Reviewer’s report

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

Version: 1 Date: 14 Sep 2018

Reviewer: Reviewer 2

Reviewer's report:

PEER REVIEWER COMMENTS: To view the full report from the academic peer reviewer, please see the attached file.

REVIEWER COMMENTS FROM REPORT: What made this study most interesting to me is the use of sub-inhibitory concentrations of antifungals, which is relevant for clinical situations in the context of tissue bioavailability of the drugs and their variable penetration into biofilms on, say, catheters (a particular problem for Candida). I am glad that the authors have looked at both secreted virulence factors and biofilms. The study seems well designed and executed. However, there are some critical issues in the analysis and interpretation, as well as in clarity, which must be addressed before this manuscript is ready for publication. I have listed the most pressing issues in the next section.

REQUESTED REVISIONS:

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.

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).

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.
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.

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.

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.

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.

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?

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.

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?

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'.

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

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.

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

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.

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

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.

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.

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).

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?

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

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'.
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.

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.

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

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'.

**Are the methods appropriate and well described?**
If not, please specify what is required in your comments to the authors.

No

**Does the work include the necessary controls?**
If not, please specify which controls are required in your comments to the authors.

No

**Are the conclusions drawn adequately supported by the data shown?**
If not, please explain in your comments to the authors.

No

**Are you able to assess any statistics in the manuscript or would you recommend an additional statistical review?**
If an additional statistical review is recommended, please specify what aspects require further assessment in your comments to the editors.

Not relevant to this manuscript

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