members of the C. parapsilosis complex (C. metapsilosis and C. orthopsilosis) tested for specificity of the C. parapsilosis primers?

Answer: Although the primers for C. albicans, C. glabrata, C. parapsilosis, C. tropicalis and C. krusei targeting the ITS region were previously employed by other authors (Bougnoux et al., 1999; Luo & Mitchell, 2002) we performed an in silico analysis to verify whether cross reactions could occur with other Candida species. This
analysis confirmed the specificity of all primers, including those designed in our study (C. lusitaniae and C. pelliculosa). Then, we performed the *in vitro* tests by means of our multiplex-PCR with some of heterologous species such as C. guilliermondii, C. kefyr, C. famata, C. dubliniensis and C. haemulonii. The amplifications were negative for all DNA samples tested, confirming the *in silico* analysis. We added this information to the revised manuscript in both Methods (lines 193-195) and Results (line 210) sections.

Regarding the *C. parapsilosis* primers, they amplify DNA samples from the three species of the complex as we have verified both *in silico* and *in vitro* analysis, although in our study only the *C. parapsilosis sensu stricto* was identified by the automated sequencing. In the revised manuscript we added the words “*sensu stricto*” to *C. parapsilosis* detected in patients’ blood samples (line 228) as well as in the legend of Table 2 (highlighted in yellow). Likewise, in the line 152 and in the Table 1 we added the word **complex** to *C. parapsilosis* primers.

3. *Figure 1* lane 7 is missing control bands for *C. albicans* and *C. lusitaniae*.

4. *Figure 1* lane 14: the *C. pelliculosa* control band is very faint – does this indicate lack of PCR efficiency or low starting template concentration?

**Answer:** The original Figure 1 that has been submitted appears below. Unfortunately, during the uploading and processing to the PDF file in the BMC infectious Diseases site it has lost most of its resolution. The missing bands in Lane 7 although less intense clearly appear in the original figure. It is important to say that although the control bands may appear slightly less intense in the agarose gels, it does not mean lack of efficiency since the detection limit values for these *Candida* species were not affected.

The faint line in lane 14 actually appears as intense as the other two bands in the assay 2. We are making efforts with the editor to provide a better final resolution of this figure.
5. The authors find 3 patients with dual infection with C. parapsilosis and C. tropicalis. Further discussion is warranted. Is this pairing common and clinically significant? It is unusual that C. albicans DNA was not detected amongst the mixed infections given the higher proportion of disease caused by this species.

**Answer:** We added to the revised version of the manuscript a paragraph addressing dual candidemia and its clinical significance. Although the isolation of more than one species has been described in the literature, there is no evidence that a greater morbidity and mortality is associated with this condition (Jensen et al., *Clin Infect Dis*, 2007; Pulimooood et al., *Diagn Microbiol Infect Dis*, 2002). However, there is a possibility that one of the species detected by molecular methods rather than by blood cultures to be resistant to the most frequently employed antifungal agents. These informations were added to the revised manuscript (lines 273-280). We cannot state if the detection of both *C. parapsilosis* and *C. tropicalis* in mixed *Candida* infections is common because despite its poor sensitivity, blood culture is still the current method for candidemia diagnosis in our institutions. The isolation of more than one *Candida* species from blood samples occurs in less than 5% of the cases of candidemia in our nosocomial settings and takes place mainly in adults probably because of the more significant blood volume (10 ml) obtained for the cultures, whereas in pediatric patients the blood volume varies from 1 to 3 ml. Furthermore, in these three particular cases of dual candidemia *C. albicans* was not present, although in the 13 positive patients this species was detected.
as frequently as *C. tropicalis* and *C. parapsilosis*. This may be related to the fact that in Brazil, studies carried out in tertiary public hospitals have demonstrated that *C. parapsilosis* and *C. tropicalis* are the non-*C. albicans* species more frequently isolated from candidemia episodes, particularly in pediatric intensive care units (Colombo et al., *Braz J Infect Dis* 2013; Santolaya et al., *Pediatr Infect Dis J* 2014). We added these informations to the revised manuscript (lines 280-284).

6. *The authors should discuss their assay in the context of other new emerging technologies such as T2 biosystems, which address the poor sensitivity of blood culture for detecting candidemia using NMR.*

**Answer:** We accept the referee comment and we added to the revised manuscript some information concerning the use of the new T2MR technology in the detection of *Candida* species in clinical samples (lines 309-316).

7. *Although the poor sensitivity of blood culture is well known, the authors do not address the potential of transient Candida DNAemia which can be a likely cause of false positives and unnecessary treatment.*

**Answer:** We added to the revised manuscript some information regarding this issue (lines 296-301).
**Referee 2**

1. Since 38 patients (70.4%) received empiric antifungal therapy and only 13 had candidemia the authors should discuss whether antifungal therapy could have been discontinued in the other 25 patients and consider the utility of their assay in improving antifungal stewardship. This is particularly relevant since all 13 patients with candidemia were already receiving antifungal therapy.

**Answer:** We accept the referee comment and we added to the revised manuscript some information regarding this issue (lines 289-293).

2. Would provide median and range of time to positivity for the positive blood cultures. This will provide for a more precise comparison to the 24h for the assay.

**Answer:** According to information provided by the microbiology laboratories of the two hospitals, the mean time for the release of culture results is 72 hours, ranging from 48 hours to up five days in most cases. This information was included in the revised manuscript (lines 294-296).

3. Would comment on whether their assay is currently only performed as a research tool or if it is used for clinical purposes. And if used clinically, is it performed 7 days a week?

**Answer:** The results presented in this study were the first effort towards the validation of this multiplex PCR assay, so it was only performed as a research. We believe that this methodology will be applied in a short time to the routine laboratory diagnosis in our tertiary public hospitals, depending on economic and human resources.

4. In the discussion would comment on how their assay performs compared to similar assays (e.g. the assay in reference 14).

**Answer:** It is difficult to compare our results with those of previously developed PCR methodologies due to differences in the assays design: the very few nested or semi-nested assays reported to detect Candida species in blood samples are single DNA target. Carvalho et al. reported (reference 14) a semi-nested multiplex PCR assay comparable to ours. However the assay was tested only for Candida species detection in positive blood culture bottles. Regarding novel platforms such as real time-PCR and T2 magnetic resonance-based technology, a comment on them was added to the discussion (lines 309-316).

**DISCRETIONARY REVISIONS**

1. On line 118 would favor "Cultures were obtained" rather than "established."
In the revised version of the manuscript we replaced the word *established* by *obtained* that is highlighted in yellow (line 124).