Author’s response to reviews

Title: Influence of subinhibitory antifungal concentrations on extracellular hydrolases and biofilm production in Candida albicans recovered from Egyptian patients

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Authors’ Responses to Reviewers’ Comments

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Dear Dr. Cecilia Devoto,

Managing Editor, BMC Infectious Diseases

Thank you very much for your e-mail regarding the reviewers’ report of our manuscript and your kind permission to submit a revised version of it. Also, we would like to express our deep gratitude to the reviewers for their time, effort, valuable comments and corrections to consolidate and improve the scientific quality of our manuscript. Attached, please, find a revised version of the manuscript, taking into account all the reviewers' comments. The changes were indicated in the text using track changes and new references were highlighted in yellow color. Also, you will kindly find responses for the reviewers answering their remarks and comments point-by-point.

Yours sincerely,

Dr. Hebatallah Nasser, Lecturer of Microbiology and Public Health

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All comments of the editor and reviewers were taken into consideration as follows:

- Editor’s Comments: We note some significant overlap in text with this previously published study: Anil, S., Hashem, M., Vellappally, S. et al. Mycopathologia (2014) 178: 207. https://doi.org/10.1007/s11046-014-9802-0. Please go over your manuscript and ensure that the text used is your own original phrasing. In addition, please be aware that we may send your submission for additional review, but are asking you to revise your manuscript first.

- Authors’ response: Rephrasing has been considered.

- Reviewer 1 Comments:

1. Title

a) I suggest removing the dot located at the end of the title, since it is not indicated the use of points, hyphen or indentation after the title.

- Authors’ response: Dot has been removed.

2. Abstract

a) I suggest replacing lines 7 to 10 for better understanding: "Extracellular hydrolytic enzymes (phospholipase, aspartyl protease and haemolysin), and biofilm formation are considered to be major virulence factors of the opportunistic pathogenic fungus Candida albicans" by "Extracellular hydrolytic enzymes (phospholipase, aspartyl protease and haemolysin), and biofilm formation are considered one of the major virulence factors of the opportunistic pathogenic fungus Candida albicans", considering that hyphae are able to invade tissues and are also related to virulence and not only biofilms or extracellular hydrolytic enzymes.

- Authors' response: Lines have been replaced according to the reviewer’s suggestion.

3. Introduction

a) In the clinical setting, C. albicans is considered a model microorganism, therefore, several papers are published annually. Therefore, I suggest the use of more up-to-date references with a maximum of five years.
- Authors' response: References have been updated.

3. Introduction

b) In the line 11 "When host defenses become compromised". What factors can lead to compromising host defenses and consequently C. albicans infections? (See: Candida albicans cell-type switching and functional plasticity in the mammalian host).

- Authors' response: Risk factors have been added.

3. Introduction

c) In paragraph: "Despite the widespread use of antifungal agents, their impact on candidate virulence determinants has been little studied" I suggest rewriting the justification, since there are already some articles published on the subject (see: Effects of Sub- Inhibitory Concentrations of Antifungal Agents on Adherence of Candida spp. to buccal epithelial cells in the vitro Enhancement of Secretory Aspartyl Protease production in biofilms of Candida albicans exposed to subinhibitory concentrations of fluconazole, Caspofungin modulates in vitro adherence of Candida albicans to plastic coated with extracellular matrix proteins). Therefore, I suggest to the authors that they rewrite the justification of the manuscript.

- Authors' response: Justification of the manuscript has been rephrased.

4. Methodology

Antifungal susceptibility testing

a) Describe how the interpretation of the results was performed and what percentage of inhibition was considered to evaluate the minimum inhibitory concentration?

- Authors' response: Interpretation of the results has been added and described in this section of methodology.

4. Methodology

Antifungal susceptibility testing

b) To validate the assay, it is not used reference strain?
Authors' response: A reference strain Candida albicans ATCC 90028 was used and added to the manuscript.

5. Results

a) At the end of Tables 2 and 3 add the acronyms and names of the antifungals.

Authors' response: Acronyms have been added.

6. Discussion

In paragraph "There is scarcity of data on the impact of antifungal therapy on secretion of extracellular hydrolases as well as biofilm production by C. albicans". I suggest rewriting the same, as there are several reports of biofilm production of C. albicans in the literature, as well as the effect of antifungal therapy on it.

Authors' response: The sentence has been rephrased.

Reviewer's 2 comments:

1. Overall, the manuscript as written has a lot of unnecessary verbiage which is affecting clarity of the statements made. I have indicated a few occasions, but please go through the whole text and revise for clarity, sentence structure, elimination of repetitions, and punctuation.

Authors' response: The manuscript has been thoroughly revised as per the reviewer’s recommendation.

2. For a reader who gets to read only (or first) the abstract, the logic of using sub-inhibitory concentrations of the antifungals is not immediately clear; the abstract could benefit from the addition of at least one explanatory sentence (or even a clause). The introduction also needs the same explanation in one or two sentence(s).

Authors' response: Explanatory sentences have been added in abstract and introduction sections.
3. Results in Abstract, last sentence of the paragraph: the statement re Fluconazole is superfluous. Shorten the sentence, for example, as "nystatin and micafungin, but not fluconazole, had a noticeable significant impact on inhibiting biofilm formation of C. albicans clinical isolates"... or something to that effect.

- Authors' response: The statement has been shortened as suggested by the reviewer.

4. Section 'Background'. Please remove the verbiage - 'on one hand', 'on the other hand', 'furthermore' and so forth. There is no need to string these independent concepts.

- Authors' response: The verbiage has been removed.

5. Second paragraph, 'Background': change 'exposition' to 'exposure'. In the same sentence, it is important to define at this point the context of the 'sub-inhibitory concentrations'. One sentence or a clause would do, but the definition is needed because the terms 'inhibitory' and 'sub-inhibitory' often refer to different parameters in respect of individual microbes vs. biofilms.

- Authors' response: The word has been changed according to the reviewer’s suggestion. The sentence has been simplified and rephrased appropriately.

6. One sentence later, the statement about azoles being fungistatic is not necessarily correct. Fluconazole is fungistatic, yes, but the newer azoles like voriconazole have significant in vitro fungicidal activities. Please consider rewriting this sentence.

- Authors' response: The sentence has been rewritten according to the reviewer’s recommendation.

7. Methods: Yeast suspension prep & antifungal exposure. If I am getting this correctly, the final volume of the test solutions was 5 mL (=1 mL yeast suspension + 4 mL medium with drug). So the drugs (at 2X or 200% of MIC) were diluted 4/5. In the resultant solution, the drug concentrations were around 160% of the MICs? So this was not sub-MIC then (compared to your biofilm experiments, where the exposure was to 50% of the MIC)? Please clarify.

- Authors' response: The sentence has been rephrased appropriately.
8. Methods: Biofilm production assay: If I understand this correctly, the biofilm production assay is contingent upon the trapping of crystal violet by the biofilm and elution by 95% ethanol (both of which are sources of variability). How many times was this measured in how many wells, to account for random variabilities via statistical means?

- Authors' response: The assay was done in triplicates.

9. Next sentence: Half the volume of the total colored solution (CV in EtOH) is assayed to measure the color intensity as a surrogate for the quantity of the biofilm. If I am correct about the assay principle, this needs to be explained in an additional sentence -- what exactly is being measured and how that parameter relates to the biofilm.

- Authors' response: Concept of the assay has been explained in the manuscript.

10. In the penultimate sentence of Biofilm method, the wavelength of measurement is indicated as 590 nm, whereas two sentences above it is mentioned as 595 nm. Which is correct?

- Authors' response: The wavelength of measurement equals to 590 nm.

11. Next section in Methods: Effect of sub-MIC antifungals on biofilm. In the sentence starting with "Volumes of 1 mL of test agents (2 × final concentrations) in RPMI 1640 medium..." change the verb from 'was' to 'were' for agreement with the plural subject 'volumes'.

- Authors' response: The verb has been changed.

12. In the next sentence "biofilms grown... as control", please change the sentence to something more descriptive as, say, "Wells with only medium were used as control (untreated) wells" or something to that effect for clarity.

- Authors' response: The sentence has been changed according to the reviewer’s suggestion.

13. Biofilms can be mentioned in the next sentence; say, something like "... incubated for the development of biofilms at 37 deg C for 48h."

- Authors' response: The sentence has been changed according to the reviewer’s recommendation.
14. Method: Statistical analysis: in the penultimate sentence, the controls are written as "unexposed to antifungal sub-MICs". But more accurately, they are unexposed to any antifungal, correct? Please clarify that.

- Authors' response: Correct, controls were not subjected to antifungal agent. It has been clarified in the manuscript.

15. Results, first paragraph. The word is 'proteinaceous' - please correct the spelling

- Authors' response: The spelling has been corrected.

16. Results, Table 1: There are no p-values here, so that footnote is superfluous. And the table is unnecessary; what is currently written can easily be described in a single sentence in the text. It'd be better and more helpful to see the median MICs and spread of MIC values amongst the isolates for each antifungal.

- Authors' response: Table (1) has been removed as suggested by the reviewer and the other two tables have been re-numbered consequently. The data have been attached as “Additional file 1”. It has been also referred to in the manuscript.

17. Results: please remove the verbiage "To start with", "Moving to", "Finally speaking of", "In an effort", and so forth.

- Authors' response: The verbiage has been removed.

18. In Tables 2 and 3, what does the negative sign (-) mean in the context of signal reduction? This does not match what is written in the preceding text. Please clarify. Additionally, you may or may not want to include the formula for calculating the signal reduction in the Methods, but I'd recommend it.

- Authors' response: (-) was not intended to express a negative sign in the columns of control in table 2 and 3. It was used to express no reduction in the production of secreted hydrolases nor biofilm formation for the assays controls. They have been replaced by (---). Formulae have been added to the methods as suggested by the reviewer.
19. Paragraph immediately after Table 2: Merge the clause "... while the rest... capacities." with the first sentence in the paragraph, which is saying the same thing.

- Authors' response: Clause has been merged.

20. Table 3: In addition to the summary data (presented), it would be interesting to see the individual data for each of the 7 isolates (No drug, NYS, FLU, MCF), especially since there appear to be significant variations in the biofilm making abilities amongst the isolates (as mentioned in the text).

- Authors' response: The data have been attached as “Additional file 2”. It has been also referred to in the manuscript.

21. The differences in the SEM images are rather striking, particularly the striations (present or absent) in the backgrounds of the adherent cells. How many wells were used for each isolate? Which isolate is represented in the figure? Are these striations truly biofilm-associated or are they normal well variants? Did you have (or can you add) a 'No yeast' control for the wells?

- Authors' response: One well was used for each isolate.

- The used isolate was the one that showed strong biofilm producing ability amongst the tested isolates.

- These striations are not biofilm-associated as it appears in 'No yeast’ control. Also, 'No yeast' control image has been attached as “Additional file 3”. It has been also referred to in the manuscript.

22. Figure 1B in the text appears to have been marked in the figures as '2A'. Please correct.

- Authors' response: Figure number has been corrected.

23. Discussion: 2nd paragraph end. No need to repeat the statements about the antifungals and their mechanisms of actions; those have been already mentioned in the 'Background'.

- Authors' response: The statements have been removed as suggested by the reviewer.
24. 3rd paragraph: Regarding 'suppressive effect on PL-ase production following exposure to FLU'. Since all the isolates were resistant to FLU, it would have been interesting to see if a high (>MIC) concentration of FLU (say 128 ug/mL, 2X the breakpoint for FLU resistance) achieved anything in terms of extracellular hydrolase activity or biofilm formation.

- Authors' response: The authors highly appreciate the reviewer’s comment, but the main objective of the current study was to evaluate the effect of sub-MIC values of the tested antifungal agents.

25. In fact, the FLU results perhaps deserve a separate paragraph, given that the isolates were resistant, and yet, sub-MIC drugs managed to demonstrate some biological effects in vitro. This calls into question the clinical or biological relevance of these virulence factors.

- Authors' response: FLU results have been mentioned in separate paragraph according to the reviewer’s comment.

26. Page 18: bottom paragraph. Second sentence: change the semicolon to a comma. "The fact remained, however, that..."

- Authors' response: The semicolon has been changed to comma.

27. Page 19, bottom paragraph on SEM. Regarding the NYS treatment showing "internally collapsed... cell wall", this is not clear at all from the images supplied. Do you have a higher mag zooming in on the individual 'collapsed' cells? Otherwise, if you have observed this consistently but do not have the images, mention 'data not shown'.

- Authors' response: A zoomed in SEM image for the collapsed cells has been attached as “Additional file 4”. It has been also referred to in the manuscript.